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CRIOPRESERVAÇÃO DE FRAGMENTOS DE TECIDO OVARIANO DE PEIXES

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Tese apresentada como um dos requisitos à obtenção do Grau de Doutora em Zootecnia
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Mestre em Zootecnia

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“Great things in business are never done by one person. They're done by a team of people.”

(Steve Jobs)

CRIOPRESERVAÇÃO DE FRAGMENTOS DE TECIDO OVARIANO DE PEIXES[1]

Autor: Lis Santos Marques
Orientador: Danilo Pedro Streit Jr.

RESUMO

Apesar dos avanços no manejo reprodutivo de peixes para programas de melhoramento, pouco se sabe sobre o uso potencial de biotecnologias reprodutivas, como a criopreservação de tecido ovariano. A criopreservação de tecido ovariano em peixes tem como objetivo preservar o germoplasma feminino de espécies selvagens e ameaçadas de extinção, assim como de peixes de alto valor zootécnico e importantes modelos de pesquisa biomédica. Em um estudo prévio realizado pelo nosso grupo de pesquisa, obtivemos um elevado percentual de viabilidade de oócitos imaturos após vitrificação de fragmentos de tecido ovariano. Esse estudo mostrou que oócitos imaturos de peixes têm maiores chances de sobrevivência após a vitrificação do que oócitos em estádios mais avançados de maturação. No entanto, até o momento nenhum estudo comparou qual técnica de criopreservação, vitrificação ou congelamento lento, apresenta melhores resultados na criopreservação de fragmentos de tecido ovariano de peixes teleósteos. Assim, no primeiro estudo utilizando o modelo biológico zebrafish (*Danio rerio*), o objetivo principal foi comparar os efeitos de dois protocolos de criopreservação, vitrificação e congelamento lento, na viabilidade do tecido ovariano por meio da avaliação de parâmetros de estresse oxidativo, de danos ao DNA, atividade mitocondrial e integridade morfológica dos oócitos imaturos. Os resultados obtidos mostraram que o congelamento lento apresentou maiores níveis de estresse oxidativo e menor atividade mitocondrial do que o tecido ovariano vitrificado. Além disso, as observações ultraestruturais dos oócitos congelados apresentaram ruptura da membrana plasmática, perda de conteúdo intracelular e um grande número de mitocôndrias danificadas, enquanto os oócitos vitrificados tinham mitocôndrias e membranas plasmáticas celulares intactas. Portanto, a vitrificação foi utilizada no segundo estudo que teve como objetivo verificar se a biotécnica utilizada em zebrafish é aplicável em uma espécie de peixe de importância econômica. A alta viabilidade de oócitos obtida após a vitrificação de tecido ovariano contendo folículos imaturos de pacu (*Piaractus mesopotamicus*) sugere que o protocolo aplicado pode ser utilizado com sucesso em outras espécies de peixes teleósteos.

Palavras-chave: vitrificação, congelamento lento, teleósteos, oócitos.

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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 (114 p.). Março, 2019.

CRYOPRESERVATION OF OVARIAN FISH TISSUE FRAGMENTS [1]

Author: Lis Santos Marques
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ABSTRACT

Despite advances in reproductive management of fish for breeding programs, little is known about the potential use of reproductive biotechnologies, such as cryopreservation of ovarian tissue. Cryopreservation of ovarian tissue in fish aims to preserve female germplasm of wild and/or endangered species, as well as fish of high zootechnical value and important models of biomedical research. In a previous study conducted by our research group, we obtained a high percentage of viability of immature oocytes after vitrification of fragments of ovarian tissue. This study showed that immature fish oocytes are more likely to survive after vitrification than oocytes at more advanced stages of maturation. However, to date, no study has compared which cryopreservation technique, vitrification or slow freezing, presents better results in the cryopreservation of ovarian tissue fragments of teleosteal fish. Thus, in the first study using the zebrafish (*Danio rerio*) biological model, the main objective was to compare the effects of two protocols of cryopreservation, vitrification and slow freezing, on the viability of ovarian tissue through the evaluation of parameters of oxidative stress, damage to DNA, mitochondrial activity and morphological integrity of immature oocytes. The results showed that slow freezing presented higher levels of oxidative stress and lower mitochondrial activity than vitrified ovarian tissue. In addition, ultrastructural observation of frozen oocytes showed rupture of the plasma membrane, loss of intracellular content and a large number of damaged mitochondria, while vitrified oocytes had intact mitochondria and plasma cell membranes. Therefore, vitrification was used in the second study that had the objective to verify if the biotechnique used in zebrafish is applicable in a species of economic importance. The high viability of oocytes obtained after vitrification of ovarian tissue containing immature pacu (*Piaractus mesopotamicus*) follicles suggests that the protocol applied can be successfully used in other species of teleosteos fish.

Keywords: vitrification, slow freezing, teleosts, oocytes.

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LISTA DE ABREVIATURAS

%	percentagem
±	mais ou menos
=	igual a
<	menor que
>	maior que
μg	micrograma(s)
μm	micrômetro(s)
µL	microlitro(s)
ºC	graus célsius
ATP	adenosina trifosfato
CIV	cultivo *in vitro*
DAF	diacetato de fluoresceína
DNA	ácido desoxirribonucleico
E2	17β estradiol
ERO	espécies reativas de oxigênio
ES	equilibrium solution
FDA	fluorescein diacetate
g	grama(s)
h	hora
HE	hematoxilina-eosina
IVC	*in vitro* culture (cultivo *in vitro*)

IP	iodeto de propídio
M	molar
Me_2SO	dimetilsulfóxido
mg	miligrama(s)
min	minuto(s)
mL	mililitro(s)
mm	milímetro(s)
OW	ovarian wall (parede ovariana)
P	probabilidade de erro
PBS	phosphate buffered saline (tampão fosfato-salino)
PG	primary growth (crescimento primário)
pH	potencial hidrogeniônico
PI	propidium iodide
POF	postovulatory follicle (complexo folicular pós-ovulatório)
s	segundo(s)
VS	vitrification solutions
Vtg1	oócito vitelogênico primário
Vtg2	oócito vitelogênico secundário
Vtg3	oócito vitelogênico terciário

CAPÍTULO I

1. INTRODUÇÃO GERAL

A criopreservação é um importante método de preservação do material genético que contribui diretamente nos programas de reprodução. Além disso, essa biotecnologia permite a preservação da diversidade de espécies ameaçadas de extinção e de modelos de peixes utilizados na pesquisa biomédica. A criopreservação do material genético inclui armazenamento em baixas temperaturas de sêmen, oócitos, embriões, blastômeros e tecido gonadal.

Embora a criopreservação de espermatozoides de peixes seja eficiente e criobancos de sêmen já estejam operando em vários países (Rahman et al., 2013), o mesmo ainda não ocorre com oócitos. Em zebrafish, uma baixa viabilidade celular tem sido relatada após a criopreservação de oócitos em estágios avançados de maturação (<50%; Guan et al., 2008, Zampolla et al., 2008) quando comparada à oócitos imaturos (>50%; Tsai et al., 2009). Possivelmente, as principais razões para esses resultados são o alto teor de lipídios nos oócitos em maturação e a baixa permeabilidade de membrana, limitando a desidratação celular e passagem do crioprotetor. Durante a maturação, os oócitos de peixes de água doce tornam-se menos permeáveis, adquirindo resistência ao ambiente hipotônico antes de serem liberados na água. Nosso grupo de pesquisa, em um estudo prévio com vitrificação de oócitos de zebrafish em diferentes estágios de maturação (Marques et al., 2015), mostrou que os oócitos imaturos inclusos no tecido ovariano (*in situ*) foram mais resistentes ao processo de criopreservação do que os oócitos em maturação e maduros. Os oócitos imaturos *in situ* possuem características que os tornam mais tolerantes à criopreservação, tais como o menor tamanho do oócito, baixa taxa metabólica, número restrito de células de suporte e pequena quantidade de lipídios intracitoplasmáticos sensíveis às baixas temperaturas.

Um dos principais danos ocasionados durante a criopreservação de oócitos é a formação de gelo intracelular. Para contornar este obstáculo, a técnica de vitrificação tem sido desenvolvida, pois na presença de soluções crioprotetoras altamente concentradas, permite a redução brusca de temperatura proporcionando a passagem do líquido para um estado vítreo amorfo, evitando a formação de cristais de gelo. A técnica de vitrificação tem mostrado resultados promissores na preservação de oócitos mamíferos (Vajta & Kuwayama, 2006), entretanto, a vitrificação de oócitos de peixes encontra-se em fase experimental, longe de aplicações práticas em aquicultura.

Assim, a hipótese do presente estudo é que existe diferença significativa na viabilidade do tecido ovariano de peixes contendo oócitos imaturos entre as técnicas de vitrificação e de congelamento lento após aquecimento.

2. REVISÃO BIBLIOGRÁFICA

2.1. Zebrafish (*Danio rerio*) como modelo biológico

As técnicas atuais de engenharia genética e biologia molecular permitiram identificar mecanismos moleculares e celulares semelhantes entre as espécies de vertebrados, tornando possíveis estudos comparativos entre zebrafish (Figura 1) e humanos, por exemplo. A maior classe de vertebrados são os Osteichthyes (do grego osteos = osso, e ichthyos = peixe), os chamados peixes ósseos, os quais oferecem versatilidade quase ilimitada para a pesquisa. Entre as principais características dos peixes modelo estão o pequeno tamanho, tempo de geração rápido, alta fecundidade, facilidade de criação e manejo. Atualmente, os modelos de peixes mais utilizados são o zebrafish (*Danio rerio,* Driever et al., 1994, Kari et al., 2007), medaka japonês (*Oryzias latipes*, Wittbrodt et al., 2002), vivíparos do gênero *Xiphophorus* (Walter & Kazianis, 2001), e *Fundulus heteroclitus* (Burnett et al., 2007). O zebrafish, o medaka e o gênero *Xiphophorus* são peixes de aquário, entretanto, eles apresentam algumas diferenças. O zebrafish e o *Xiphophorus* são espécies de água doce estrita (Hawkins et al., 2001), enquanto o medaka é anfídromo, ou seja, passa da água salgada para a doce em algum momento de sua vida (Inoue & Takei, 2002). Além disso, essas espécies apresentam duas diferentes formas de reprodução, fecundação externa (zebrafish e medaka) e fecundação interna (*Xiphophorus*).

Em 2013, Howe et al. (2013) publicaram na revista científica internacional *Nature Research* a sequência do genoma do zebrafish, revelando que esse peixe e os humanos apresentam 12.719 genes em comum, ou seja, 70% dos genes humanos são encontrados no zebrafish. Assim, quando genes causadores de doenças em humanos são injetados em embriões de zebrafish, os peixes em crescimento acabam por adquirir a mesma doença. Por essa razão, a espécie tem sido amplamente utilizada em estudos visando à cura de doenças genéticas, tais como depressão, esquizofrenia e mal de Parkinson. Além de outras tantas vantagens de sua morfologia e fisiologia semelhantes ao do humano, tais como fibras musculares, sistema nervoso central, sistema esquelético complexo, células hematopoiéticas múltiplas e sistema cardiovascular (Lieschke & Currie, 2007). Assim, o zebrafish possui grande importância na pesquisa mundial, resultando na criação de um site especializado nessa espécie, o The Zebrafish Information Network (ZFIN, http://zfin.org), no qual são depositadas todas as informações conhecidas relacionadas ao zebrafish.

Com extensos estudos utilizando esses modelos de peixes, dezenas de milhares de linhagens específicas foram criadas e atualmente são armazenadas ao redor do mundo como animais vivos em centros de recursos, como o Zebrafish International Resource Center (Universidade de Oregon, Eugene, OR), que detém cerca de 1.080 linhagens endogâmicas, transgênicas, knockout e mutantes; a University of Georgia (Athens, GA), que possui várias linhas de medaka endogâmicas e transgênicas; e o *Xiphophorus* Genetic Stock

Center (Texas State University, San Marcos, TX), o qual possui 61 linhagens de 24 espécies (Yang & Tiersch, 2009). Assim, a preservação do material genético destes e de outras espécies de peixes apresenta desafios significativos e urgentes. A criopreservação de gametas, embriões e gônadas é uma abordagem útil para enfrentar esses desafios.

Figura 1. Macho (acima) e fêmea (abaixo) de zebrafish (*Danio rerio*).

2.2. Criopreservação de gametas, embriões e tecido ovariano de zebrafish

A criopreservação não tem sido bem sucedida para oócitos e embriões de peixes devido as seguintes características dessas células: grande tamanho, alto teor lipídico intracitoplasmático (Blesbios & Labbe, 2003) e impermeabilidade à membrana (Hagedorn et al., 1998). Por outro lado, a criopreservação de espermatozoides em peixes foi relatada pela primeira vez há mais de 50 anos (Blaxter, 1953), e, desde então, protocolos de criopreservação de espermatozoides foram desenvolvidos em mais de 200 espécies de peixes, das quais aproximadamente 40 são espécies marinhas (Tiersch & Mazik, 2000; Gwo et al., 2011). No entanto, a maior parte da pesquisa tem se concentrado em espécies de grande porte, tais como salmonídeos e carpas, e poucos estudos abordaram peixes de aquário (Tiersch, 2001). Os fatores que dificultam estudos de criopreservação de espermatozoides de peixes de aquários, como o zebrafish, são o tamanho pequeno do animal e a disponibilidade limitada de sêmen, em que geralmente se obtem 3,3 µL de sêmen em uma coleta (Draper & Moens, 2009). Consequentemente, a criopreservação de espermatozoides nessas espécies apresenta desafios, como o desenho experimental, a coleta de gametas e a fecundação artificial, especialmente utilizando peixes vivos.

O primeiro estudo, com o uso de congelamento lento ou vitrificação, na criopreservação de zebrafish foi publicado por Zhang e colaboradores no ano de 1996 (Tabela 1). Os resultados deste estudo demonstraram que, embora o córion dos embriões fosse permeável à água e aos crioprotetores, a permeabilidade da membrana vitelina ao crioprotetor era baixa. Os autores observaram que os embriões se tornaram temporariamente opacos durante o aquecimento, indicando formação de gelo intracelular. Dois outros estudos posteriores, buscando desenvolver protocolos de criopreservação de embriões de zebrafish, também relataram não haver sobrevivência celular após a técnica de vitrificação (Janik et al., 2000; Robles et al., 2004). Por outro lado, Martínez-

Páramo et al. (2009) relataram que a incorporação de proteínas anticongelantes (AFP) aumentou a viabilidade de blastômeros congelados e embriões vitirificados. Outro estudo com células germinativas primordiais criopreservadas (CPG) de zebrafish relatou que a remoção parcial do vitelo de embriões antes da vitrificação melhorou as taxas de sobrevivência (67%) de CPG recuperadas de embriões vitrificados com etilenoglicol (Higaki et al., 2013). No entanto, até o momento nenhum estudo conseguiu um protocolo bem sucedido de criopreservação de embriões de peixes, incluindo o zebrafish. As principais barreiras apontadas como entraves para o sucesso na criopreservação de embriões de peixes são: (1) o tamanho grande do ovo; (2) elevado conteúdo do vitelo, aumentando as chances de rompimento durante a congelação; (3) a baixa permeabilidade das membranas que envolvem o embrião; (4) e a alta sensibilidade ao resfriamento.

Os primeiros estudos com congelamento de oócitos ovarianos de zebrafish foram publicados em 2008, em que os autores registraram entre 14,9 e 29,5% de viabilidade por meio da avaliação com azul de trypan (Guan et al., 2008; Zhang et al., 2008). Tsai et al. (2009) relatam que a criopreservação de oócitos ovarianos pode oferecer vantagens quando comparada aos embriões de peixes, principalmente devido à ausência de um córion completamente formado. Além disso, os oócitos ovarianos em estádio inicial apresentam um menor tamanho, o que resulta em uma maior relação superfície/volume e, portanto, podem ser mais permeáveis à água e solutos, aumentando as chances de sobrevivência durante a criopreservação. Após esses estudos outros pesquisadores começaram a desenvolver protocolos de criopreservação de oócitos ovarianos de zebrafish. Sendo que a maioria dos trabalhos utilizou o método de congelamento lento (Guan et al., 2008; Zhang et al., 2008; Tsai et al., 2009; Tsai et al., 2010; Zampolla et al., 2012). O único estudo que investigou a eficiência da vitrificação na criopreservação de oócitos ovarianos isolados de zebrafish relatou uma alta perda da viabilidade folicular após o aquecimento (Guan et al., 2010), no entanto, apenas oócitos em estágio vitelogênico (em maturação) foram utilizados no experimento. Além disso, esses autores utilizaram apenas a técnica de coloração com azul de trypan para avaliação da viabilidade celular.

A criopreservação de oócitos *in situ*, ou seja, a criopreservação de tecido ovariano foi proposta pela primeira vez em zebrafish por Zampolla et al. (2011). Neste estudo foi utilizada a técnica de congelamento lento, em que a criopreservação causou danos às proteínas do citoesqueleto, bem como na atividade mitocondrial e distribuição de mitocôndrias. Esses autores sugeriram, após avaliação da viabilidade utilizando azul de trypan e sondas fluorescentes, que os fragmentos ovarianos contendo oócitos nos estádios I e II (imaturos) eram menos sensíveis à criopreservação do que o oócito ovariano de estádio III (vitelogênico). A eficiência da técnica de vitrificação na criopreservação de tecido ovariano de peixes foi investigada em 2013, em que os autores relataram alta perda da viabilidade folicular após o aquecimento (Godoy et al., 2013). No entanto, o estudo utilizou somente fragmentos de tecido ovariano contendo oócitos em estádio III (vitelogênico). Recentemente, nosso grupo, após vitrificação de fragmentos de tecido ovariano, obteve um elevado

percentual de viabilidade de oócitos imaturos (76% nos estádios I e 43% nos estádios II), comprovada por análise com sondas fluorescentes iodeto de propidio e diacetato de fluoresceína (Marques et al., 2015). Esse estudo mostrou que oócitos imaturos de peixes têm maiores chances de sobrevivência após a vitrificação do que oócitos em estádios mais avançados de maturação (III, IV e V). No entanto, até o momento nenhum estudo comparou qual técnica de criopreservação, vitrificação ou congelamento lento, apresenta melhores resultados na criopreservação de fragmentos de tecido ovariano de peixes (Tabela 1).

Tabela 1. Estudos em congelamento e/ou vitrificação de gametas, embriões e tecido ovariano de zebrafish (*Danio rerio*).

