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

THAIZA RODRIGUES DE FREITAS

VITRIFICAÇÃO DE TECIDO OVARIANO DE ZEBRAFISH (*Danio rerio*) ENCAPSULADO EM HIDROGEL DE ALGINATO DE SÓDIO

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Vitrificação de tecido ovariano de zebrafish (*Danio rerio*) encapsulado em hidrogel de alginato de sódio

Autor: Thaiza Rodrigues de Freitas
Orientador: Danilo Pedro Streit Jr.
Co-orientadora: Andrea Gianotti Galuppo

Resumo: O zebrafish (*Danio rerio*) é um importante modelo animal e tem se destacado na pesquisa biomédica por sua homologia fisiológica e genética aos humanos. Com isso, diversas linhagens e animais de grande valor genético têm sido desenvolvidos, possuindo assim, grande necessidade de sua preservação. No entanto, a criopreservação de oócitos de peixe ainda é limitada e necessita aprimoramento. O hidrogel de alginato de sódio além de fornecer suporte para as células, tem demonstrado ser um potencial crioprotetor. Portanto, o objetivo deste estudo foi avaliar a eficiência da técnica de encapsulamento em hidrogel de alginato de sódio durante o procedimento de vitrificação do tecido ovariano de zebrafish. No Experimento 1, foram avaliados a forma de encapsulamento (imersão ou em 30 µL de alginato), o momento de exposição ao crioprotetor (antes ou após o encapsulamento) e a temperatura de aquecimento (28, 37 e 50ºC) e foi avaliada a integridade da membrana pela coloração azul de tripan. Os dados mostraram que o tecido ovariano encapsulado por imersão, exposto ao crioprotetor após o encapsulamento e aquecido a 28ºC, apresentou maior integridade de membrana em todas as fases de desenvolvimento dos oócitos (PG: 37.71 ± 3.86 %; CA: 29.93 ± 4.18 %; Vtg1: 18.61 ± 4.69 %) e foi utilizado no Experimento 2. No Experimento 2 foram avaliados quatro grupos vitrificados (VS: Metanol 1,5 M + Me_2SO 5,5 M + sacarose 0,5 M; VS1-A: Metanol 1,5 M + Me_2SO 5,5 M + sacarose 0,5 M - encapsulado em alginato; VS2-A: Metanol 0,75 M + Me_2SO 2,75 M + sacarose 0,25 M - encapsulado em alginato; VA: encapsulado em alginato) e foram avaliados a integridade da membrana (SYBR-14/PI), morfologia (histologia – HE), atividade mitocondrial (MTT) e peroxidação lipídica (TBARS). O tratamento VA demonstrou menor percentagem de integridade de membrana, enquanto VS demonstrou maior integridade de membrana no qual não diferenciou do tratamento VS1-A. A atividade mitocondrial foi maior no tratamento não encapsulado (VS) e os tratamentos encapsulados tiveram menor valor. O tratamento VA obteve o maior nível de peroxidação lipídica, enquanto VS1-A e VS obtiveram os menores valores no qual VS não se diferenciou do tratamento VS2-A. Os resultados obtidos neste trabalho demonstram que a técnica de encapsulamento em hidrogel de alginato de sódio não teve ação crioprotetora e não permitiu a redução da concentração de crioprotetores. No entanto, a partir de observações durante os experimentos, foi encapsulamento dos fragmentos de tecido ovariano em hidrogel de alginato, possibilitou suporte e evitou perda de células durante o processo de vitrificação.

Palavras-chave: Alginato; Tecido ovariano; Zebrafish; Criopreservação; Oócito de peixe.

[1]Dissertação de mestrado em Zootecnia – Produção Animal, Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil. (90 p.) Abril, 2021.

Vitrification of zebrafish (*Danio rerio*) ovarian tissue encapsulated in sodium alginate hydrogel

Author: Thaiza Rodrigues de Freitas
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Co-supervisor: Andrea Gianotti Galuppo