Estudo	Amostra	Método	Solução de criopreservação	Principais considerações
Zhang & Rawson (1996)	Embrião	Vitrificação	DMSO[1] (2M) + PD[2] (3M) + PG[3] (0,5M); MET[4] (2M) + PD[2] (5M) + PG[3] (0,15M); BD[5] (2M) + PD[2] (3M) + PG[3] (0,15M)	Não houve sobrevivência embrionária após vitrificação.
Janik et al. (2000)	Embrião	Vitrificação	PG[3] (2, 3, 4, 6 e 8M)	Dano mitocondrial, ribossomos desorganizados e ruptura da membrana plasmática do saco vitelino; Sem sobrevivência dos embriões.
Robles et al. (2004)	Embrião	Vitrificação	DMSO[1] (5M) + EG[6] (1M) + MET[4] (2M) + SAC[16]	Menor atividade das enzimas LDH e G6PDH após vitrificação. Não houve sobrevivência dos embriões
Yang et al. (2007)	Sêmen	Congelamento	DMA[7] (8%); DMSO[1] (8%); MET[4] (4 e 8%)	Metanol (8%) proporcionou melhores resultados na motilidade e fertilidade do sêmen criopreservado.
Guan et al. (2008)	Oócito ovariano	Congelamento	MET[4] (4M) + KCL[9]; MET[4] (4M) + L-15[10]	Maior viabilidade (coloração com AT[22]) em KCL; Viabilidade de 29,5% após 2 horas de incubação a 22°C.
Zhang et al. (2008)	Oócito ovariano	Congelamento	DMSO[1] (2M) + SH[20]; MET[4] (2M) + SH[20]	Menor atividade das catepsinas após criopreservação. A viabilidade (coloração com AT[22]) foi de 14,9 ± 2,6% (DMSO) e 1,4 ± 0,8% (MET)
Tsai et al. (2009)	Oócito ovariano	Congelamento	MET[4] (4M) + L-15[10] ou KCL[9]; DMSO[1] (3M) + L-15[10] ou KCL[9]	L-15 apresentou melhores resultados que KCL; O MET foi mais eficaz que o DMSO; Oócitos ovarianos do estágio iniciais (I e II) são menos sensíveis ao congelamento. Os resultados mostraram que a coloração diacetato de fluorescencia + iodeto de

				propídio, e a relação de ADP/ATP são mais sensíveis do que a coloração com AT[22].
Martínez-Páramo et al. (2009)	Embrião; Blastômero	Congelamento e vitrificação	Embrião: DMSO[1] (5M) + MET[4] (2M) + EG[6] (1M) + SAC[16]; Blastômeros: SFB[19] (10%); DMSO[1] (2M) + SFB[19] (10%); DMSO[1] (2M) + SFB[19] (10%) + AFP[11] (10mg/ml)	A adição de proteínas anti-congelantes (AFP) aumentou a viabilidade de blastômeros congelados e embriões vitirificados.
Draper & Moens (2009)	Sêmen	Congelamento	MET[4] (1mL) + leite em pó desnatado (1,5g) + GPR[12]	Taxas de fecundação de 25 a 40% após descongelamento
Guan et al. (2010)	Oócito ovariano	Vitrificação	DMSO[1] (3M) + MET[4] (2M) + PG[3] (2M); DMSO[1] (2,5M) + MET[4] (1M) + PG[3] (0,75M)	A viabilidade (coloração com AT[22]) foi elevada após o aquecimento em KCL, no entanto, no entanto, oócitos inchados e translúcidos indicaram danos causados pela vitrificação.
Zampolla et al. (2011)	Tecido ovariano	Congelamento	MET[4] (1 e 4M)	MET (1M) apresentou maior viabilidade (coloração com AT[22] e coloração de diacetato de fluorescencia + iodeto de propídio), após criopreservação.
Zampolla et al. (2012)	Oócito ovariano	Congelamento	MET[4] (1M) ou MET[4] (2M) ou MET[4] (4M) + SFB[19] (10%)	A adição de 10% de SFB[19] aos meios utilizados no congelamento de oócitos ováricos não permitiu uma criopreservação bem sucedida.
Higaki et al. (2013)	Embrião/ Célula Germinativa Primordial	Vitrificação	EG[6] (5M); DMSO[1] (5M); PG[3] (5M); EG[6] (3M) + DMSO[1] (2M); EG[6] (3M) + PG[3] (2M); DMSO[1] (3M) + EG[6] (2M); DMSO[1] (3M) + PG[3] (2M); PG[3] (3M) + DMSO[1] (2M); PG[3] (3M) + EG[6] (2M)	A remoção parcial do vitelo e utilizando solução com EG (3M) + DMSO (2M) melhorou a viabilidade (coloração com AT[22]) e eficiência na fecundação por transplante de CGP[21] criopreservadas, em cerca de 3% (7 quimeras férteis / 236 embriões receptores)

Godoy et al. (2013)	Tecido ovariano	Vitrificação	MET[4] (8; 8,5; 9; 9,5; 10; 10,5 e 11M); ET[15] (8; 8,5; 9; 9,5; 10; 10,5 e 11M); DMSO[1] (3; 3,5; 4; 4,5; 5; 5,5 e 6M); PG[3] (2,5; 3; 3,5; 4; 4,5; 5 e 5,5M); EG[6] (4; 4,5; 5; 5,5; 6; 6,5 e 7M) + SAC[16] ou GLI[17]	A integridade mitocondrial das células da camada da granulosa foi danificada pela vitrificação e o nível de ATP nos oócitos ovarianos diminuiu após o aquecimento.
Bai et al. (2013)	Sêmen	Congelamento	DMSO[1] (8%); MET[4] (4%)	Melhores resultados com o uso da solução contendo DMSO (8%).
Marques et al. (2015)	Tecido ovariano	Vitrificação	MET[4] (1,5M) + PG[3] (4,5M); MET[4] (1,5M) + DMSO[1] (5,5M); MET[4] (1,5M) + PG[3] (4,5M) + SAC[16] (0,5M); MET[4] (1,5M) + DMSO[1] (5,5M) + SAC[16] (0,5M)	Oócitos imaturos apresentaram maior viabilidade celular que oócitos em maturação e maduros (coloração AT e diacetato de fluorescencia + iodeto de propídio). Sobrevivência de oócitos imaturos foi observada após 24 h de cultivo *in vitro*.
Wang et al. (2015)	Sêmen	Congelamento	DMSO[1] (8%)	A exposição aguda ao frio (18°C por 24 h) antes da criopreservação de espermatozoides é benéfica para a sobrevivência espermática pós-descongelamento.
Yang et al. (2016)	Sêmen	Congelamento	MET[4] (8%)	A fertilidade dos espermatozoides pós-descongelamento em 4 h (62% ± 14%) e 24 h (73% ± 21%) em uma população de zebrafish de pesquisa bem caracterizada foi significativamente maior do que em 4 h (27% ± 15%) e 24 h (32% ± 18%) em uma população de zebrafish cultivada em fazenda.
Matthews et al. (2018)	Sêmen	Congelamento	MET[4] (6,67%)	A motilidade obtida foi de 20% ± 13% e uma taxa de fertilização pós-descongelamento de 68% ± 16%.
Marinović	Testículos	Vitrificação	Solução de equilíbrio: MET[4] (1,5M) e PG[3] (1,5 M); Solução	Viabilidade acima de 50% em cinco diferentes cepas

et al. (2018)	inteiros	de vitrificação: DMSO[1] (3 M) e PG[3] (3 M).	de zebrafish.

1: Dimetilsulfóxido (C_2H_6OS); 2: Propano-1,3-diol ($C_3H_8O_2$); 3: Propilenoglicol ($C_3H_8O_2$); 4: Metanol (CH_4O); 5: Butanodiol ($C_4H_{10}O_2$); 6: Etilenoglicol ($C_2H_4(OH)_2$); 7: Dimetilacetamida (C_4H_9NO); 8: Glicerol ($C_3H_8O_3$); 9: Cloreto de potássio (KCL); 10: Solução L-15 (Leibovitz); 11: Proteína anti-congelante; 12: Solução Ginsberg Fish Ringers (Ginsburg, 1963); 13: Butilenoglicol; 14: Polivinilpirrolidona $(C_6H_9NO)_n$; 15: Etanol (C_2H_6O); 16: Sacarose ($C_{12}H_{22}O_{11}$); 17: Glicose $C_6H_{12}O_6$; 18: Trealose $C_{12}H_{22}O_{11}$ (anidrido); 19: Soro fetal bovino; 20: Solução de Hanks; 21: Célula Germinativa Primordial; 22: Azul de Trypan; 23: Adenosina trifosfato.

2.3. Princípios básicos da criopreservação

A criopreservação é definida como a preservação da viabilidade do material biológico em temperaturas extremamente baixas, abaixo de -80°C ou de -140°C (Baust et al., 2009). Em nitrogênio líquido, a uma temperatura de -196°C, todas as reações químicas, processos biológicos e atividades físicas intra e extracelular são suspensas. Assim, teoricamente, nessas condições, células e tecidos podem ser armazenados por um período de tempo indeterminado. Os dois métodos de criopreservação mais utilizados para a preservação do germoplasma animal são o congelamento lento e a vitrificação. Esses métodos são bastante diferentes, no entanto, referem-se às mesmas relações físico-químicas.

2.3.1. Aspectos físico-químicos da formação de cristais de gelo

Danos celulares ocorrem quando a temperatura reduz de 37°C para -196°C. Isto se deve principalmente a perda de 95% de água intracelular, ao aumento considerável da concentração de eletrólitos nos meios intra e extracelulares e a possível formação de cristais de gelo nos espaços intracelulares que deformam e comprimem as células, podendo destruir as estruturas intracelulares (Mazur, 1984). Nas células, a água em condições isotônicas torna-se termodinamicamente instável á temperaturas inferiores a 0°C e tende a cristalizar (Mazur, 1963; Mazur, 1984). A água ocupa uma proporção considerável das células e tecidos viáveis, assim a preocupação central durante a criopreservação é a transição de fase do estado líquido para o estado sólido. Tanto o congelamento lento quanto a vitrificação baseiam-se nos mesmos princípios de proteger as células dos danos devido à formação de gelo intracelular, da desidratação celular excessiva e das alterações nas concentrações de solutos. As soluções líquidas quando submetidas a temperaturas abaixo do seu ponto de fusão tendem para um estado sólido (Korber, 1988). A maioria dos líquidos congela por meio da formação de um sólido cristalino a partir do líquido uniforme. Este processo de solidificação corresponde ao fenômeno de cristalização. Durante esse processo, a porção da solução ainda em estado líquido é excluída da matriz cristalina e torna-se cada vez mais concentrada. Esse aumento da concentração diminui o ponto de fusão da solução restante. Quando a porção líquida não é mais super-resfriada, a cristalização cessa, uma vez que o estado cristalino (sólido) está em equilíbrio termodinâmico com a solução líquida (Karlsson & Toner, 1996). A quantidade de cristais de gelo que se forma a uma determinada temperatura depende da composição inicial da solução e pode ser previsto por meio de fórmulas matemáticas (Hossain et al., 2014).

A formação de gelo também depende de fenômenos cinéticos. Uma solução super-resfriada e termodinamicamente estável pode iniciar um processo de agregação de moléculas de água, em que os cristais de gelo se propagam por toda a solução. Este é o processo chamado de nucleação (Turnbull & Fisher, 1949). Como a temperatura cai progressivamente, a condição denominada “super-resfriada” surge. A formação de gelo em soluções super-resfriadas inicia pelo processo de nucleação, em que ocorre a agregação

de moléculas de água em grupos (clusters) termodinamicamente estáveis onde os cristais de gelo podem crescer (Hossain et al., 2014). Assim, a taxa de cristalização depende tanto da cinética de nucleação, quanto da cinética do subsequente crescimento do cristal de gelo. O aumento da viscosidade da solução bem como o declínio da temperatura pode contribuir para reduzir a taxa de cristalização. Uma viscosidade suficientemente elevada inibe a difusão molecular e bloqueia a transição de um estado líquido para um sólido cristalino. A solução tenderá para um estado meta-estável, não cristalino, amorfo. Assim, em um resfriamento rápido, o limiar de fusão da solução é atingido antes da cristalização iniciar. Mesmo se a cristalização ocorrer, a quantidade de cristais de gelo é limitada e é inferior a 10^{-6} (Uhlmann, 1972). Esse processo de solidificar sem cristalização por meio do aumento da viscosidade da solução e utilizando altas velocidades de resfriamento é a chamada "vitrificação". Portanto, o aumento da concentração de solutos na solução irá aumentar a viscosidade da solução, consequentemente, reduzindo as taxas de nucleação e de crescimento de cristais de gelo.

2.3.2. Efeitos biofísicos da formação de cristais de gelo

A primeira consequência da formação de cristais de gelo no espaço extracelular é o aumento da concentração iônica do líquido extracelular. A pressão osmótica aumentada no espaço extracelular cria um gradiente osmótico com efluxo de água através da membrana plasmática (Mazur, 1963, Mazur et al., 1984). Os fatores que influenciam a velocidade do efluxo de água são a taxa de resfriamento, a permeabilidade inerente da célula e a relação superfície-volume celular (Mazur et al., 1984). A desidratação pode causar um aumento na concentração de sais no meio extracelular, o qual modifica e desnatura as proteínas e lipoproteínas que compreendem a maior parte das membranas celulares. Além disso, a desidratação pode levar a cristalização de sais tampões que resulta em importantes variações de pH com a conseqüente desnaturação irreversível de certas proteínas (Karow & Webb, 1965).

A formação de gelo extracelular produz uma tensão mecânica que deforma a célula. A matriz congelada-concentrada, ou também chamada de matriz cristalina, seqüestra as células dos canais que ainda contêm líquido residual. O volume ocupado pelo líquido é em função do volume da célula. À medida que a temperatura reduz, a água é convertida em gelo, consequentemente, o diâmetro dos canais diminui, deformando progressivamente as células (Mazur et al., 1981; Mazur & Cole, 1985).

A formação de gelo extracelular, além de causar estresse mecânico e químico, também está envolvida na cristalização intracelular (Mazur, 1965). O fluxo de água através da membrana celular durante congelamento/ descongelamento pode induzir formação de gelo intracelular causando lesões (Muldrew & McGann, 1994). Quando as células são resfriadas lentamente, o fluxo de água para o meio extracelular é aumentado, o que limita a cristalização intracelular. A desidratação celular domina o processo de cristalização intracelular. Em contraste, no resfriamento rápido, a difusão de água é relativamente baixa e predomina a cristalização intracelular.

Uma mudança brusca de temperatura durante o resfriamento pode causar lesões nas membranas celulares, mesmo na ausência de formação de cristais de gelo (Morris & Farrant, 1973; Farrant & Morris,1973). O denominado choque térmico ocorre entre 37°C e 15°C, e entre 0°C e -80°C. As lesões de membrana causadas por choque térmico estão relacionadas ao aumento da força iônica, resultante das alterações na composição aniônica do meio extracelular, especialmente de ânions acetato, cloreto, nitrato, iodo e sulfato. O choque térmico pode ser reduzido pela adição de agentes crioprotetores e fosfolípidos específicos (fosfatidilserina) combinados com um resfriamento controlado e relativamente lento.

2.3.3. Efeitos biológicos da formação de cristais de gelo

Mazur et al. (1972) propuseram uma hipótese para explicar a ligação entre a velocidade de congelamento e seu efeito sobre as células. De acordo com essa hipótese, em baixas velocidades de resfriamento, as lesões celulares são causadas em parte pela exposição a solutos celulares altamente concentrados, o que é intensificado pela desidratação celular, o chamado "efeito de solução". As interações mecânicas entre células e cristais de gelo extracelulares também causam danos celulares em resfriamentos lentos. Em altas velocidades de resfriamento, os danos celulares estão relacionados à formação de cristais de gelo intracelulares. As lesões celulares de origem mecânica ocorrem na membrana plasmática ou nas membranas das organelas intracelulares. Os cristais intracelulares podem ser pequenos e relativamente instáveis. Assim, durante o aquecimento lento, eles podem se agregar formando cristais maiores, os quais são prejudiciais. Esse é o fenômeno denominado "nucleação".

Portanto, a velocidade de resfriamento ótima deve ser adaptada a fim de permitir uma desidratação suficiente da célula e evitar a cristalização intracelular antecipada.

Outros efeitos prejudiciais de natureza não mecânica são a indução de formação de bolhas de gás no espaço intracelular (Morris & McGrath, 1981) e os efeitos osmóticos relacionados à cristalização durante o aquecimento (Muldrew & McGann, 1994). No entanto, os cristais de gelo intracelulares podem manter-se inofensivos caso sua extensão seja controlada (Mazur, 1977; Farrant, 1977; Rall et al., 1980).

2.3.4. Protocolos de criopreservação

Os protocolos de criopreservação podem ser classificados em dois grandes grupos como "protocolos de equilíbrio" ou lentos e "protocolos sem-equilíbrio" ou super-rápidos (vitrificação), conforme a curva de resfriamento e agentes crioprotetores utilizados (Rosato & Iaffaldano, 2013). Ambos os protocolos se baseiam nos mesmos princípios de proteger as células dos danos devido à formação de gelo intracelular, da desidratação celular excessiva e das alterações nas concentrações de solutos. O congelamento lento requer um procedimento controlado e gradual de temperatura,

geralmente, por meio da utilização de congeladores programáveis, entretanto, ocorre o risco de cristalização que está correlacionada com danos de membrana. A vitrificação, em contraste, na presença de soluções crioprotetoras altamente concentradas, permite a redução brusca de temperatura proporcionando a passagem do líquido para um estado vítreo amorfo, evitando a formação de cristais de gelo (Vajta & Kuwayama, 2006). No entanto, a ausência de cristalização, não exclui a possibilidade da ocorrência de danos celulares, como por exemplo, de origem tóxica ou osmótica. As lesões tóxicas são comuns no processo de vitrificação, devido à necessidade do uso de altas concentrações de soluções crioprotetoras, porém o grau de toxicidade vai depender das propriedades dos crioprotetores, somadas ao tempo e à temperatura de exposição (Fahy et al., 2004). O método de vitrificação, além de ser um procedimento extremamente rápido, apresenta a vantagem do custo reduzido, já que não requer a utilização de um equipamento programável de congelação (Yavin et al., 2009).

2.3.5. Agentes crioprotetores

Os agentes crioprotetores são compostos químicos naturais ou sintéticos utilizados a fim de proteger as células dos danos causados durante o congelamento e descongelamento. Entre as funções dos crioprotetores, a principal é remover e/ou substituir o líquido intracelular. As características ideais descritas por Fahy (2010) para um agente crioprotetor foram baixo peso molecular, baixa toxicidade e alta capacidade de atravessar a membrana celular.

Os crioprotetores permeáveis são substâncias orgânicas bastante solúveis e de baixo peso molecular capazes de penetrar nas células e conduzir sua ação protetora por todo o citoplasma e organelas (Hubálek, 2003). Entre os crioprotetores permeáveis mais utilizados estão o dimetilsulfóxido (Me_2SO) e o propilenoglicol, os quais possuem alta solubilidade na água e penetram rapidamente nas células (Merino et al., 2011). O Me_2SO tem peso molecular 78 e é rapidamente permeável através das membranas (Hubálek, 2003). O propilenoglicol tem peso molecular 76 e é caracterizado por uma baixa toxicidade, sendo considerado atualmente como um composto não-genotóxico ou carcinogênico (Aye et al., 2010). O uso de álcoois monovalentes é pouco frequente, devido à sua alta toxicidade. No entanto, em um teste de exposição por 30 min, o metanol foi menos tóxico que o Me_2SO para embriões de zebrafish (Zhang et al., 1996). Zhang et al. (2005) sugeriram que o metanol, devido ao seu baixo peso molecular (32), tem uma alta taxa de permeabilidade, superando significativamente o Me_2SO.

De acordo com Fahy et al. (2004) os efeitos tóxicos dos crioprotetores são próprios de cada agente, e apresentam diferentes efeitos físico-químicos e osmóticos. Estratégias, como o uso de crioprotetores menos tóxicos e/ou a combinação de dois ou três, tem sido sugeridas nos procedimentos de criopreservação (Vajta & Nagy, 2006).

Os crioprotetores impermeáveis são açúcares, como por exemplo, a

sacarose e a trealose, ou proteínas, como as presentes no soro fetal bovino, que por serem moléculas com grandes dimensões, não conseguem penetrar no interior das células (Hovatta, 2005). Entretanto, interagem com os fosfolipídios na membrana celular conferindo uma maior estabilidade à membrana (Dong et al., 2009). Além disso, por serem compostos hidrofílicos, eles se ligam às moléculas de água, aumentando a viscosidade da solução e diminuindo a formação de cristais de gelo (Fahy, 2007). Os açúcares também podem reduzir a toxicidade química dos crioprotetores, pois o aumento da viscosidade permite que se reduza a concentração de crioprotetor e, portanto, a toxicidade (Best, 2015). A sacarose é o mais comumente utilizado como crioprotetor impermeável em várias espécies. No entanto, a trealose foi menos prejudicial que a sacarose na vitrificação de oócitos bovinos imaturos (Arav et al., 1993). Após congelamento lento de tecido ovariano de coelha, autores observaram 78% de viabilidade folicular utilizando 1,5 M de Me_2SO associado a 0,2 M de trealose (Neto et al., 2008). Além disso, os autores relataram uma morfologia bem preservada de folículos primordiais e primários. A combinação de 0,5 M de trealose com 0,5 M de Me_2SO microinjetada em oócitos murinos resultou em uma alta sobrevivência celular e em uma prole saudável (Eroglu et al., 2009).

A gema de ovo é formada por lipoproteínas, livetinas e fosvitinas, encontradas em duas frações facilmente separáveis pelo método de centrifugação: o grânulo e o plasma. Acredita-se que as lipoproteínas de baixa densidade, presentes no plasma, sejam as responsáveis por sua ação crioprotetora. Os efeitos da gema de ovo na estabilização de membrana já são bem conhecidos na criopreservação de espermatozoides, no entanto, apenas um estudo avaliou o efeito na vitrificação de embriões suínos (Fujino et al., 1993) e outro na vitrificação de oócitos imaturos de camundongos (Isachenko & Nayudu, 1999). Isachenko & Nayudu (1999) obtiveram 80% de sobrevivência dos oócitos imaturos após aquecimento e uma taxa de maturação de 84%. Outro estudo mostrou uma alta taxa de viabilidade de espermatogônias após congelamento lento (1°C/min) de testículos de truta arco-íris utilizando 1,3 M de Me_2SO associado a 0,1 M de Trealose e 10% gema de ovo (Lee et al., 2013). Recentemente, ovários inteiros de truta arco-íris foram congelados em solução crioprotetora contendo 1,0 M de Me_2SO, 0,1 M trealose e 10% de gema de ovo, e após aquecimento e transplante das células germinativas ovarianas para a cavidade peritoneal de receptores, os autores obtiveram gametas funcionais (Lee et al., 2016).

2.4. Avaliação da qualidade folicular após criopreservação

Sabe-se que a exposição aos crioprotetores, a redução de temperatura e o posterior aquecimento da amostra criopreservada ocasionam danos celulares estruturais e metabólicos (Kopeika et al., 2005; Vajta & Kuwayama, 2005; Gosden, 2011). Por outro lado, de acordo com Vajta & Nagy (2006), os oócitos e os embriões possuem capacidade de reparar parcial ou totalmente os danos, possibilitando a retomada do desenvolvimento.

A qualidade folicular após a criopreservação do tecido ovariano pode ser avaliada através de diferentes parâmetros tais como: viabilidade celular, por meio de marcadores de viabilidade folicular (Chambers et al., 2010); manutenção da morfologia dos folículos e do estroma ovariano, analisados por microscopia de luz e/ou microscopia eletrônica de transmissão (Sheikhi et al., 2011; Sheikhi et al., 2013); e análise de fragmentação de DNA, utilizando sistemas de detecção de apoptose (Merdassi et al., 2011).

A microscopia de luz permite a quantificação de determinada característica em comparação ao total, no entanto, o poder de resolução é baixo. Assim, por meio dessa técnica é possível identificar somente sinais avançados de atresia, como picnose nuclear, danos citoplasmáticos, destacamento das células da granulosa e danos na membrana. No entanto, essa técnica possibilita que um grande número de oócitos seja avaliado, sendo uma importante ferramenta da análise quantitativa. A microscopia eletrônica de transmissão (MET), por outro lado, é considerado um método qualitativo, pois tem um poder de resolução mil vezes superior ao da microscopia de luz, permitindo a visualização de alterações das organelas celulares e mudanças ultraestruturais, inclusive na membrana nuclear (Salehnia et al., 2002).

Sabe-se que a criopreservação pode acarretar ruptura da membrana celular, ocasionando a morte das células. Assim, corantes vitais como azul de Trypan (Zampolla et al., 2011) ou marcadores fluorescentes, como o diacetato de fluoresceína (DAF) e o iodeto de propídio (PI) (Tsai et al., 2009; Zampolla et al., 2009) têm sido usados para análise da viabilidade celular dos oócitos após isolamento do tecido ovariano.

Apesar de haver muitos estudos que mostram os danos da criopreservação na morfologia e viabilidade celular, ainda são poucos os estudos que investigam os efeitos que a criopreservação pode causar ao DNA. Um estudo em ratas mostrou que o processo de criopreservação de células da granulosa de tecido ovariano, ocasionou estresse oxidativo, fragmentação de DNA e apoptose (Zhang et al., 2013). A apoptose, morte celular programada, é ativada por mecanismos intrínsecos e extrínsecos, como danos ao DNA. Morfologicamente é caracterizada pela condensação da cromatina, fragmentação do DNA, perda de volume celular, formação de protuberâncias na membrana plasmática e de corpos celulares condensados (Hussein, 2005). Entre as técnicas disponíveis para avaliar a estrutura da cromatina e a integridade do DNA celular, está a técnica de túnel (desoxinucleotídeo transferase terminal, mediante a determinação de encaixes terminais) e o ensaio cometa (eletroforese em gel das células). O ensaio cometa permite uma avaliação da quantidade de fragmentação do DNA em células individuais. Sendo uma técnica rápida, simples, sensível e de baixo custo para mensurar e analisar as lesões além de detectar efeitos de reparo no DNA (Singh et al., 1988).

Tanto o resfriamento quanto o aquecimento aumentam a produção de espécies reativas de oxigênio (ERO) causando alterações no metabolismo oxidativo. O aumento das ERO está relacionado com o estresse osmótico

ocasionado pelas concentrações elevadas de crioprotetores e as alterações físico-químicas da célula durante os procedimentos de criopreservação (Tatone et al., 2010). A produção desequilibrada de ERO resulta em peroxidação de grande quantidade de lipídios da membrana plasmática acarretando perda de fluidez e alteração de permeabilidade (Li et al., 2010). Os ensaios antioxidantes disponíveis podem ser classificados em duas categorias: ensaios baseados em estudos de cinética química, também denominados de métodos diretos e ensaios mediados pela transferência de elétrons, também denominados de métodos indiretos (Huang et al., 2005). Um método amplamente utilizado para estimar a peroxidação lipídica é o ensaio de TBARS (produtos reativos ao ácido tiobarbitúrico) que é de fácil execução e de baixo custo. Esse é um método direto, pois se baseia na mensuração por espectrofotometria da concentração dos produtos oriundos da peroxidação dos lipídios (Sanocka & Kurpisz, 2004). De acordo com França et al. (2013), os métodos mais utilizados na mensuração indireta de ERO são os espectrofotométricos e cromatométricos, que medem a atividade enzimática (superóxido dismutase - SOD, catalase, glutationa peroxidase - GSH-Px e glutationa redutase - GSH- Rd) e/ou a concentração de tripeptídeos (glutationa reduzida - GSH) e aldeídos (malondialdeido - MDA). Um método indireto relativamente simples e muito utilizado é o ensaio FRAP (Ferric Reducing Antioxidant Power, Oyawoye et al., 2003; Talebi et al., 2012; Hatami et al., 2014; Zavareh et al., 2016), em que a atividade antioxidante é avaliada quanto ao poder de redução do Ferro (Benzie, et al., 1996). Este ensaio está baseado na capacidade dos fenóis em reduzir o Fe^{3+} em Fe^{2+}. Quando isto ocorre, na presença de 2,4,6-tripiridils-triazina (TPTZ), a redução é acompanhada pela formação de um complexo corado com o $Fe^{2+.}$ O ensaio é expresso em ácido ascórbico equivalente. Menor FRAP indica menor capacidade de ligação da ferritina ao ferro e, consequentemente, maior quantidade de ferro livre, capaz de catalisar a geração de radicais OH, por meio das reações de Fenton e Haber-Weiss (Welch et al., 2002).

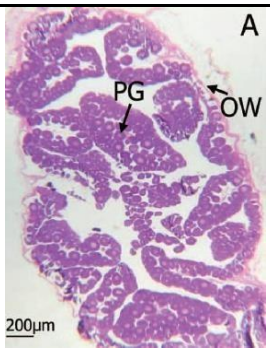
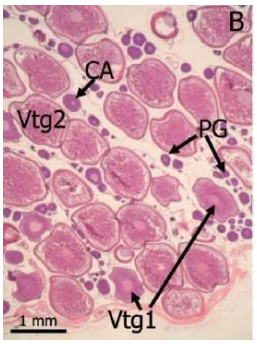
Zampolla et al. (2011) relatou que a criopreservação causou decréscimo do nível de ATP e da atividade mitocondrial de fragmentos de tecido ovariano contendo oócitos em estágios I, II e III. Entretanto, os autores sugerem que novos estudos sobre os níveis de ATP e atividade mitocondrial sejam realizados em oócitos ovarianos contendo isoladamente os diferentes estágios, a fim de avaliar qual estágio é menos afetado nesse parâmetro pela criopreservação. O teste do MTT (3-(4,5-dimetiltiazol-2yl)-2,5-difenil brometo de tetrazolina) é um teste amplamente usado para avaliar viabilidade celular (Van Meerloo et al., 2011). O sal de MTT é um composto hidrosolúvel, que em solução apresenta coloração amarelo-pálido, e é facilmente incorporado pelas células viáveis, que reduzem esse composto em suas mitocôndrias através da enzima desidrogenase succínica. Ao ser reduzido o MTT é convertido em cristais de formazan, um composto de coloração azul-escuro, não solúvel em água e que fica armazenado no citoplasma celular. Dessa forma, a redução do MTT a formazan, será diretamente proporcional à atividade mitocondrial e a viabilidade celular. Em um estudo avaliando a sensibilidade ao resfriamento relatou que a atividade da enzima desidrogenase succínica aumentou em oócitos zebrafish após a exposição a baixas temperaturas (Isayeva et al.,

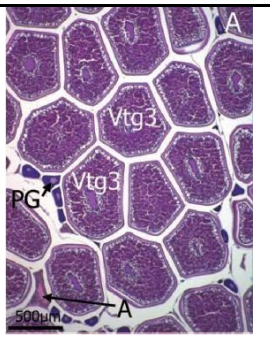
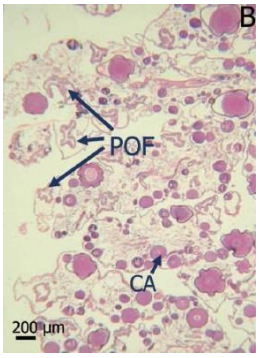
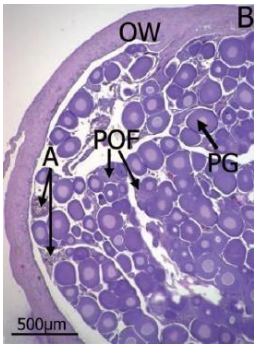
2004). Altos níveis dessa enzima, também, foram registrados após congelamento-descongelamento em oócitos murinos (Boerjan et al., 1991).

2.5. Fases reprodutivas em fêmeas de peixes teleósteos

O grupo teleósteo possui cerca de 27.000 espécies, e representa aproximadamente 50% do total de vertebrados e 96% do total de espécies de peixes identificados no planeta; sendo subdividido em 40 ordens, 448 famílias e 4.064 gêneros (Vazzoler, 1996). O desenvolvimento gonadal, na maioria dos teleósteos, é cíclico e sazonal. Ao longo de cada ciclo reprodutivo, ocorre a renovação das células germinativas, sua diferenciação, desenvolvimento, maturação e liberação que resultam em alterações gonadais que caracterizam diferentes fases reprodutivas. No presente estudo será utilizada a terminologia proposta por Brown-Peterson et al. (2011), pois, além de simples, é universal para as fases do ciclo reprodutivo e pode ser aplicada a todos os peixes teleósteos. De acordo com esses autores, quatro fases são reconhecidas ao longo do ciclo reprodutivo: Desenvolvimento; Apto à Desova; Regressão e Regeneração. A fase Imatura corresponde aos peixes que ainda não estão aptos para a reprodução, ou seja, são os peixes que ainda não entraram no ciclo reprodutivo. Apesar de a seqüência temporal de eventos durante o desenvolvimento de gametas em cada fase possa variar entre as espécies, cada fase possui marcadores histológicos e fisiológicos específicos (Tabela 2).

Tabela 2. Descrição geral das fases dos ciclos reprodutivos em fêmeas de peixes.

Fase	Características macroscópicas e microscópicas	Histologia ovariana ilustrando as fases
Imaturo: Nunca desovado	Ovários pequenos, muitas vezes claros, vasos sanguíneos não distinguíveis. Apenas oogônias e oócitos pré-vitelogênicos em crescimento primário presentes, sem atresia. Parede do ovário fina e pouco espaço entre os oócitos	
Desenvolvimento: Ovários em início ou em desenvolvimento, não prontos para desova.	Ovários em expansão, vasos sanguíneos tornando–se mais evidentes. Oócitos em crescimento primário; com alvéolos corticais e em início de vitelogênese presentes. Não evidência de folículos desovados ou oócitos completamente desenvolvidos. Alguns oócitos atrésicos podem estar presentes	

Apto à desova: Os peixes encontram-se aptos para desovar neste ciclo, tanto em termos de desenvolvimento como fisiológicos**.**	Ovários grandes, vasos sanguíneos evidentes. Oócitos individuais visíveis macroscopicamente. Presença de oócitos vitelogênicos finais/completamente desenvolvidos. Algumas atresias e folículos desovados tardios podem estar presentes.	
Regressão: Término da desova.	Ovários flácidos, vasos sanguíneos proeminentes. Folículos atrésicos e folículos pós-ovulatórios presentes. Oócitos com alvéolos corticais e/ou em vitelogênese podem estar presentes.	
Regeneração: Maduro sexualmente, reprodutivamente inativo**.**	Ovários pequenos, parede ovariana espessa, vasos sanguíneos reduzidos. Presença apenas de oogônias e oócitos em crescimento primário. Vasos sanguíneos dilatados, folículos atrésicos ou folículos pós-ovulatórios em degeneração podem estar presentes.	

Adaptado de Brown-Peterson et al. (2011). PG = oócito em crescimento primário; OW= parede ovariana; A = atresia; POF = complexo folicular pós- ovulatório; Vtg1 = oócito vitelogênico primário; Vtg2 = oócito vitelogênico secundário; Vtg3 = oócito vitelogênico terciário.

Na fase Imatura, a diferenciação gonadal, a proliferação e o crescimento de gametas são gonadotropinas independentes (oogônias e oócitos em estágio perinuclear). A fase Imatura é histologicamente semelhante em todos os teleósteos. Esta fase pode ser distinguida histologicamente pela presença de oogônias e de oócitos em estágio perinuclear (também chamados de oócitos em crescimento primário - CP). Além disso, há escasso tecido conjuntivo entre os folículos, pouco espaço entre os oócitos, e a parede ovariana é geralmente fina. Não há evidência de gotas lipídicas nos oócitos em CP ou feixes musculares em ovários imaturos. Raramente, atresia dos oócitos CP pode estar presente.

Os peixes entram no ciclo reprodutivo quando o crescimento gonadal e o desenvolvimento de gametas se tornam dependentes de gonadotropina. Um peixe que atingiu a maturidade sexual nunca sairá do ciclo

reprodutivo e nem retornará à fase imatura. Assim, a fase de Desenvolvimento indica a entrada no estádio dependente de gonadotropina da oogênese e resulta em crescimento gonadal. A fase de Desenvolvimento pode ser considerada uma fase de preparação de desova caracterizada pela produção de oócitos vitelogênicos. Os peixes entram nesta fase com o aparecimento de oócitos alvéolo cortical (CA), também chamados de oócitos em crescimento secundário, e pelo aparecimento tardio de oócitos vitelogênicos primários (Vtg1) e secundários (Vtg2). A vitelogênese é normalmente um processo longo durante o qual ocorrem mudanças importantes e visíveis dentro do oócito: o tamanho do oócito aumenta de forma notável, o vitelo se acumula progressivamente no citoplasma e várias inclusões citoplasmáticas aparecem (vacúolos, gotículas lipídicas). Os oócitos Vtg2 são o estágio mais avançado presente na fase de Desenvolvimento. Em espécies com desenvolvimento gonadal assíncrono, como o zebrafish, oócitos em vários estádios de desenvolvimento estão presentes no ovário durante a fase de Desenvolvimento. Por outro lado, espécies com desenvolvimento gonadal síncrono, como os reprodutores totais, tendem a ter oócitos em apenas um estágio de desenvolvimento, além de oócitos em CP.

A fase de Apto à Desova é caracterizada pelo aparecimento de oócitos vitelogênicos terciários (Vtg3). Peixes nesta fase são capazes de desovar durante o ciclo reprodutivo atual devido ao desenvolvimento de receptores para o hormônio indutor de maturação nos oócitos Vtg3. Os peixes submetidos a estágios iniciais de maturação oocitária (MO) também são considerados fase de Apto à Desova.

O fim do ciclo reprodutivo é indicado pela fase de Regressão que é caracterizada por atresia, presença de complexos foliculares pós-ovulatórios (POFs) e poucos (quando houver) oócitos Vtg2 ou Vtg3 saudáveis. Os peixes permanecem na fase de regressão por um tempo relativamente curto e depois passam para a fase de Regeneração. Durante a fase de Regeneração, os gametas sofrem proliferação mitótica independente de gonadotropina (oogônias) e crescimento oocitário (oócitos em CP) para o próximo ciclo reprodutivo. Os peixes nessa fase são sexualmente maduros, mas reprodutivamente inativos. As características da fase de regeneração em fêmeas incluem oócitos em CP, atresia tardia e uma parede ovariana mais espessa do que a observada em peixes imaturos.

2.6. Espécie nativa de importância comercial

2.6.1. Piaractus mesopotamicus

Piaractus mesopotamicus (HOLMBERG, 1887), popularmente conhecido como pacu (Figura 2), pertence à ordem dos Characiformes, família Characidae e subfamília Myleinae. A espécie é originária da bacia dos rios Paraná, Paraguai e Uruguai (Calcagnotto & Desalle, 2009). O pacu é um peixe que realiza migração reprodutiva, possui hábito alimentar onívoro, na natureza sua alimentação é baseada principalmente em frutas e sementes das áreas alagáveis. Na região do Pantanal tem um importante papel ecológico como dispersor de sementes. Esse peixe está entre as espécies nativas mais

cultivadas no Brasil, especialmente nas regiões Centro Oeste e Sudeste do País, contribuindo com 2,7% das 485 mil toneladas produzidas pela piscicultura continental em 2017 (IBGE, 2017). A espécie é de fácil adaptação ao cativeiro, possui rusticidade, rápido crescimento, boa taxa de conversão alimentar e de fácil reprodução (Abreu et al., 2009). Apesar da produção da espécie em cativeiro ter aumentado nos anos recentes, existem fortes evidências de que as populações naturais de *Piaractus mesopotamicus* estão sofrendo sobrepesca, como reportado por Resende (2003) e, mais recentemente, por Calcagnotto & DeSalle (2009). Entre as diversas espécies de peixes comercialmente exploradas no Pantanal do Mato Grosso do Sul, o pacu é uma das mais ameaçadas (Peixer et al., 2007). Para tentar minimizar os efeitos da pesca excessiva, o tamanho mínimo de captura passou de 40 para 45 cm no Estado do Mato Grosso do Sul, em 1994. No baixo Paraná, a pesca do pacu é inteiramente proibida (Resende, 2003). Apesar dessas ações, os estoques naturais não têm mostrado sinais de recuperação nos últimos anos.

Figura 2. *Piaractus mesopotamicus* (HOLMBERG, 1887), popularmente conhecido como pacu.

3. HIPÓTESE E OBJETIVOS

3.1. Hipótese

Existe diferença significativa na viabilidade do tecido ovariano de peixes contendo oócitos imaturos entre as técnicas de vitrificação e de congelamento lento após aquecimento.

3.2. Objetivos

3.2.1. Objetivo principal

Desenvolver um protocolo de criopreservação de fragmentos de tecido ovariano de peixes teleósteos.

3.2.2. Objetivos específicos

(1) Selecionar a melhor solução crioprotetora para a vitrificação e o congelamento lento, testando o efeito da gema de ovo e da trealose na solução crioprotetora sobre a integridade de membrana dos oócitos imaturos após criopreservação.

(2) Comparar os efeitos de dois protocolos de criopreservação, vitrificação e congelamento lento, na viabilidade dos oócitos imaturos inclusos no tecido ovariano de zebrafish por meio da avaliação de estresse oxidativo, de danos ao DNA, atividade mitocondrial e avaliação quantitativa e qualitativa da integridade morfológica dos oócitos.

(3) Verificar se o protocolo desenvolvido em zebrafish (*Danio rerio*) é aplicável em outra espécie de peixe teleósteo (*Piaractus mesopotamicus*) utilizado na produção de alimento.

CAPÍTULO II[1]

[1] Artigo elaborado conforme as normas do periódico *Scientific Reports.*

SLOW FREEZING VERSUS VITRIFICATION FOR THE CRYOPRESERVATION OF ZEBRAFISH (*DANIO RERIO*) OVARIAN TISSUE

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Abstract

The aim of the present study was to compare the efficiency of vitrification and slow freezing techniques for the cryopreservation of zebrafish ovarian tissue containing immature follicles. In Experiment 1, assessment of cell membrane integrity by trypan blue exclusion staining was used to select the best cryoprotectant solution for each cryopreservation method. Primary growth (PG) oocytes showed the best percentage of membrane integrity (63.5 ± 2.99 %) when SF4 solution (2 M methanol + 0.1 M trehalose + 10% egg yolk solution) was employed. The vitrification solution, which presented the highest membrane integrity (V2; 1.5 M methanol + 5.5 M Me_2SO + 0.5 M sucrose + 10% egg yolk solution) was selected for Experiment 2. Experiment 2 aimed to compare the vitrification and slow freezing techniques in the following parameters: morphology, oxidative stress, mitochondrial activity, and DNA damage. Frozen ovarian tissue showed higher ROS levels and lower mitochondrial activity than vitrified ovarian tissue. Ultrastructural observations of frozen PG oocytes showed rupture of the plasma membrane, loss of intracellular contents and a large number of damaged mitochondria, while vitrified PG oocytes had intact mitochondria and cell plasma membranes. We conclude that vitrification may be more effective than slow freezing for the cryopreservation of zebrafish ovarian tissue.

Introduction

Currently, the most widely used fish model is zebrafish (*Danio rerio*), which presents 12,719 genes in common with humans[1], and shows high fertility[2] and embryonic transparency[3]. Due to the use of the species as an

animal model for scientific research, numerous specific strains and lines have been created and are stored as live animals in resource centers worldwide, such as the Zebrafish International Resource Center (University of Oregon, Eugene, OR). The preservation of the genetic resources of this important model and other valuable fish is a challenge that requires attention, and the cryopreservation of gametes, embryos and gonads is a useful approach to address these challenges.

The cryopreservation of fish oocytes or embryos has not been successful due to the large size, high intracytoplasmic lipid content[4] and membrane impermeability5 of these cells. However, the cryopreservation of fish sperm was first reported more than 50 years ago[6], and since then, sperm cryopreservation protocols have been developed in more than 200 species of fish[7]. However, most research has focused on large species, such as salmonids and carp, and few studies have addressed aquarium fish[8]. The factors that hamper studies on sperm cryopreservation in aquarium fish, such as zebrafish, include the small size of the animal and the limited semen availability, where 3.3 µL of semen is typically obtained in a single collection[9]. Consequently, sperm cryopreservation in these species presents challenges, such as experimental design, gamete collection and artificial fertilization, especially when using live fish. Ovarian tissue cryopreservation was first proposed in zebrafish by Zampolla *et al*.[10] These authors suggested that ovarian fragments containing follicles in stages I and II (immature) were less susceptible to cryoinjury than ovarian follicles in stage III (vitellogenic). Immature follicles have a smaller size, which results in a higher surface/volume ratio; hence, these tissues are more permeable to water

and solutes, increasing the chances of survival during cryopreservation. The efficiency of the vitrification technique in the cryopreservation of zebrafish ovarian tissue was investigated in 2013, where authors reported a high loss of follicular viability after warming[11]. However, this study only used fragments of ovarian tissue containing stage III follicles (vitellogenic). In a recent study, we obtained a high viability of immature follicles (76% in stages I and 43% in stages II) following zebrafish ovarian tissue vitrification[12]. These data show that immature follicles are more likely to survive after vitrification than follicles at more advanced stages of maturation (III, IV and V).

The two most commonly used cryopreservation methods for the preservation of animal germplasm are slow freezing and vitrification. Both methods are based on the same principles of protecting cells from damage due to intracellular ice formation, excessive cell dehydration and changes in solute concentrations. Slow freezing requires a controlled and gradual temperature procedure; however, there is the risk of crystallization that is correlated with membrane damage. On the other hand, highly concentrated cryoprotective solutions in vitrification, enables the sudden reduction of temperature, allowing the passage from a liquid to an amorphous vitreous state, avoiding the formation of ice crystals. However, the absence of crystallization does not exclude the possibility of cellular damage, such as oxidative stress and DNA fragmentation. Both cooling and heating procedures increase the production of reactive oxygen species (ROS) causing changes in oxidative metabolism. Thus, to evaluate oocyte quality after cryopreservation of ovarian tissue, the following assessments were performed: membrane integrity, oocyte morphology, DNA

damage and oxidative stress parameters (levels of reactive oxygen species and antioxidant capacity). To date, no study has compared vitrification and slow freezing techniques in the cryopreservation of zebrafish ovarian tissue fragments. Therefore, the major aim of the present study was to compare the efficiency of vitrification and slow freezing techniques in the cryopreservation of zebrafish ovarian tissue containing immature follicles.

Materials and Methods

Experimental design

In Experiment 1, eight cryoprotectant solutions were tested in slow freezing, and four vitrification solutions were tested in the vitrification technique. Five replicates were performed for each group (cryopreserved treatments and control/fresh ovarian tissue).

In Experiment 2, ovarian tissue fragments were distributed among treatments (slow freezing, vitrification, and control/fresh ovarian tissue). Five replicates were performed in the histology analysis and mitochondrial activity assay, and eight replicates were performed in the biochemical analysis and comet assay.

The animal experiment reported in the present study was conducted in accordance with the Conselho Nacional de Controle e Experimentação Animal - CONCEA (National Council for Control and Animal Experimentation) and approved by the Ethics Committee of the Federal University of Rio Grande do Sul. Project number: 29303.

Fish care and ovarian fragment collection

Eighteen-week-old females with 0.36 ± 0.07 g average weight were euthanized with a lethal dose of tricaine methane sulfonate (0.6 mg/mL, pH 7.4), followed by decapitation. The ovaries were immediately collected after decapitation and placed in 90% Leibovitz L-15 medium (pH 9.0). The body and ovaries were weighed (average 0.03 ± 0.01 g), and the average gonadosomatic index (GSI = [ovaries weight/body weight] × 100) was 7.20 ± 2.44. Fragments containing primary growth (PG) oocytes were carefully dissected from the ovaries and cut into thin slices (2 mm) using syringe needles.

Two or three fragments were collected from each female and randomly distributed among treatments.

Experiment 1

The aim of Experiment 1 was to select the best cryoprotectant solution according to the results of membrane integrity assay for each cryopreservation method (slow freezing and vitrification). The solution that presented the best result, in each method, was used in Experiment 2.

Cryoprotectant solutions

The composition and concentrations of the cryoprotectant solutions for slow freezing were elaborated according to the results obtained in previous studies with zebrafish follicles[13,14,15], as well as the data reported by Lee *et al.*[16] on the slow freezing of rainbow trout (*Oncorhynchus mykiss*) testicles using Bicell recipient (Nihon Freezer Co., Ltd., Tokyo, Japan).

In a previous study, where zebrafish ovarian tissue was vitrified, the solution comprised 1.5 M methanol + 5.5 M Me_2SO + 0.5 M sucrose resulted in a 64% viability of early-stage follicles[12]. Thus, this cryoprotectant solution was

used to verify the effect of substituting sucrose for trehalose and to evaluate the effects of supplementation with 10% egg yolk solution.

The egg yolk solution was prepared as described by Isachenko and Nayudu[17], with some modifications. Fresh egg yolk was mixed with 90% L-15 medium (pH 9.0) at a 1:2 ratio and then centrifuged for 30 min at 10,000 rpm at 15°C. Only the supernatant was used in the cryoprotectant solution. All cryoprotectant solutions tested in the present study are presented in Table 1.

Table 1. The composition of cryoprotectant solutions tested in vitrification (V) or slow freezing (SF).

Cryoprotectant Solution	Methanol (M)	Me_2SO (M)	Sucrose (M)	Trehalose (M)	Yolk solution (%)
V1	1.5	5.5	0.5		
V2	1.5	5.5	0.5		10
V3	1.5	5.5		0.5	
V4	1.5	5.5		0.5	10
SF1	2		0.1		
SF2	2		0.1		10
SF3	2			0.1	
SF4	2			0.1	10
SF5		2	0.1		
SF6		2	0.1		10
SF7		2		0.1	
SF8		2		0.1	10

V= cryoprotectant solution tested in the vitrification procedure. SF= cryoprotectant solution tested in the slow freezing procedure. Me_2SO= dimethyl sulfoxide.

Slow freezing

Ovarian tissue fragments were transferred to 1.2-mL cryotubes containing 500 µL of cryoprotectant solution at 4°C. Samples were incubated in

cryomedium at 4°C for 60 min and then placed in a Bicell plastic freezing container (Nihon Freezer, Japan) and subsequently stored in a -80°C freezer for 90 min, enabling a -1°C/min cooling curve before immersion in liquid nitrogen.

After seven days, the cryotubes were removed from the liquid nitrogen for warming procedure. The cryotubes were thawed in water bath for 30 s at 28°C, and then the samples were washed three times in 90% L-15 medium (pH 9.0, 22°C) to remove cryoprotectants.

Vitrification

Ovarian tissue fragments were transferred to 1.2-mL cryotubes containing 500 µL of equilibrium solution (1.5 M methanol and 2.75 M Me_2SO) for 15 min at room temperature (22°C ± 1°C). Next, the equilibrium solution was removed, and 500 µL of vitrification solution (VS) was added. After 90 sec, VS was removed, and a minimum volume of medium (~10 µL) was left in the cryotubes. Then, the bottom of the cryotubes were placed in contact with liquid nitrogen, sealed and immediately plunged in liquid nitrogen. After seven days, the cryotubes were removed from the liquid nitrogen and thawed in water bath for 4 min at 10°C. Subsequently, the ovarian tissue fragments were exposed to three warming solutions at 22°C: the first warming solution, containing 1 M sucrose, for 1 min, followed by a second solution containing 0.5 M sucrose for 3 min and finally a third solution of 0.25 M sucrose for 5 min. The samples were washed three times in 90% L-15 medium (pH 9.0, 22°C).

Membrane integrity assay

Immediately after warming, cryopreserved and control follicles were isolated by gentle pipetting in Leibovitz L-15 medium. PG oocytes were incubated in 0.4% trypan blue for 3 min and then washed three times in 90% L- 15 medium. At least 100 PG oocytes in each group (cryopreserved treatments and fresh ovarian tissue fragments) were observed under a light microscope. The unstained oocytes were considered membrane-intact oocytes (percentage of membrane integrity of PG oocytes), while the blue stained oocytes were considered membrane-damaged oocytes.

Experiment 2

The aim of Experiment 2 was to compare the vitrification and slow freezing techniques based on the following parameters: morphology, oxidative stress, mitochondrial activity, and DNA damage.

Slow freezing and vitrification procedures and the methodology for obtaining ovarian tissue fragments were the same as those described in Experiment 1. V2 (1.5 M methanol +5.5 M Me_2SO + 0.5 M sucrose+ 10% egg yolk solution) and SF4 (2 M methanol + 0.1 M trehalose + 10% egg yolk solution) cryoprotectant solutions were selected to be used in Experiment 2.

Histological analysis

Ovarian tissue fragments were fixed in 10% buffered formalin (pH 7.2- 7.4) for 24 h, then embedded in paraffin wax, sliced (5 µm), and stained with hematoxylin and eosin (HE). Microscopic evaluation was performed to assess the morphological integrity of PG oocytes following both cryopreservation procedures. In each group (vitrification, slow freezing and control/fresh ovarian tissue fragments), at least 100 PG oocytes with a visible nucleus were counted

and categorized as intact and non-intact (percentage of cell integrity). Non- intact oocytes were those with presence of pyknotic nuclei (nucleus damage; condensation of the chromatin and shrinkage of the nucleus) and/or follicular membrane rupture (membrane damage).

Ultrastructural analysis by transmission electron microscopy (TEM)

Fresh, vitrified and frozen ovarian tissue fragments were fixed in 2.5% glutaraldehyde + 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2– 7.4) for 24 h. After osmium tetroxide postfixation and alcohol dehydration, the samples were embedded in 100% resin. Ultra-thin sections were placed on copper grids, stained with 2% uranyl acetate and lead citrate, and examined using a transmission electron microscope (TEM JEM 1200 Exll, Jeol, USA). The integrity of cytoplasmic organelles and cytoplasmic and nuclear membranes of PG oocytes was investigated.

Biochemical analysis

Ovarian tissue fragments were homogenized in ice-cold (0°C to 4°C) 50 mM Tris-HCl, pH 7.4 (1/10, w/v). The homogenate fragments were centrifuged for 10 min at 3000×g. Subsequently, the pellet was discarded, and the obtained low-speed supernatant (S1) was used to determine the reactive species and antioxidant capacity and mitochondrial activity.

The levels of reactive oxygen species (ROS) were determined by a spectrofluorimetric method[18] using the dichlorodihydrofluorescein diacetate (DCHF-DA) assay. The samples (20 µL) were incubated with DCHF-DA (1 mM), and the oxidation of DCHF-DA to fluorescent dichlorofluorescein was measured

using the fluorescence intensity at 520 nm emission (with 480 nm excitation) at 120 min after the addition of DCHF-DA to the medium. Each sample was analyzed in triplicate.

The total antioxidant capacity was determined by the “ferric reducing antioxidant potential” (FRAP), where the antioxidants present on samples were evaluated as reducers of Fe^{+3} to Fe^{+2}, which is chelated by 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ), forming the complex Fe+2-TPTZ, with max absorption at 593 nm[19]. An ascorbic acid standard curve was realized, and the results were expressed in µg equivalent of ascorbic acid. Each sample was analyzed in triplicate.

Mitochondrial activity

Mitochondrial activity was assessed by thiazolyl blue tetrazolium bromide (MTT) staining using spectrophotometry[20]. The principle of this method is based on the reduction of MTT to formazan crystals by the mitochondrial succinyl dehydrogenase enzyme, active only in living cells. Immediately after warming, ovarian tissue fragments were homogenized in phosphate-buffered saline (PBS) and incubated with MTT (5 mg/mL) for 120 min at 25°C. The supernatant (200 µL) was carefully removed and dimethyl sulfoxide (200 µL) was added to dissolve the formed crystals. Next, 200 µL of the colored solution were transferred for absorbance analysis at 570 nm on the SpectraMax® 250 Microplate Spectrophotometer. The greater the color intensity was, the higher the mitochondrial activity. Each sample was analyzed in triplicate.

DNA damage

The comet assay was performed under alkaline conditions, according to the procedure of Singh *et al*.[21], Collins[22] and adaptations from Da Silva *et al*.[23]. Oocytes were isolated by density centrifugation (30 min, 18°C, 400×g) on Ficoll-Paque™ Plus. To examine basal DNA damage, aliquots (20 µL) of the oocytes were immediately collected after centrifugation, mixed with 90 □L of low melting point agarose (0.7% in phosphate buffer) and added to microscope slides precoated with 1.5% agarose. The slides were then incubated in ice-cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 20 mM NaOH, pH 10.2, 1% Triton X-100, and 10% DMSO). For the DNA repair study, the cells were treated with H_2O_2 (10 µM, 5 min on ice) or with methyl methane sulfonate (MMS) (8 × 10− 5 M, 1 h at 37°C).

After treatment, the cells were washed with PBS (centrifugation, 5 min, 4°C, 200×g) and incubated in RMPI 1640 (200 µL) with 10% fetal calf serum. Aliquots of the suspension (20 µL) were immediately collected or after 60 and 180 min of post-incubation, mixed with (80 µL) 0.75% low melting point agarose and cast onto microscope slides precoated with 1.5% normal agarose. After 24 h at 4°C, the slides were removed from the lysis solution and placed in an electrophoresis unit filled with fresh electrophoresis buffer at 4°C. In the alkaline version of the comet assay (10 M NaOH, 1 mM EDTA, pH > 13), 20 min of denaturation and 15 min of electrophoresis were used. For the neutral version of the comet assay (3 M sodium acetate, 1 M Tris, and pH = 8.5), 1 h of denaturation and 1 h of electrophoresis were used. In both versions of the comet assay, after electrophoresis, the slides were neutralized (0.4 M Tris, pH 7.5) and washed in water. The slides were dried overnight at room temperature,

then fixed and stained with 20 µL of 4,6-diamidino-2-phenylindole. Each slide was viewed by fluorescence microscopy, and the degree of damage was visually scored. A total of 100 comets on each slide were assigned a score from 0 to 4, depending on the fraction of DNA extracted from tail. The overall score for each slide was between 0 (undamaged) and 400 (completely damaged).

Statistical Analyses

Normality (Kolmogorov-Smirnov) and homogeneity (Levene's test) were previously verified. Variables ROS showed non-normal distribution; therefore, all data were log-transformed to show a normal distribution prior to analysis. Statistical analysis was performed by using one-way analysis of variance (ANOVA). Tukey's test, at 95%, was used to assess significant differences among the means. Student's t test was applied for biochemical analysis data and mitochondrial activity. The results of the comet assay did not show normal distribution, even after data transformation; thus, a non-parametric Kruskal-Wallis analysis was applied, followed by Dunn's test.

Results

Experiment 1

The membrane integrity of PG oocytes in the control group (fresh ovarian tissue fragment) was significantly higher compared to all vitrification solutions; however, there was no significant difference among the treatments (Figure 1).

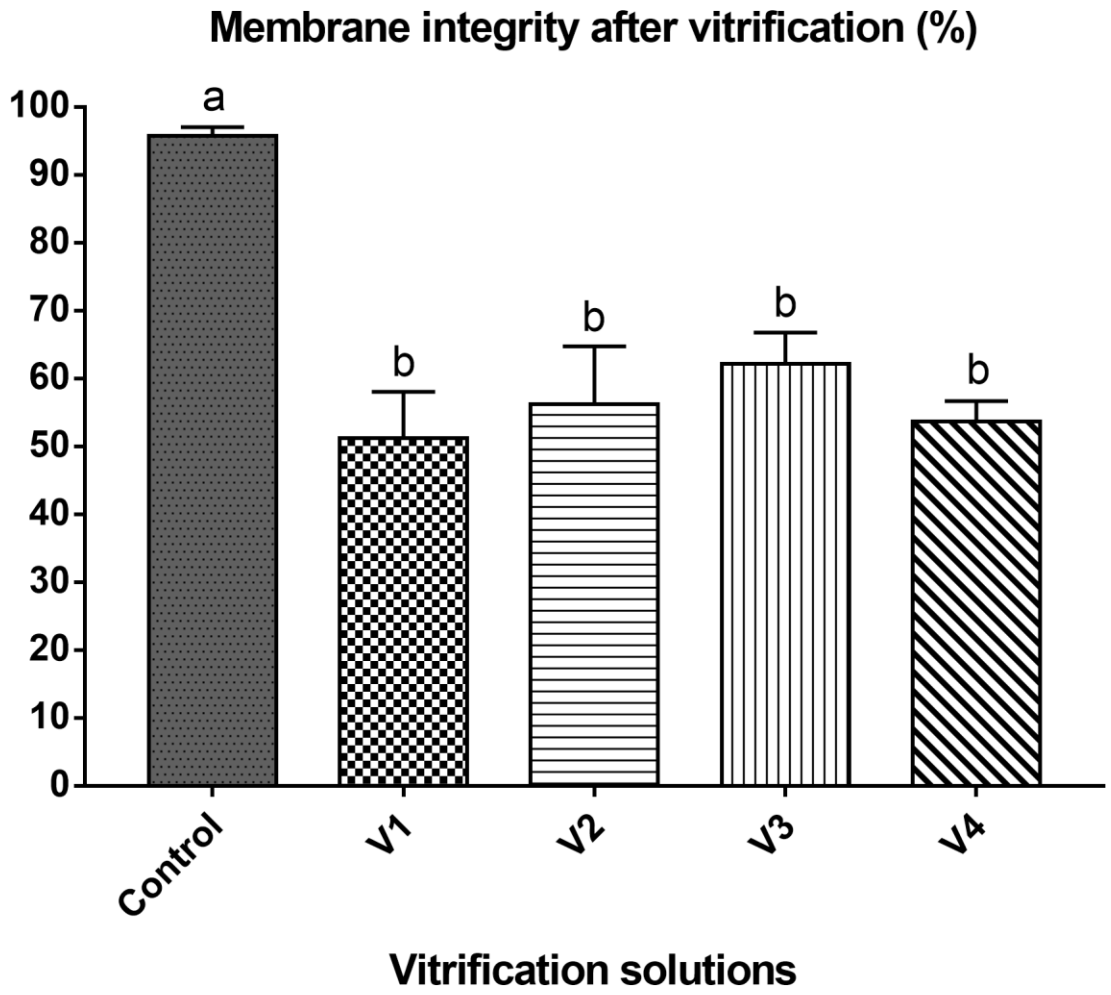

Figure 1. Membrane integrity after vitrification. V1 (1.5 M methanol + 5.5 M Me2SO + 0.5 M sucrose); V2 (1.5 M methanol + 5.5 M Me2SO + 0.5 M sucrose + 10% egg yolk solution); V3 (1.5 M methanol + 5.5 M Me2SO + 0.5 M trehalose); V4 (1.5 M methanol + 5.5 M Me2SO + 0.5 M trehalose + 10% egg yolk solution). Control = fresh ovarian tissue fragments. Mean ± SE followed by different letters differ by Tukey's test (P = 0.0004).

The membrane integrity after slow freezing with the eight cryoprotectant solutions is shown in Figure 2. There was a significant difference in membrane integrity between the control group and the slow freezing treatments (Figure 2). PG oocytes showed the best percentage of membrane integrity (63.5 ± 2.99%) when SF4 solution was employed. When ovarian tissue fragments were frozen in SF1 and SF3, the membrane integrity decreased to 41.5 ± 21.0% and 43.75

(P < 0.05), respectively. Based on these results, SF4 solution was used for the subsequent experiments. As there was no significant difference among vitrification solutions, the solution containing egg yolk that presented the highest membrane integrity percentage (V2) was selected for Experiment 2.

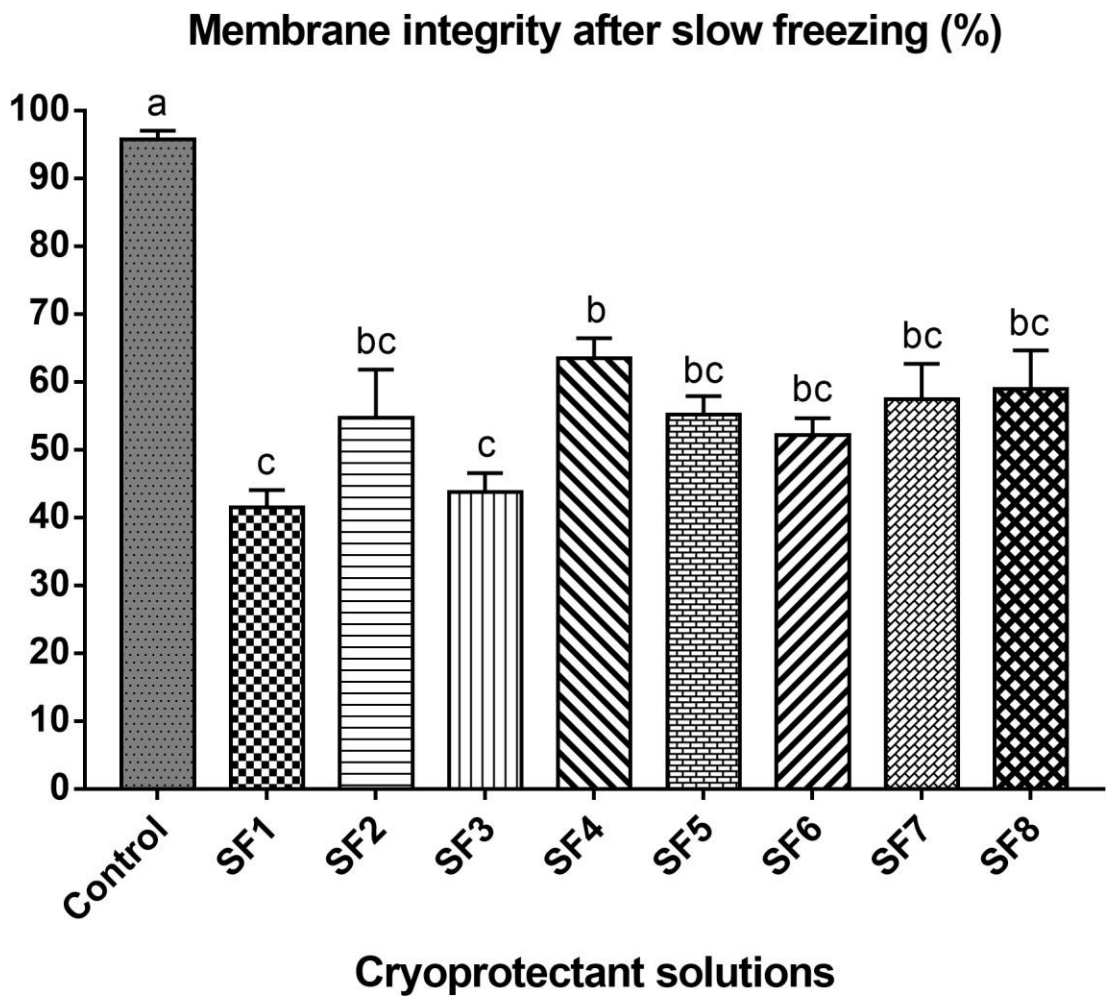

Figure 2. Membrane integrity after slow freezing. SF1 (2 M methanol + 0.1 M sucrose); SF2 (2 M methanol + 0.1 M sucrose + 10% egg yolk solution); SF3 (2 M methanol + 0.1 M trehalose); SF4 (2 M methanol + 0.1 M trehalose + 10% egg yolk solution); SF5 (2 M Me2SO + 0.1 M sucrose); SF6 (2 M Me2SO + 0.1 M sucrose + 10% egg yolk solution); SF7 (2 M Me2SO + 0.1 M trehalose); SF8 (2 M Me2SO + 0.1 M trehalose + 10% egg yolk solution). Control = fresh ovarian tissue fragments. Means ± SE followed by different letters differ by Tukey's test (P<0.0001).

Experiment 2

There was no significant difference (P>0.05) among control, slow freezing and vitrification in the histological analysis (Figure 3). In the evaluation of nuclear damage (Figure 4), vitrification showed an increase (19.64%) in relation to freezing (12.04%); however, this difference was not statistically significant (P = 0.4627).

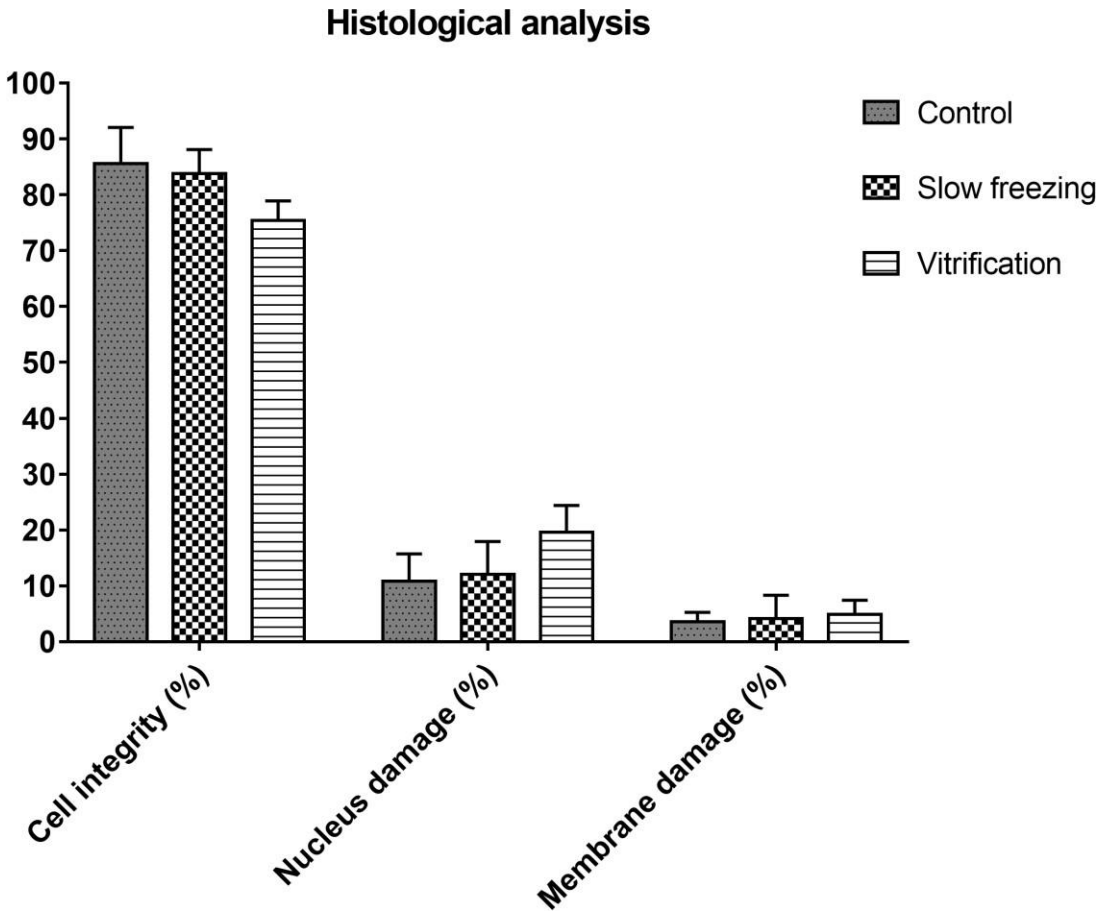

Figure 3. Histological analysis after slow freezing and vitrification. Not significant (P>0.05). Cell integrity (%): P = 0.3452; Nuclear damage (%): P = 0.4627; Membrane damage (%): P = 0.9517.

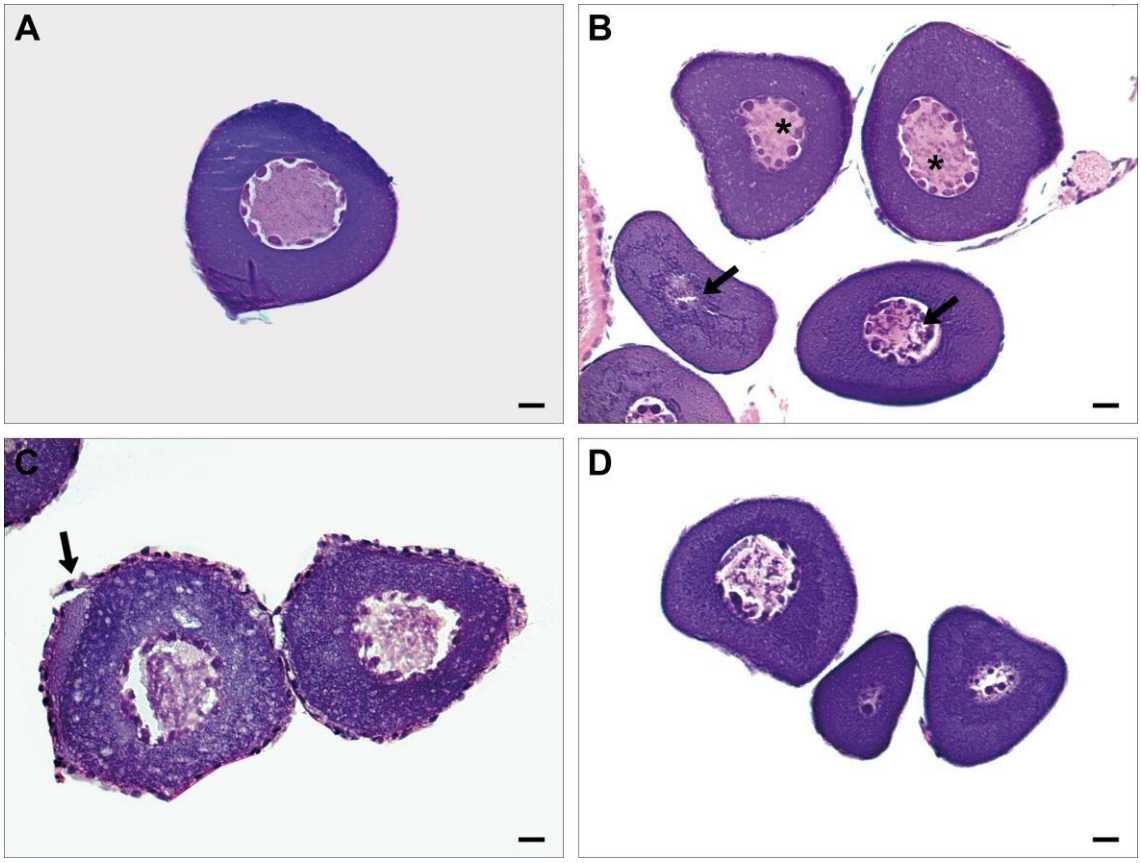

Figure 4. Histology of fresh (A), vitrified (B) and frozen (C, D) primary growth (PG) oocytes of zebrafish (*Danio rerio*) after thawing. In A, fresh PG oocyte with intact nucleus and cell membrane. In B, vitrified PG oocytes with condensed chromatin (arrow) and oocytes with intact nucleus (asterisk). In C, frozen PG oocyte with follicular membrane rupture (arrow). In D, three frozen PG oocytes with nucleus damage. Light microscope 40x. Stain: HE. Bar=10 µm.

Figure 5 shows morphological ultrastructural features of fresh, vitrified and frozen PG oocytes by TEM. The ultrastructural observations of fresh PG oocytes showed a normal aspect of the organelles and intact plasma and nuclear membranes (Figure 5). In addition, fresh PG oocytes had a large number of mitochondria in the cytoplasm (Figure 5.B). Although the vitrified PG oocytes had intact plasma membranes (Figure 5.D), there was nuclear condensation (pyknosis) and signs of a ruptured nuclear membrane (Figure 5.F). The TEM also showed a lower number of mitochondria in the cytoplasm of vitrified PG oocytes (Figure 5.E). The ultrastructural observations of frozen PG

oocytes showed the rupture of the plasma membrane and the loss of intracellular contents (Figure 5.G and H). In addition, a large number of damaged mitochondria were observed in frozen PG oocytes (Figure 5.I).

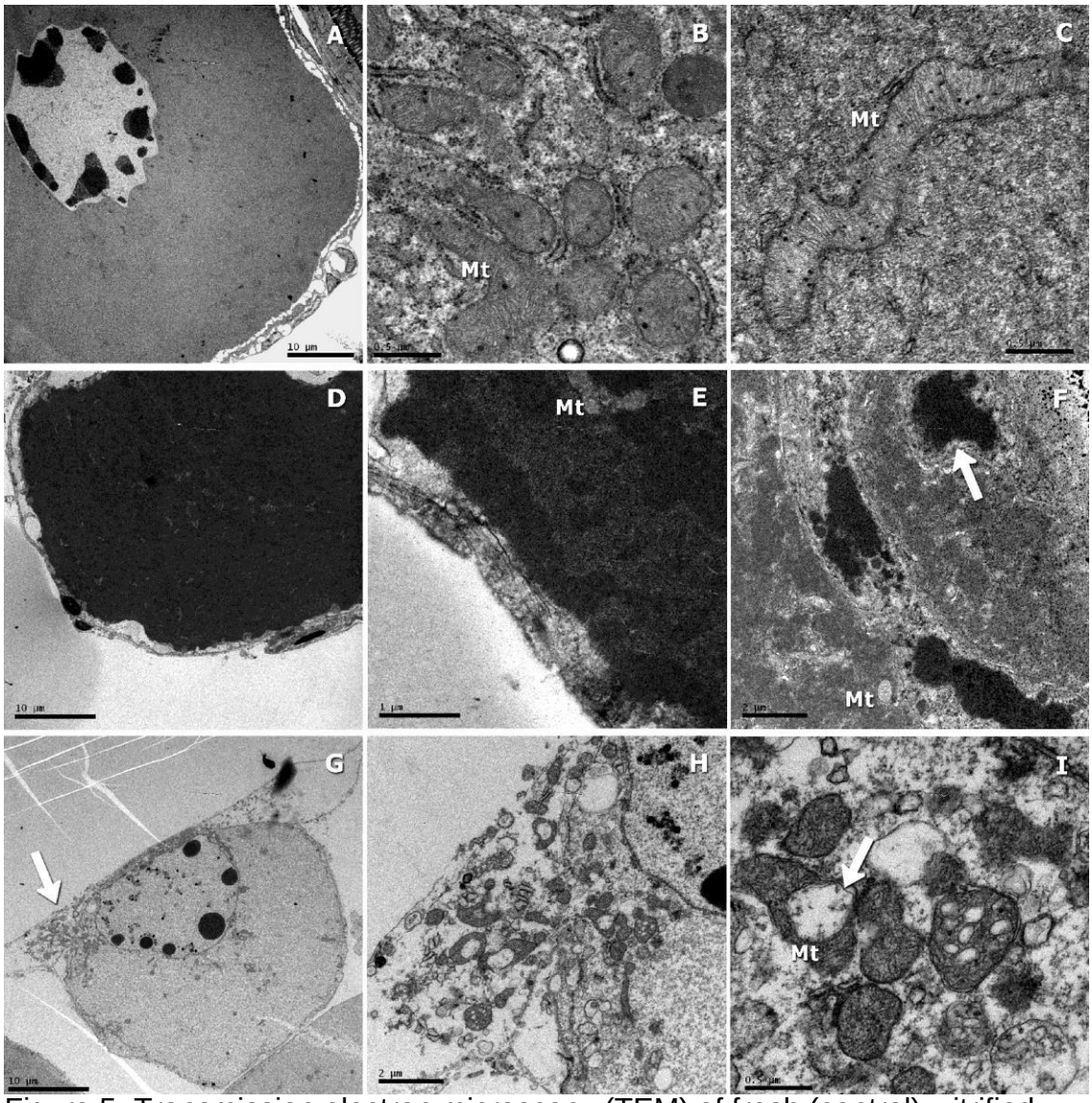

Figure 5. Transmission electron microscopy (TEM) of fresh (control), vitrified and frozen primary growth (PG) oocytes of zebrafish (*Danio rerio*). (A, B, C) Fresh PG oocytes with normal cell organelles and intact plasma and nuclear membranes. (B, C) Presence of large number of mitochondria in the cytoplasm. (D, E, F) Vitrified PG oocyte showing an intact plasma membrane, chromatin condensation (arrow), signs of a ruptured nuclear membrane and a lower number of mitochondria. (G, H) Frozen PG oocyte showing a ruptured plasma membrane and a loss of intracellular contents (arrow). (I) Cytoplasm of a frozen PG oocyte with swollen mitochondria (arrow). PG = primary growth, Mt = mitochondria.

Frozen ovarian tissue showed higher ROS levels than vitrified ovarian tissue (P<0.0001; Figure 6.A). Compared to frozen ovarian tissue, vitrified ovarian tissue showed a reduced antioxidant capacity as measured by FRAP (P<0.0001; Figure 6.B).

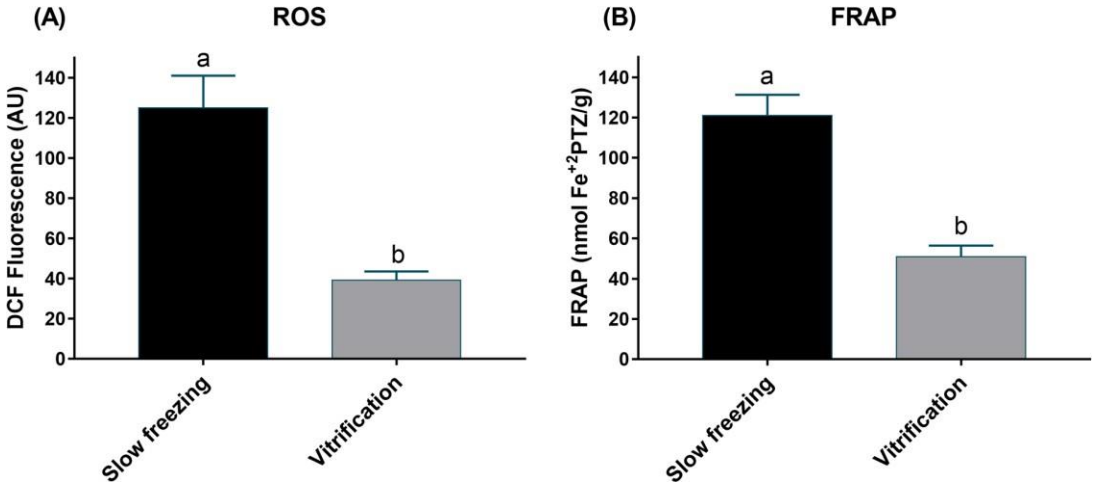

Figure 6. (A) Reactive oxygen species (ROS) by DCFH, and (B) total antioxidant capacity by FRAP (Ferric reducing/antioxidant power) after slow freezing and vitrification. Means ± SE followed by different letters differ by Student's t test. ROS: P < 0.0001; FRAP: P < 0.0001.

The mitochondrial activity in vitrified ovarian tissue was significantly higher when compared to that in frozen ovarian tissue (P = 0.0081; Figure 7).

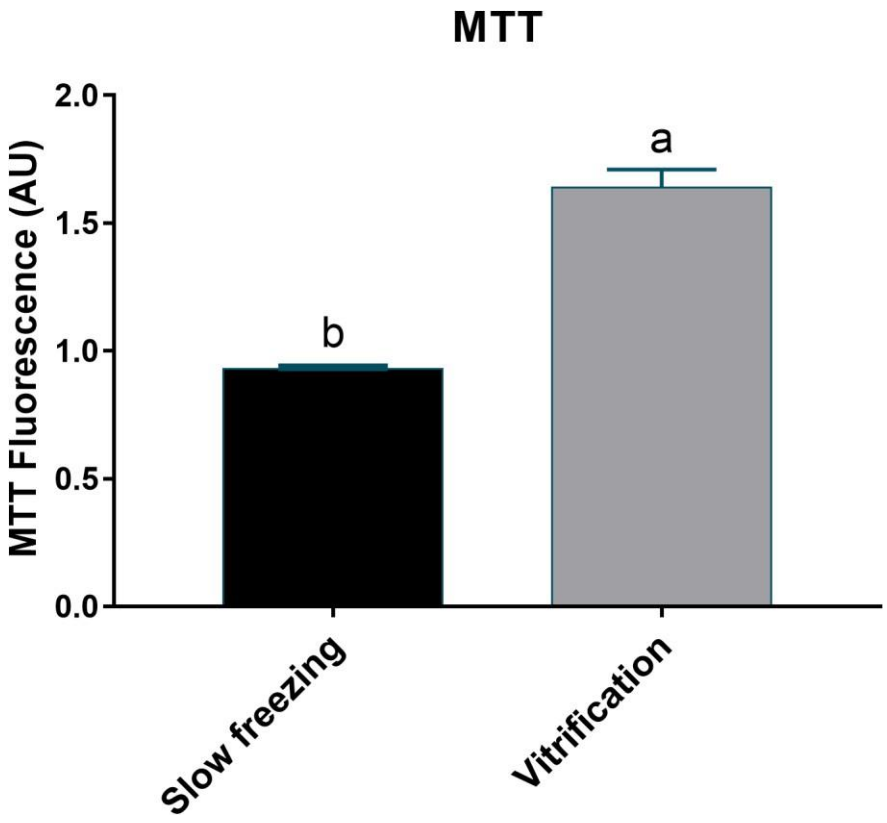

Figure 7. Mitochondrial activity by thiazolyl blue tetrazolium bromide (MTT). Means ± SE followed by different letters differ by Student's t test (P = 0.0081).

Comet assays showed no difference between vitrification (61.86%) and slow freezing (69.63%; P>0.05; Figure 8). However, slow freezing showed a more significant difference in relation to the control (39.6%; P = 0.0013), whereas vitrification showed the least significant P in relation to the control (39.6%; P = 0.0382).

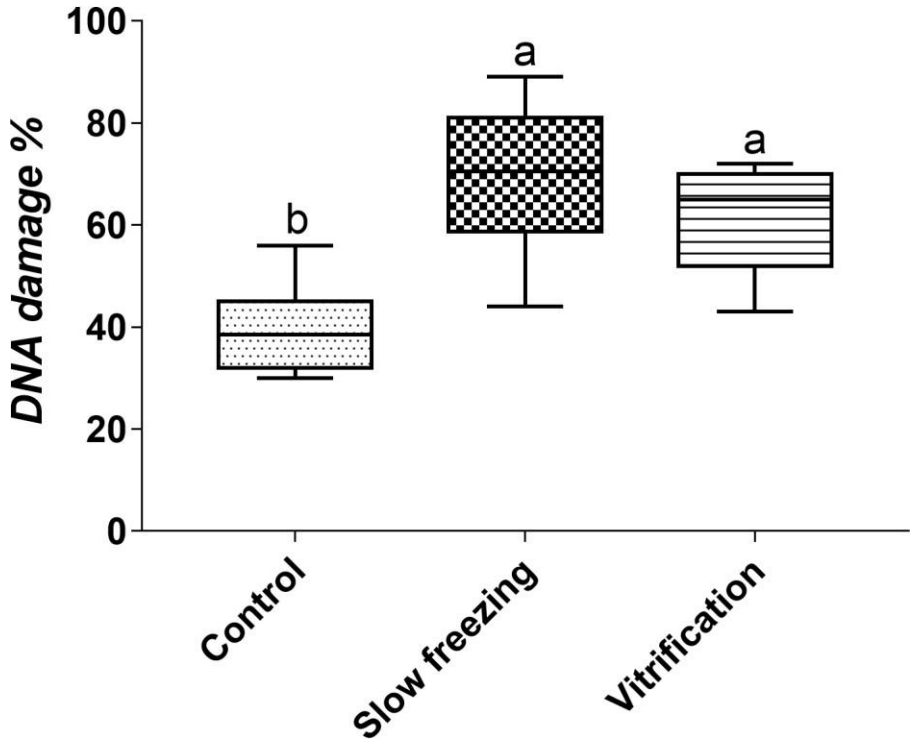

Figure 8. DNA damage (%) by comet assay of fresh (control), frozen and vitrified primary growth (PG) oocytes of zebrafish (*Danio rerio*). Means ± SE followed by different letters differ by the analysis of Kruskal-Wallis, followed by Dunn's test (P = 0.0013).

Discussion

Cryopreservation promotes changes in the physical and chemical properties of oocytes, including cell membrane damage, increased production of reactive oxygen species, DNA damage, and mitochondrial depolarization[24,25,26,27,28]. Therefore, cell morphology, oxidative stress, DNA damage, mitochondrial activity and structure were measure after slow freezing and vitrification.

Part of the damage caused by the cryopreservation of fish oocytes occurs at the plasma membrane. Therefore, the development of more efficient cryopreservation methods depends on the use of substances that protect the

integrity of the oocyte membrane during cooling. Previous studies have reported that the addition of egg yolk in cryoprotectant solution had a protective effect on the integrity of both mouse oocytes and cumulus cells[17]. In a study with the frozen testes of rainbow trout, type A spermatogonia (ASGs) obtained from testes whose cryoprotectant solution contained 10% (vol/vol) egg yolk displayed the highest rates of survival[16]. The present study provides the first evidence of the effect of egg yolk on oocyte membrane integrity after vitrification and slow freezing of fish ovarian tissue. Although the egg yolk did not show a positive effect after vitrification, when added to freezing solution containing 2 M methanol and 0.1 M trehalose, the membrane integrity after slow freezing was 63.5%. The major concern during the slow cooling of cells is the potential formation of intracellular ice that may cause cell and membrane damage and directly affect viability[29]. The action of egg yolk may be attributed to phospholipids[30], cholesterol[31] and low-density lipoprotein[32] contents, which are incorporated in the cell membrane, providing protection to the oocyte membrane against osmotic shock and cryoinjuries during slow freezing[33,34].

Morphological evaluation by histology showed no difference between the slow freezing and vitrification techniques, as well as no difference between fresh and cryopreserved PG oocytes in membrane and nuclear damage. The cryopreservation of immature oocytes may reduce membrane damage because PG oocytes are surround by a single layer of squamous follicle cells that lie on a distinct basement membrane. While the cortical alveolar stage presents three layers, the newly formed inner layer is thickest and more architecturally complex[35].

Several stress factors associated with cryopreservation are known as initiators of apoptotic cell death. Further, the combination of cell physical damage with cell stresses experienced during the freeze-thaw procedure can result in necrosis[36]. Apoptosis describes a specific morphological aspect of cell death and is accompanied by pyknosis (reduction of cellular volume and chromatin condensation), nuclear fragmentation (karyorrhexis), and little or no ultrastructural modifications of cytoplasmic organelles[37]. In the present study, these morphological features were observed in the ultrastructural analysis of vitrified PG oocyte, indicating the occurrence of cellular apoptosis during vitrification. The ultrastructural observations of frozen PG oocytes showed organelle swelling, plasma membrane rupture and intracellular content loss, which are characteristic of cellular necrotic cell death.

The number and distribution of mitochondria and energy (ATP) production are critical factors that influence the oocyte maturation, fertilization, and embryo development[38]. Zampolla *et al.*[38] showed that higher concentrations of methanol (3 and 4 M) induced a decrease in mitochondrial membrane potential and the loss of mitochondrial distributional arrangement in zebrafish ovarian follicles at stage III, compromising mitochondrial function. In the present study, the ultrastructural analysis of fresh PG oocytes showed a large number of mitochondria in the cytoplasm. Despite the smaller number of mitochondria in the cytoplasm of vitrified PG oocytes, the structure of these mitochondria appeared well preserved. In contrast, in frozen PG oocytes showed a large number of damaged mitochondria. Probably for this reason, mitochondrial activity in vitrified ovarian tissue was higher when compared to that in frozen

ovarian tissue. In addition, frozen ovarian tissue showed higher ROS levels and total antioxidant capacity than vitrified ovarian tissue. These results can be explained by the mitochondrial damage observed in frozen PG oocytes. Mitochondrial antioxidant enzymes detoxify ROS; however, excess ROS generation may overwhelm the capacity of these defenses, leading to mitochondrial damage[39].

The comet assay is a simple method for measuring deoxyribonucleic acid (DNA) strand breaks in individual cells[22], and this assay is widely used to measure DNA damage in reproductive cells[40,41,42,43,44,45,46]. Freezing and thawing increase DNA strand breaks, and previous studies have associated this increased damage with the oxidative stress that occurs during cryopreservation[47]. In addition, studies showed that vitrification was associated with significantly less DNA fragmentation in human primordial follicles compared with that in slow freezing[48]. However, in the present study, there was no difference between vitrification and slow freezing techniques on the cryopreservation of zebrafish ovarian tissue containing immature follicles.

Conclusion

The present study suggests that vitrification is more effective than slow freezing for the cryopreservation of zebrafish ovarian tissue containing immature follicles, for resulting in less ROS production and better preserved PG oocytes, which should lead to improved tissue function during *in vitro* maturation.

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Author Contributions

Conception and design of the study: LSM, DPS. Acquisition of data: LSM, AANF, RBR, HTR, API, JBR, JCF, FWS. Analysis and interpretation of data: LSM, AANF, RBR, HTR, API, JBR. Drafting the article or revising it critically for important intellectual content: LSM, DPS, TZ, JCF, FWS. All authors read and approved the final version to be submitted.

Competing interests

The author(s) declare no competing interests.

CAPÍTULO III[1]

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VIABILITY ASSESSMENT OF PRIMARY GROWTH OOCYTES FOLLOWING OVARIAN TISSUE VITRIFICATION OF NEOTROPICAL TELEOST PACU (*PIARACTUS MESOPOTAMICUS*)

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ABSTRACT

Vitrification of ovarian tissue containing immature oocytes provides an important tool for protecting the endangered species and genetic diversity in aquatic species. Therefore, the main objective was to assess primary growth (PG) oocytes viability following ovarian tissue vitrification using histological analysis, two staining protocols (trypan blue or fluorescein diacetate combined with propidium iodide) and mitochondrial activity assay (MTT assay). In addition, oocyte histomorphometry was performed to evaluate the morphometric parameters after vitrification and the relationship with the occurrence of damage (nucleus and/or membrane) in PG oocytes. There was no significant difference among the vitrified oocytes using trypan blue dye or FDA + IP staining. Oocyte viability assessed using histological analysis showed that vitrification solution

2.0 M Me_2SO + 2.5 M etilenoglycol + 0.5 M sucrose (VS3; 66.43 ± 4.68%) and 1.5 M methanol + 5.5 M Me_2SO + 0.5 M sucrose (VS5; 74.14 ± 3.71%) had the lowest viability rate. Similar results were observed in MTT assay where VS3 (1.63 ± 0.12) and VS5 (1.58 ± 0.09) had the lowest averages when compare with VS1 (2.39 ± 0.14), VS2 (1.78 ± 0.06) and VS4 (2.34 ± 0.19) (P=0.0002). In membrane damage evaluation by histology, there was no difference among vitrified oocytes and control. However, the highest percentages of nucleus damage were observed in treatments VS3 (26.00 ± 5.55) and VS5 (26.00 ± 5.55). Oocyte diameter did not change after vitrification; however, nucleus diameter was significantly higher in control group (49.03 ± 1.07). Oocyte viability by histological analysis was positive-correlated to the occurrence of nucleus (r^2= 0.78) and membrane (r^2= 0.45) damage after vitrification/warming. The high viability of PG oocytes obtained after ovarian tissue vitrification of *Piaractus mesopotamicus* suggests that the protocol applied here might be used successfully in other teleost species for food production.

Keywords: ovarian tissue cryopreservation; immature oocytes; ovarian follicle; histomorphometry.

1. Introduction

Fish stocks are globally threatened mainly due to overfishing and environmental pollution. Aquaculture has become an important activity for food production in order to provide protein for the growing demand; and at same time preserves the native populations from overfishing. Therefore, the importance of gene banks or cryobanks has become increasingly evident for wildlife

maintenance as well as agricultural production. Cryopreservation of gametes is a promising biotechnology in preservation of fish genetic resources, which is particularly beneficial for threatened species and fish breeding programs. Furthermore, to cultivated species that reproduce only once a year, cryopreservation would allow the production of fingerlings throughout the year and, consequently, would increase commercial production. More than 200 fish species with external fertilization have been tested for sperm cryopreservation, and many of them seem to be adequate for gene banking [2]. On the other hand, oocyte and embryo cryopreservation is not successful in fish species, mainly because of the large cell size; low membrane permeability; and high lipid content, which increases the probability of membrane rupture during freezing [21,22,4]. Cryopreservation of ovarian tissue fragments containing immature oocytes is an alternative, since immature oocytes have smaller size resulting in higher surface area to volume ratio; higher membrane permeability; and absence of lipid droplets. In an earlier study, we showed the feasibility of cryopreservation of ovarian tissue fragments using zebrafish as model [10]. Post-thaw survival of primary growth (PG) oocytes was demonstrated following 24 h of culture. Therefore, the aim of the present study was to verify if the protocol previously developed in zebrafish model is applicable in other fish species used for food production.

Piaractus mesopotamicus (Holmberg, 1887), popularly known as pacu, is a teleost fish from the Parana-Paraguay Basin, South America. This fish has seasonal reproductive cycle and migrates large distances upstream in rivers to spawn. The first gonadal maturation occurs at four years of age in nature and at

three years of age in captivity [15]. Their reproduction does not occur under artificial conditions, except when hormonally induced. *P. mesopotamicus* is the most captured fish in Pantanal, Mato Grosso do Sul State, Brazil; and its stock is currently overexploited [13]. Pacu together with the hybrids patinga (*P. mesopotamicus* × *Piaractus brachypomus*), tambacu (*P. mesopotamicus* male × *C. macropomum* female), and tambatinga (*Piaractus brachypomus* male x *C. macropomum* female) amounted to 58,013 tons, accounted for 13.2% of national aquaculture production in 2016 [7]. Therefore, due to the ecological, social and economic importance of *P. mesopotamicus*, this species was chosen for the study.

2. Materials and Methods

2.1. Fish and experimental design

Pacu (*Piaractus mesopotamicus*) were kept at the Instituto de Pesquisa em Aquicultura Ambiental (InPAA) (Paraná, Brazil) under a natural photoperiod at a water temperature of 27°C. The experiment was carried out during the autumn of 2016, after completion of the spawning season, when pacu was in the regenerating phase [1]. Fish in the regenerating phase are sexually mature but reproductively inactive [3]. Ten four-year-old females (341.26 ± 58.37 g of average weight and 27.5 ± 1.35 cm of average total length) were used in the experiment following the Brazilian Guide for the Care and Use of animals in scientific research activities of the National Council of Animal Experimentation, presided by the Ministry of Science and Technology.

Fish were individually anesthetized with 150 mg L^{-1} of clove oil [16]. Clove oil was dissolved in 95% ethanol at a 1：9 ratio (clove oil:ethanol) and then further diluted with tank holding water. After opercular movement ceased (3 - 5 min), fish were placed in ventral recumbency on a flat surface, and euthanized through a firm sharp blow on the head in order to cause brain death. Ovaries were collected, weighed and placed in 90% Leibovitz L-15 medium (pH 9.0) supplemented with L-glutamine. Fragments containing only primary growth (PG) oocytes were carefully dissected from the ovaries and cut into thin slices (2-3 mm) using syringe needles. Ovarian tissue fragments were distributed into five treatments (ES1/VS1, ES2/VS2, ES3/VS3, ES4/VS4 and ES5/VS5; Table 1 and 2) and one control group (non-vitrified). Twelve ovarian tissue fragments were collected from each female. One half (n= 6) was used in histological analysis and the other half (n= 6) for vital staining tests and mitochondrial activity assay. One fragment was obtained for ultrastructural analysis of primary growth (PG) oocytes.

Table 1. Cryoprotectant concentration (mol/L) in each vitrification solution (VS) treatment.

Vitrification Solution	Methanol	Propylene glycol	Dimethyl sulfoxide	Ethylene glycol	Sucrose
VS1	1.5	4.5			
VS2	1.5		5.5		
VS3			2.0	2.5	0.5
VS4	1.5	4.5			0.5
VS5	1.5		5.5		0.5

Table 2. Cryoprotectant concentration (mol/L) in each equilibrium solution (ES) treatment.

Equilibrium Solution	Methanol	Propylene glycol	Dimethyl sulfoxide	Ethylene glycol
ES1	1.5	2.25		
ES2	1.5		2.75	
ES3			1.0	1.5

ES4	1.5	2.25	
ES5	1.5		2.75

2.2. Ultrastructural analysis of primary growth (PG) oocytes

The ultrastructural study was carried out using fresh control fragment with the objective of identifying the main organelles present in the PG oocyte and confirming the absence of intracytoplasmic lipids. A sample of fresh ovarian tissue fragment was pre-fixed with 2.5% glutaraldehyde + 2% paraformaldehyde in 0.1 mol/l phosphate buffer, pH 7.4, for 24 h at 4°C and post-fixed with 1% osmium tetroxide for 60 min at 4°C. Sample was then dehydrated in alcohol at progressively higher concentrations and embedded in 100% resin. Ultrathin sections were placed on copper grids, stained with 2% uranyl acetate and lead citrate, finally observed in a transmission electron microscope (TEM JEM 1200 Exll, Jeol, USA).

2.3. Ovarian tissue vitrification

Fragments were exposed to equilibrium solution (ES, Table 2) for 15 minutes at room temperature (RT) (25°C ± 1°C). Following ES exposure time, the fragment was kept in the vitrification solution (VS) treatment (Table 1) for 90 sec, and then gently transferred with a minimum volume of medium to the 2 mL cryotube (external threaded polypropylene cryogenic vial with round bottom, Corning®). Cryotubes were plunged directly into liquid nitrogen and stored for 24 hours.

Warming was performed by exposing the cryotubes to water bath for 1 min at 28°C. Cryoprotectants were removed by a sequence of three warming

solutions: the first warming solution containing 1 M sucrose for 1 min, then to the second solution containing 0.5 M sucrose for 3 min and finally to the third solution of 0.25 M sucrose for 5 min, at RT (25 ± 1°C).

2.4. Assessing oocytes viability after vitrification

2.4.1. Live/dead oocyte viability stain protocols

Two staining protocols were used to determine the number of viable cells present in the ovarian tissue fragments following warming procedure. Trypan blue is a dye exclusion method. It is based on the principle that live cells possess intact cell membranes that exclude trypan blue dye. Thus, viable cells are able to repel the dye and do not stain. The use of fluoresce in diacetate (FDA) and propidium iodide (PI) is a rapid fluorometric method to test the integrity of the membrane simultaneously using inclusion (FDA) and exclusion dyes (PI). FDA is a nonpolar ester, which passes through plasma membranes and is hydrolyzed by intracellular esterases to produce free fluorescein. In contrast, PI cannot pass through a viable cell membrane. Thus, viable cells are bright green stained with FDA and non-viable cells are bright red stained with PI. One ovarian tissue fragment from each female was used for both staining protocols. Half of the fragment was incubated in 0.2% of Trypan Blue for 3-5 min at RT (25 ± 1°C) and then washed in L-15 medium. Viability assays using FDA with PI was performed by the method described by Jones and Senft [8]. Other half of the fragment was incubated with FDA (2.0 µg) and PI (0.6 µg) in the dark for 3-4 min at RT (25 ± 1°C). At least 100 oocytes in each group (treatments and control) were evaluated in both protocols.

2.4.2. Histological analysis and histomorphometry

Samples were fixed in 10% buffered formalin (pH 7.2 - 7.4) for 24 h, then embedded in paraffin wax, sliced (5 µm), and stained with hematoxylin and eosin (HE). Microscopic evaluation was performed to assess morphological integrity of PG oocytes after ovarian tissue vitrification/warming procedure. In each group, at least 100 PG oocytes with a visible nucleus were counted and categorized in viable and non-viable. Non-viable oocytes were those with presence of a pyknotic nuclei (nucleus damage) and/or granulosa membrane rupture (membrane damage). For the morphometric study, images were captured with a light microscope (Carl Zeiss GmbH, 20x objective lens) equipped with a digital camera (AxioVision LE64 V 4.9.1.0). Oocyte and nucleus diameters (µm) were measured, using the Image-Pro Plus® v. 4.5.0.29 software, by drawing a horizontal (maximum diameter) and perpendicular line making a 90° angle (Figure 1).The relationship between oocyte and nucleus was determined using the diameter measurements. Oocyte histomorphometry was performed to evaluate the morphometric parameters after vitrification/warming, and the relationship with the occurrence of damage (nucleus and/or membrane) in PG oocytes.

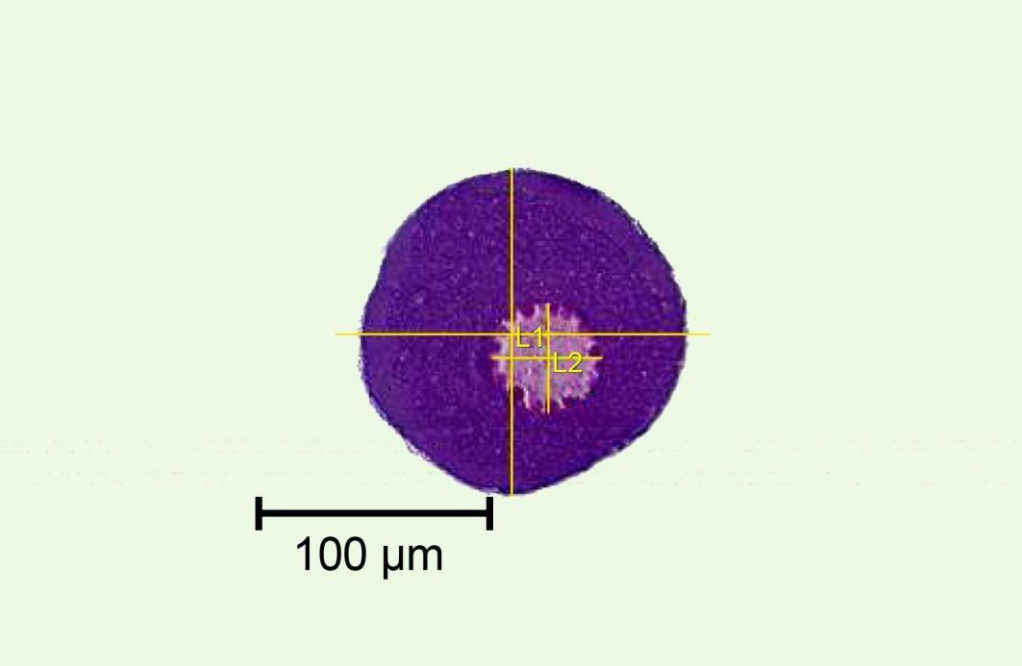

Figure 1. Oocyte (L1) and nucleus (L2) histomorphometry. Stain: HE.

2.4.3. Measurement of cell viability using the MTT assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) assay evaluates the cell metabolic activity through the absorbance values, correlated to living cell numbers [14]. Ovarian tissue fragments were homogenized in phosphate-buffered saline (PBS) and incubated with MTT (5 mg/mL) for 120 min at 25°C. The crystals were dissolved in dimethyl sulfoxide (1:1), resulting in a purple solution, measured by spectrophotometry at 570 nm. The greater the color intensity, the higher the mitochondrial activity it is.

2.5. Statistical analysis

All data are presented as mean values ± standard error. Statistical significance was determined by using one-way ANOVA followed by Tukey multiple comparisons test using a statistical significance level of $P<0.05$. Normality (Kolmogorov-Smirnov) and homogeneity (Levene's test) were

previously verified. Non-normality data (plasma membrane damage and mitochondrial activity by MTT assay) were log-transformed. Duncan's test was used for the means comparison among viability techniques of pacu oocytes. Correlation analysis were performed using used linear correlation Pearson ($P<0.05$) and considering all the vitrified treatments, excluding the control. Correlation was performed to evaluate the relationship with the occurrence of damage (nucleus and/or membrane) and diameter (oocyte and/or nucleus) after vitrification/warming.

3. Results

Ovaries were empty and presented a pinkish-orange color (3.87±1.87 g of average weight). Average gonadosomatic index (GSI = [ovaries weight/body weight] × 100) was 1.11±0.47. These parameters along with the data obtained by the histological analysis (PG oocytes) confirm the regeneration phase of the reproductive cycle of the females.

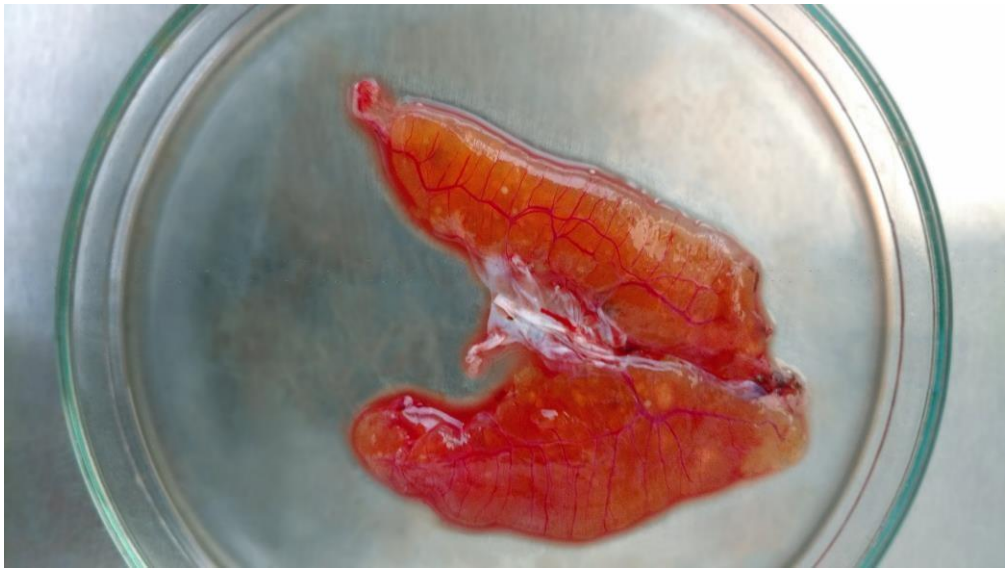

Figure 2. Empty ovaries of *Piaractus mesopotamicus.*

The detailed characterization of the fresh PG oocytes was carried out with transmission electron microscopy (TEM). At this stage, the nucleus is spherical and in a central position. The nucleoli are located on the periphery of the nucleus, close to the nuclear envelope (Figure 3.A). Organelles were uniformly distributed throughout the homogeneous cytoplasm, with mitochondria being the most evident organelle (Figure 3.B). No lipid droplets were observed in the cytoplasm.

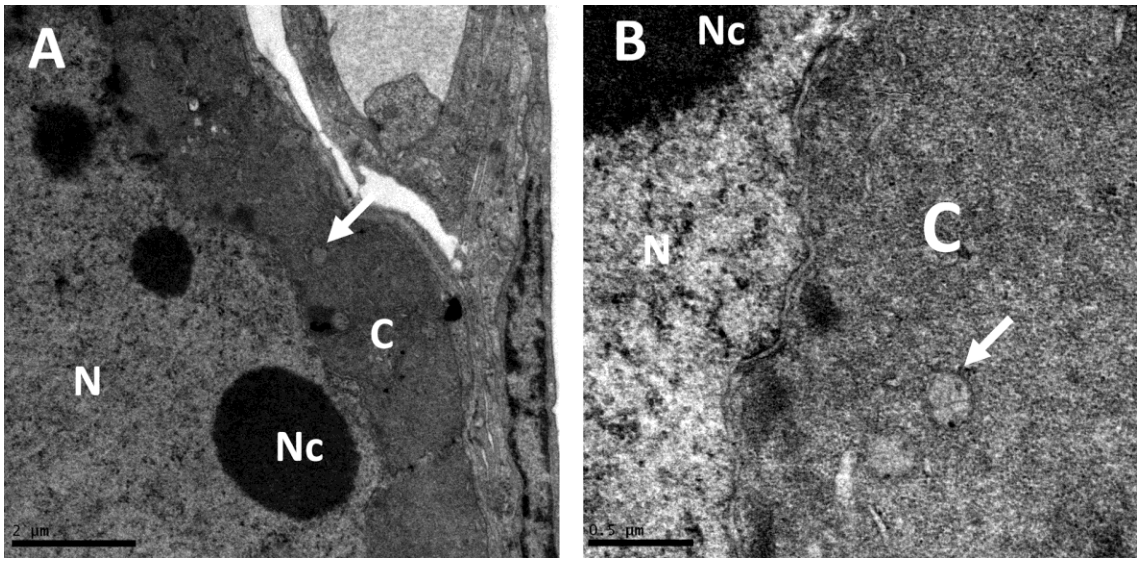

Figure 3. Transmission Electron Microscopy (TEM) showing ultrastructural details of fresh (control) primary growth (PG) oocytes of *Piaractus mesopotamicus.* (A) Section of the nucleus showing the nucleoli close to the nuclear envelope. (B) Section of the cytoplasm showing the rounded mitochondria (arrow) and absence of lipid droplets. N= nucleus; Nc= nucleoli; C= cytoplasm.

Oocyte viability in control group was significantly higher compared to all five vitrified treatments (Figure 2). There was no significant difference among the

vitrified treatments using trypan blue dye or FDA + IP staining. Oocyte viability assessed using histological analysis showed that treatments VS3 (66.43 ± 4.68%) and VS5 (74.14 ± 3.71%) had the lowest viability rate.

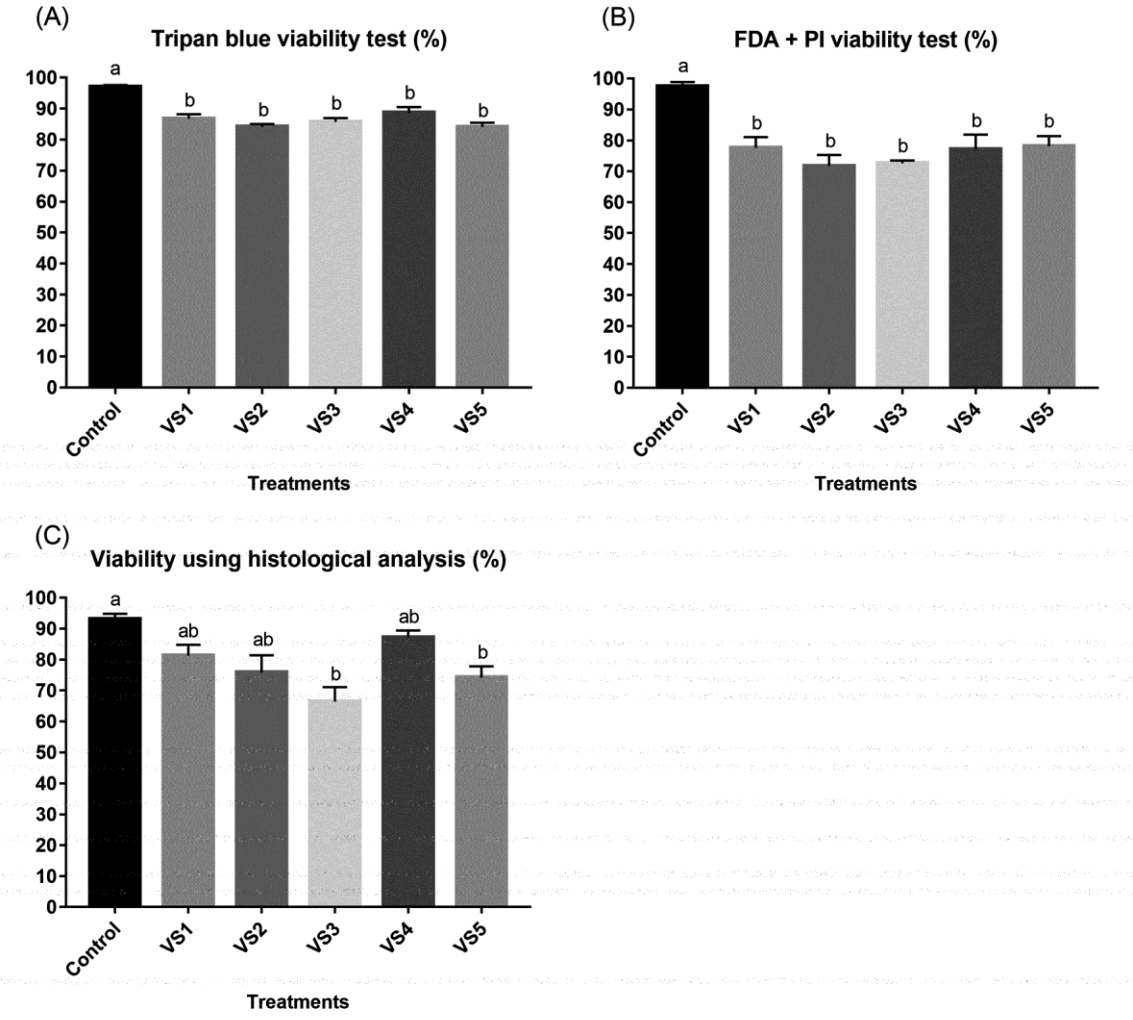

Figure 4. Oocyte viability after vitrification/warming. (A) Oocyte viability was assessed using trypan blue 0.2% (P<0.001). (B) Oocyte viability was assessed using Fluorescein diacetate (FDA) and propidium iodide (PI) staining (P<0.0003). (C) Oocyte viability was assessed using histological analysis (P<0.0024). Each value is mean ± SE. Means with different letters are significantly different. VS1 (1.5 M of methanol and 4.5 M of propylene glycol),

VS2 (1.5 M of methanol and 5.5 M of Me_2SO), VS3 (2.0 M of Me_2SO, 2.5 M of ethylene glycol and 0.5 M of sucrose), VS4 (1.5 M of methanol, 4.5 M of propylene glycol and 0.5 M of sucrose) and VS5 (1.5 M of methanol, 5.5 M of Me_2SO and 0.5 M of sucrose). Control = fresh ovarian tissue fragments.

As well as histological analysis, measurement of cell viability using the MTT assay showed that VS3 (1.63 ± 0.12) and VS5 (1.58 ± 0.09) had the lowest viability rates (Figure 5). The highest values of mitochondrial activity were observed in VS1 (2.39 ± 0.14) and VS4 (2.34 ± 0.19) treatments.

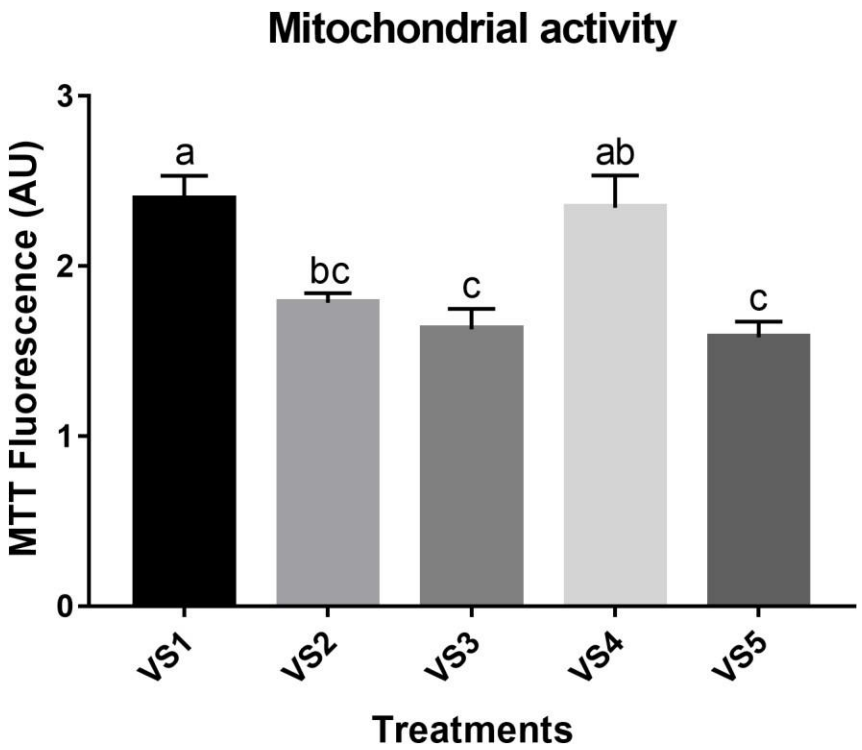

Figure 5. Mitochondrial activity by MTT assay. Mean ± SE followed by different letters differ by Tukey test (P= 0.0002). VS1 (1.5 M of methanol and 4.5 M of propylene glycol), VS2 (1.5 M of methanol and 5.5 M of Me_2SO), VS3 (2.0 M of Me_2SO, 2.5 M of ethylene glycol and 0.5 M of sucrose), VS4 (1.5 M of

methanol, 4.5 M of propylene glycol and 0.5 M of sucrose) and VS5 (1.5 M of methanol, 5.5 M of Me_2SO and 0.5 M of sucrose).

The comparisons of the three viability assessment methods used in this study are shown in Table 3. There was no significant difference among trypan blue dye, FDA + PI staining, and HE-stained histology in the control group and in VS1 and VS2 treatments. However, in VS3, VS4 and VS5 treatments there was significant difference among the three viability assessment methods.

Table 3. Comparison among the different oocyte viability assessment methods after vitrification.

Treatment	Oocyte viability assessment methods			P value*
	Histological analysis (%)	FDA + PI (%)	Trypan blue (%)	
Control	93.00±1.83	97.39 ± 1.44	96.89 ± 0.71	0.0678
VS1	81.29±3.53	77.61 ± 3.44	86.68 ± 1.52	0.1009
VS2	75.86±5.60	71.63 ± 3.64	84.10 ± 0.86	0.0833
VS3	66.43±4.68^{b}	72.57 ± 0.90^{b}	85.67 ± 1.26^{a}	0.0005
VS4	82.71±4.85^{a}	77.06 ± 4.82^{b}	88.63 ± 1.89^{a}	0.0281
VS5	74.14±3.71^{b}	78.10 ± 3.24^{ab}	84.04 ± 1.37^{a}	0.0377

*Mean ± SE followed by different letters on the same line differ significantly from each other by the Duncan's test ($P<0.05$).

The percentage of nucleus and membrane damage indicated by histological analysis is shown in Table 4. There was no significant difference among treatments in membrane damage evaluation. However, there was difference among control (2.43 ± 1.94%, $P<0.05$) and treatments in nucleus damage evaluation. The highest percentages of nucleus damage were observed in treatments VS3 (26.00 ± 5.55) and VS5 (26.00 ± 5.55). There was no difference between membrane and nucleus damage in control, VS2 and VS4 ($P>0.05$).

Table 4. Evaluation of the damages observed in the histological analysis among the treatments and within the treatment.

Treatment	Nucleus damage (%)	Membrane damage (%)	P value[2]
Control	2.43±1.94^{B}	4.57±1.59	0.4090
VS1	17.43±3.29^{Aba}	2.71±1.19^{b}	0.0012
VS2	14.57±3.60^{AB}	9.57±4.99	0.4322
VS3	26.00±5.55^{Aa}	7.57±2.95^{b}	0.0126
VS4	12.00±4.15^{AB}	5.29±1.84	0.1649
VS5	26.00±5.55^{Aa}	4.86±2.47^{b}	0.0045
P value[1]	0.0035	0.5883	-

[1]Mean ± SE followed by different uppercase letters on the same column differ significantly from each other by the Tukey's test (P<0.05).
[2] Mean ± SE followed by different lowercase letters on the same line differ significantly from each other by t-test (P<0.05).

Oocyte and nucleus diameter mean, and the relationship between oocyte and nucleus are shown in Table 5. There was no significant difference among all treatments, including control, in oocyte diameter. However, nucleus diameter was significantly higher in control group (49.03 ± 1.07, P<0.0001). After vitrification/warming procedure, nucleus diameter reduced mainly in the treatment VS4 (40.13 ± 1.02, P<0.0001). The relationship between oocyte and nucleus was higher in control (40.51 ± 0.54) when compared to vitrified treatments.

Table 5. Oocyte and nucleus histomorphometry, and relationship between oocyte and nucleus.

Treatment	Oocyte (µm)	Nucleus (µm)	Relationship (%)
Control	121.29±2.12	49.03±1.07^{a}	40.51±0.54^{a}
VS1	126.08±3.04	43.05±1.10^{bc}	34.73±0.51^{c}
VS2	119.78±2.48	41.40±1.01^{bc}	34.79±0.51^{c}
VS3	121.93±2.68	41.80±1.06^{bc}	34.86±0.68^{c}
VS4	120.43±2.74	40.13±1.02^{c}	33.65±0.50^{c}
VS5	123.76±2.47	45.25±0.99^{ab}	37.14±0.58^{b}
P value*	0.5439	<0.0001	<0.0001

*Mean ± SE followed by diferente letters on the same column differ significantly from each other by the Tukey's test (P<0.05).

Oocyte viability by histological analysis was positive-correlated to the occurrence of nucleus (r^2= 0.78, P<0.01) and membrane (r^2= 0.45, P<0.01) damage after vitrification/warming (Table 6). The percentage of nucleus damage was negative-correlated to the oocyte diameter (r^2= 0.68, P<0.01). The percentage of membrane damage was positive-correlated to the nucleus diameter (r^2= 0.54, P<0.04).

Table 6. Correlations among viability by histological analysis, oocyte and nucleus diameter, and occurrence of damage (nucleus or membrane) in all vitrified treatments.

Variables	Nucleus damage	Membrane damage	Oocyte diameter	Nucleus diameter
Viability	r= -0.78 (<0.01)**	r= -0.45 (<0.01)**	r= -0.46 (0.09)	r= 0.21 (0.46)
Nucleus damage		r= -0.20 (0.25)	r= 0.68 (<0.01)*	r= 0.34 (0.22)
Membrane damage			r= -0.12 (0.66)	r= -0.54 (0.04)**
Oocyte diameter				r= 0.21 (0.46)

Correlation Coefficient (r); P value between parenthesis; *Positive correlation; ** Negative correlation.

4. Discussion

A study of pacu embryo cooling reported a hatching rate of 64.0% after 12 h storage at -8°C [6]. Despite this good result in the cooling of pacu embryos, the same does not occur after embryo freezing. Authors reported a high intracellular ice formation after frozen (-33°C) and thawing pacu embryos [5,11]. According to these authors, crystallization caused irreversible morphological damage and embryonic death. To date success in fish embryo cryopreservation has not been achieved due to their high yolk content, large size, low permeability to

water and cryoprotectants, and their high sensitivity to chilling [22]. On the other hand, oocyte cryopreservation offers several advantages such as the smaller size, much lower water content and absence of a fully developed chorion. Besides oocyte has more permeability to water and solutes than embryo. In the present study, high percentage of PG oocyte viability was observed after warming of vitrified ovarian tissue fragments in all treatments. This result is in agreement with the previously found in zebrafish using the same cryopreservation protocol [10]. PG oocytes are more permeable to water and solutes, and, in contrast to mature oocytes, they do not have lipid droplets, increasing survival rate after cryopreservation.

Oocyte viability following vitrification/warming was evaluating using four methods; however, trypan blue dye and FDA + PI staining did not detect differences among vitrification solutions. A previous study reported that FDA + PI test is more sensitive than trypan blue dye to measure zebrafish oocyte viability after cryopreservation [20]. This is due to the fact that the FDA + IP test, unlike trypan blue, evaluates two cellular functions, i.e. esterase activity and membrane integrity, after staining with fluorescent diacetate (FDA) and propidium iodide (PI) respectively. The FDA stain is transported across the cell membrane into the cytoplasm and in the process the esterase enzymes decoupled the FDA to produce fluorescein, which was retained within the cell [8]. However, these authors used vitellogenic oocytes, in contrast to our study, where the PG oocytes were evaluated. In vitellogenic oocyte, besides the lipid accumulation and the development of a chorion, there are two follicular layers (external theca and internalgranulosa) surrounding the oocyte [9,3]. Thus, in

vitellogenic oocytes membrane damage is more frequent, unlike what occurs in PG oocytes, where the nucleus is more damage by cryopreservation.

Tests on mitochondria metabolism includes MTT and ATP assay [14]. Tsai et al. [18,19] reported that FDA+IP staining and ADP/ATP assay were more sensitive than trypan blue test. In our study, although the histological analysis detected differences among the treatments, the MTT assay was more sensitive to separate the treatments with greater viability (VS1 and VS4) from the ones with the lowest viability (VS3 and VS5). It is likely that this MTT assay response is due to the large number of mitochondria groups present in the PG oocyte cytoplasm.

During primary growth, occurs a continuous synthesis and transport of nuclear RNAs [9]. Probably for this reason, the main damage observed in the present study, during vitrification of PG oocytes, was the pycnotic nucleus. Therefore, the association of histological analysis and MTT assay makes the evaluation of oocyte viability more thorough; since histology allows a detailed evaluation of membranes and nucleus, and MTT assay assessed cell metabolic activity.

Apoptotic volume decrease, leading to cell shrinkage, is one of the major events during the apoptosis process [12]; however, in the present study, oocyte diameter did not decrease after cryopreservation. One possible explanation for this is that the PG oocyte has low water content; hence, it does not suffer significant alteration in cellular volume. On the other hand, nucleus diameter was lower in all treatments when compared to non-cryopreserved oocytes. In

healthy cells, the genomic DNA is packaged into the nucleus in few micrometers in diameter. In apoptosis process, the DNA attains a level of condensation even greater than that observed in mitosis, and it is packaged together with nuclear proteins into "apoptotic bodies" [17]. It is likely that for this reason cryopreserved oocytes showed a diameter decrease in the nucleus.

Although ethylene glycol has a lower formula weight (62.07 g/mol) than either propylene glycol (76.10 g/mol) or Me_2SO (78.13 g/mol), VS3 (2.0 M of Me_2SO, 2.5 M of ethylene glycol and 0.5 M of sucrose) showed the lowest viability rate (63.43%) by histological analysis. VS3 was the only solution that did not contain methanol in its composition. The use of monovalent alcohols is unusual in mammals due to their high toxicity. However, in a 30 min exposure test, methanol was less toxic than Me_2SO for zebrafish embryos [22]. Zhang et al. [21] suggested that methanol, due to its low molecular weight (32), has a high permeability rate, significantly exceeding Me_2SO. Hence, it is suggested that methanol enters and leaves the cell very rapidly due to both its low formula weight and high permeation ability. Thereby cells do not undergo osmotic shock during the freezing and warming procedure.

In the present study, the high permeation, low toxicity and high efficiency of methanol on vitrification of pacu PG oocytes was confirmed. The high viability of PG oocytes obtained after ovarian tissue vitrification of pacu suggests that protocol applied here might be used successfully in other teleost species used for food production. Since immature follicles have smaller size resulting in higher surface area to volume ratio and absence of lipid droplets.

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CAPÍTULO IV

CONSIDERAÇÕES FINAIS

Os Os dados obtidos na criopreservação de tecido ovariano utilizando o modelo biológico zebrafish sugerem que a vitrificação é mais eficaz que o congelamento lento, pois resulta em menor produção de espécies reativas de oxigênio e uma melhor preservação da morfologia dos oócitos em crescimento primário, acarretando uma melhor função tecidual durante a maturação *in vitro*. Assim, o protocolo de vitrificação foi aplicado na criopreservação de tecido ovariano de pacu, em que se obteve um alto percentual de oócitos morfologicamente íntegros. O sucesso obtido na vitrificação de tecido ovariano contendo oócitos em crescimento primário tanto de zebrafish quanto de pacu se deve principalmente à ausência de gotículas lipídicas e ao menor tamanho dos oócitos nesta fase. Portanto, o protocolo de vitrificação desenvolvido aqui é uma alternativa promissora para a criopreservação do genoma materno de peixes teleósteos e deve ser testado em outras espécies de teleósteos de importância econômica, ecológica e cientifica.

As linhagens de peixes utilizadas como modelos de pesquisa de diversas doenças humanas são crescentes, e esses animais são armazenados e mantidos vivos em diversos centros de pesquisas, como por exemplo, o Zebrafish International Resource Center (Universidade de Oregon, Eugene, OR), que detém cerca de 1.080 linhagens endogâmicas, transgênicas, knockout e mutantes. Deste modo, os avanços na criopreservação de gametas, embriões e gônadas são urgentes, pois reduzem os custos de armazenamento e manutenção dessas linhagens. As biotécnica de criopreservação de tecido ovariano desenvolvidas no presente estudo são abordagens úteis para enfrentar esse desafio.

O próximo passo, a fim de tornar a criopreservação de oócitos de peixes uma ferramenta de aplicações práticas em aquicultura, é associar as biotécnicas utilizadas aqui em estudos de maturação *in vitro* de peixes. Um protocolo eficiente de maturação *in vitro* de oócitos de peixes, irá tornar possível maturar esses oócitos previamente criopreservados, para então serem fertilizados e cultivados. Assim, os dados obtidos nesse trabalho são importantes para auxiliar no desenvolvimento de protocolos de preservação do material genético de peixes.

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APÊNDICES

APÊNDICE 1 - *SCIENTIFIC REPORTS* instruções para autores

General information for preparing manuscripts

Format of articles

Scientific Reports publishes original research in one format, Article. In most cases we do not impose strict limits on word count or page number. We do, however, strongly encourage authors to write concisely and to adhere to the guidelines below.

Articles should ideally be no more than 11 typeset pages in length. As a guide, the main text (not including Abstract, Methods, References and figure legends) should be no more than 4,500 words. The maximum Article title length is 20 words. The Abstract — which must be no more than 200 words long and contain no references — should serve both as a general introduction to the topic and as a brief, non-technical summary of the main results and their implications.

For the main body of the text, there are no explicit requirements for section organization. According to the authors' preference, the text may be organized as best suits the research. As a guideline and in the majority of cases, however, we recommend that you structure your manuscript as follows:

Introduction

Results (with subheadings)

Discussion (without subheadings)

Methods

A specific order for the main body of the text is not compulsory and, in some cases, it may be appropriate to combine sections. Figure legends are limited to 350 words. As a guideline references should be limited to 60 (this is not strictly enforced). Footnotes should not be used.

We suggest that Articles contain no more than 8 display items (figures and/or tables). In addition, a limited number of uncaptioned molecular structure graphics and numbered mathematical equations may be included if necessary. To enable typesetting of papers, the number of display items should be commensurate with the word length — we suggest that for Articles with less than 2,000 words, no more than 4 figures/tables should be included. Please note that schemes are not used and should be presented as figures.

Format of manuscripts

In most cases we do not impose strict limits on word counts and page numbers, but we encourage authors to write concisely and suggest authors adhere to the guidelines below. For a definitive list of which limits are mandatory please visit the submission checklist page.

Articles should be no more than 11 typeset pages in length. As a guide, the main text (not including Abstract, Methods, References and figure legends) should be no more than 4,500 words. The maximum title length is 20 words. The Abstract (without heading) - which must be no more than 200 words long and contain no references - should serve both as a general introduction to the topic and as a brief, non-technical summary of the main results and their implications.

The manuscript text file should include the following parts, in order: a title page with author affiliations and contact information (the corresponding author should be identified with an asterisk). The main text of an Article can be organised in different ways and according to the authors' preferences, it may be appropriate to combine sections.

As a guideline, we recommend that sections include an Introduction of referenced text that expands on the background of the work. Some overlap with the Abstract is acceptable. This may then be followed by sections headed Results (with subheadings), Discussion (without subheadings) and Methods.

The main body of text must be followed by References, Acknowledgements (optional), Author Contributions (names must be given as initials), Additional Information (including a Competing Interests Statement), Figure Legends (these are limited to 350 words per figure) and Tables (maximum size of one page). Footnotes are not used.

Methods

Where appropriate, we recommend that authors limit their Methods section to 1,500 words. Authors must ensure that their Methods section includes adequate experimental and characterization data necessary for others in the field to reproduce their work. Descriptions of standard protocols and experimental procedures should be given. Commercial suppliers of reagents or instrumentation should be identified only when the source is critical to the outcome of the experiments. Sources for kits should be identified. Experimental protocols that describe the synthesis of new compounds should be included. The systematic name of the compound and its bold Arabic numeral are used as the heading for the experimental protocol. Thereafter, the compound is represented by its assigned bold numeral. Authors should describe the experimental protocol in detail, referring to amounts of reagents in parentheses, when possible (eg 1.03 g, 0.100 mmol). Standard abbreviations for reagents and solvents are encouraged. Safety hazards posed by reagents or protocols should be identified clearly. Isolated mass and percent yields should be reported at the end of each protocol. Any manuscript reporting an experiment/s on live vertebrates (or higher invertebrates), humans or human samples must

include a statement of ethical approval in the Methods section (see our detailed requirements for further information on preparing these statements).

References

References will not be copy edited by Scientific Reports. References will be linked electronically to external databases where possible, making correct formatting of the references essential.

References should be numbered sequentially, first throughout the text, then in tables, followed by figures; that is, references that only appear in tables or figures should be last in the reference list. Only one publication is given for each number. Only papers or datasets that have been published or accepted by a named publication, recognized preprint server or data repository should be in the numbered list; preprints of accepted papers in the reference list should be submitted with the manuscript. Published conference abstracts and numbered patents may be included in the reference list. Grant details and acknowledgements are not permitted as numbered references. Footnotes are not used.

BibTeX (.bib) bibliography files cannot be accepted. LaTeX submission must either contain all references within the manuscript .tex file itself, or (for authors using the Overleaf template) can include the .bbl file generated during the compilation process as a ‘related manuscript file’ (see the "Format of manuscripts" section for more details).

Scientific Reports uses standard Nature referencing style. All authors should be included in reference lists unless there are six or more, in which case only the first author should be given, followed by 'et al.'. Authors should be listed last name first, followed by a comma and initials (followed by full stops) of given names. Article and dataset titles should be in Roman text, only the first word of the title should have an initial capital and the title should be written exactly as it appears in the work cited, ending with a full stop. Book titles should be given in italics and all words in the title should have initial capitals. Journal and data repository names are italicized and abbreviated (with full stops) according to common usage. Volume numbers and the subsequent comma appear in bold. The full page range should be given (or article number), where appropriate.

Published papers:

Printed journals

Schott, D. H., Collins, R. N. & Bretscher, A. Secretory vesicle transport velocity in living cells depends on the myosin V lever arm length. J. Cell Biol. 156, 35-39 (2002).

Online only

Bellin, D. L. et al. Electrochemical camera chip for simultaneous imaging of multiple metabolites in biofilms. Nat. Commun. 7, 10535; 10.1038/ncomms10535 (2016).

For papers with more than five authors include only the first author’s name followed by ‘et al.’.

Books:

Smith, J. Syntax of referencing in How to reference books (ed. Smith, S.) 180-181 (Macmillan, 2013).

Online material:

Babichev, S. A., Ries, J. & Lvovsky, A. I. Quantum scissors: teleportation of single-mode optical states by means of a nonlocal single photon. Preprint at https://arxiv.org/abs/quant-ph/0208066 (2002).

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Acknowledgements

Acknowledgements should be brief, and should not include thanks to anonymous referees and editors, or effusive comments. Grant or contribution numbers may be acknowledged. Assistance from medical writers, proof-readers and editors should also be acknowledged here.

Figure legends

Figure legends begin with a brief title sentence for the whole figure and continue with a short description of what is shown in each panel in sequence and the symbols used; methodological details should be minimised as much as possible. Each legend must total no more than 350 words. Text for figure legends should be provided in numerical order after the references.

Tables

Please submit tables in your main article document in an editable format (Word or TeX/LaTeX, as appropriate), and not as images. Tables that include statistical analysis of data should describe their standards of error analysis and ranges in a table legend.

Statistical guidelines

Every article that contains statistical testing should state the name of the statistical test, the n value for each statistical analysis, the comparisons of interest, a justification for the use of that test (including, for example, a discussion of the normality of the data when the test is appropriate only for normal data), the alpha level for all tests, whether the tests were one-tailed or two-tailed, and the actual P value for each test (not merely "significant" or "P < 0.05"). It should be clear what statistical test was used to generate every P

value. Use of the word "significant" should always be accompanied by a P value; otherwise, use "substantial," "considerable," etc.

Data sets should be summarized with descriptive statistics, which should include the n value for each data set, a clearly labelled measure of centre (such as the mean or the median), and a clearly labelled measure of variability (such as standard deviation or range). Ranges are more appropriate than standard deviations or standard errors for small data sets. Graphs should include clearly labelled error bars. Authors must state whether a number that follows the ± sign is a standard error (s.e.m.) or a standard deviation (s.d.).

Authors must justify the use of a particular test and explain whether their data conform to the assumptions of the tests. Three errors are particularly common:

- Multiple comparisons: When making multiple statistical comparisons on a single data set, authors should explain how they adjusted the alpha level to avoid an inflated Type I error rate, or they should select statistical tests appropriate for multiple groups (such as ANOVA rather than a series of t-tests).

- Normal distribution: Many statistical tests require that the data be approximately normally distributed; when using these tests, authors should explain how they tested their data for normality. If the data do not meet the assumptions of the test, then a non-parametric alternative should be used instead.

- Small sample size: When the sample size is small (less than about 10), authors should use tests appropriate to small samples or justify their use of large-sample tests.

APÊNDICE 2 - *CRYOBIOLOGY* instruções para autores

Manuscripts must be prepared in double or triple line spacing and lines must be numbered. Pages should be numbered in consecutive order.

Subdivision

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.

Introduction

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.

Material and methods

Provide sufficient details to allow the work to be reproduced by an independent researcher. Methods that are already published should be summarized, and indicated by a reference. If quoting directly from a previously published method, use quotation marks and also cite the source. Any modifications to existing methods should also be described.

Results

Results should be clear and concise.

Avoid redundant tables and figures illustrating the same data.

Discussion

This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Essential title page information

• Title. Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.

• Author names and affiliations. Please clearly indicate the given name(s) and family name(s) of each author and check that all names are accurately spelled. You can add your name between parentheses in your own script behind the English transliteration. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower- case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.

• Corresponding author. Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. This responsibility includes answering any future queries about Methodology and Materials. Ensure that the e-mail address is given and that contact details are kept up to date by the corresponding author.

• Present/permanent address. If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Abstract

A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon

abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

Abstracts of Regular Papers should not exceed 250 words, abstracts of Brief Communications should not exceed 150 words

Keywords

Immediately after the abstract, provide a maximum of 10 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, "and", "of"). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Abbreviations

Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

Use the latest version of the American Chemical Society Style Guide, available at http://pubs.acs.org/styleguide/.

The preferred abbreviation for dimethyl sulfoxide is Me2SO rather than DMSO.

Acknowledgements

Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Formatting of funding sources

List funding sources in this standard way to facilitate compliance to funder's requirements:

Funding: This work was supported by the National Institutes of Health [grant numbers xxxx, yyyy]; the Bill & Melinda Gates Foundation, Seattle, WA [grant number zzzz]; and the United States Institutes of Peace [grant number aaaa].

It is not necessary to include detailed descriptions on the program or type of grants and awards. When funding is from a block grant or other resources available to a university, college, or other research institution, submit the name of the institute or organization that provided the funding.

If no funding has been provided for the research, please include the following sentence:

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Units

Follow internationally accepted rules and conventions: use the international system of units (SI). If other units are mentioned, please give their equivalent in SI.

Temperatures should be expressed on the Celcius scale. Where relevant, Kelvin units may be used, but the equivalent in degrees Celcius must be added in parentheses

Color artwork

Please make sure that artwork files are in an acceptable format (TIFF (or JPEG), EPS (or PDF), or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color online (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in color in the printed version. For color reproduction in print, you will receive information regarding the costs from Elsevier after receipt of your accepted article. Please indicate your preference for color: in print or online only. Further information on the preparation of electronic artwork.

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Ensure that each illustration has a caption. Supply captions separately, not attached to the figure. A caption should comprise a brief title (not on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

Tables

Please submit tables as editable text and not as images. Tables can be placed either next to the relevant text in the article, or on separate page(s) at the end. Number tables consecutively in accordance with their appearance in the text and place any table notes below the table body. Be sparing in the use of tables and ensure that the data presented in them do not duplicate results described elsewhere in the article. Please avoid using vertical rules and shading in table cells.

References

Citation in text

Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these

references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication.

Web references

As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.

Reference style

Text: Indicate references by number(s) in square brackets in line with the text. The actual authors can be referred to, but the reference number(s) must always be given.

Example: '..... as demonstrated [3,6]. Barnaby and Jones [8] obtained a different result ....'

List: The list of references is arranged alphabetically and then numbered (numbers in square brackets).

Examples:

Reference to a journal publication:

[1] J. van der Geer, J.A.J. Hanraads, R.A. Lupton, The art of writing a scientific article, J. Sci. Commun. 163 (2010) 51–59. https://doi.org/10.1016/j.Sc.2010.00372.

Reference to a journal publication with an article number:

[2] J. van der Geer, J.A.J. Hanraads, R.A. Lupton, The art of writing a scientific article, Heliyon 19 (2018) e00205, https://doi.org/10.1016/j.heliyon.2018.e00205.

Reference to a book:

[3] W. Strunk Jr., E.B. White, The Elements of Style, fourth ed., Longman, New York, 2000.

Reference to a chapter in an edited book:

[4] G.R. Mettam, L.B. Adams, How to prepare an electronic version of your article, in: B.S. Jones, R.Z. Smith (Eds.), Introduction to the Electronic Age, E-Publishing Inc., New York, 2009, pp. 281–304.

Journal names should be abbreviated according to CAS (Chemical Abstracts Service): http://www.cas.org/sent.html

VITA

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