Abstract: Zebrafish (*Danio rerio*) is an important animal model and has stood out in biomedical research for its physiological and genetic homology to humans. Thereby, several lines and animals of great genetic value have been developed, thus having a great need for their preservation. However, the cryopreservation of fish oocytes is still limited and needs improvement. The sodium alginate hydrogel, in addition to providing support for the cells, has been shown to be a potential cryoprotectant. Therefore, the aim of this study was to evaluate the efficiency of the sodium alginate hydrogel encapsulation technique during the zebrafish ovarian tissue vitrification procedure. In Experiment 1, were evaluated the encapsulation form (immersion or 30 µL alginate bead), the moment of exposure to vitrification solution (before or after encapsulation) and the warming temperature (28, 37 and 50 ºC) and was evaluated the membrane integrity by trypan blue stain. Data showed that ovarian tissue encapsuled by immersion, exposed to vitrification solution after encapsulation and warmed at 28ºC, had higher membrane integrity of all oocyte developmental stage (PG: 37.71 ± 3.86 %; CA: 29.93 ± 4.18 %; Vtg1: 18.61 ± 4.69 %) and was used in Experiment 2. In Experiment 2 were evaluated four vitrified groups (VS: 1.5M Methanol + 5.5M Me_2SO + 0.5M sucrose; VS1-A: 1.5M Methanol + 5.5M Me_2SO + 0.5M sucrose – encapsulated in alginate; VS2-A: 0.75M Methanol + 2.75M Me_2SO + 0.25M sucrose – encapsulated in alginate; VA: encapsulated in alginate) and were evaluated the membrane integrity (SYBR-14/PI), morphology (histology – HE), mitochondrial activity (MTT), and lipid peroxidation (TBARS). The VA treatment showed a lower percentage of membrane integrity, while VS demonstrated a higher membrane integrity in which it did not differ from the VS1-A treatment. Mitochondrial activity was greater in the non-encapsulated treatment (VS) and the encapsulated treatments had less value. The VA treatment obtained the highest level of lipid peroxidation, while VS1-A and VS obtained the lowest values in which VS was not different from the VS2-A treatment. The results obtained in this study demonstrate that the sodium alginate hydrogel encapsulation technique did not have a cryoprotective action and did not allow the reduction of the CPA concentration. However, the encapsulation of the fragments of ovarian tissue in alginate hydrogel, enabled support and prevented loss of cells during the vitrification process.

Key words: Alginate; Ovarian tissue; Zebrafish; Cryopreservation; Fish oocyte.

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RELAÇÃO DE FIGURAS

CAPÍTULO I

CAPÍTULO II

LISTA DE ABREVIAÇÕES

%	percentagem
±	mais ou menos
=	igual a
<	menor que
>	maior que
μg	micrograma(s)
μm	micrômetro(s)
μL	microlitro(s)
^{o}C	graus célsius
ATP	adenosina trifosfato
CA	alvéolo cortical
CPA	agente crioprotetor
CP	crescimento primário
DNA	ácido desoxirribonucleico
ERO	espécies reativas de oxigênio
FDA	diacetato de fluoresceína
g	grama(s)
h	hora
HE	hematoxilina-eosina
IP	iodeto de propídio
M	molar
Me_2SO	dimetilsulfóxido
mg	miligrama(s)

min	minuto(s)
mL	mililitro(s)
mm	milímetro(s)
P	probabilidade de erro
PBS	phosphate buffered saline (tampão fosfato-salino)
PG	primary growth (crescimento primário)
pH	potencial hidrogeniônico
PI	propidium iodide
s	segundo(s)
Vtg1	oócito vitelogênico primário
Vtg2	oócito vitelogênico secundário
Vtg3	oócito vitelogênico terciário

SUMÁRIO

CAPÍTULO I

1. INTRODUÇÃO

O zebrafish (*Danio rerio*) é um organismo modelo importante em diversos campos de pesquisa (Kalueff et al., 2014), uma vez que seu genoma é aproximadamente 70% semelhante ao dos humanos (Howe et al., 2013). Além disso, laboratórios em todo o mundo produziram diversas linhagens zebrafish mutantes, transgênicos e selvagens na última década (Hagedorn & Carter, 2011). A preservação desses valiosos genótipos é particularmente importante e apresenta desafios significativos e urgentes e para isso, a criopreservação de tecidos e gametas tem se mostrado uma ferramenta eficiente capaz de suprir essa demanda. Particularmente em peixes, a criopreservação de gametas é uma biotecnologia promissora para a conservação de material genético que também pode ser aplicada a espécies ameaçadas de extinção e espécies de importante valor comercial. Protocolos de criopreservação de espermatozoides têm sido amplamente estudados para muitas espécies de peixes diferentes, principalmente para salmonídeos de água doce e ciprinídeos, tornando a criopreservação de gametas masculinos bem estabelecida e comercializada (Asturiano et al*.,* 2017). Porém, a criopreservação espermática preserva apenas o genoma paterno o que não é suficiente para manter a diversidade genética, que também depende do genoma de origem materna (Zhang et al., 2007). Estudos anteriores mostraram que a criopreservação de fragmentos de tecido ovariano é uma alternativa promissora para a conservação de oócitos de peixes (Marques et al., 2015; Lujić et al*.*, 2017; Marques et al., 2018; Marques et al*.*, 2019). A principal vantagem da criopreservação do tecido ovariano é a maior permeabilidade da membrana oocitária quando comparada ao embrião, que possui um córion totalmente formado (Zhang et al., 2005 Tsai et al., 2009b). Recentemente, foi demonstrado que a vitrificação foi mais eficiente do que o congelamento lento na prevenção de danos mitocondriais, além de evitar o estresse oxidativo no tecido ovariano do zebrafish (Marques et al., 2019). A vitrificação é uma técnica de criopreservação a partir de resfriamento ultrarrápido, que evita a formação de cristais de gelo por meio de alta concentração de agentes crioprotetores (CPAs) (Rall & Fahy; 1985). Durante o procedimento de criopreservação, o CPA tem o papel de proteger as células de danos por congelamento (ou seja, devido à formação de gelo). No entanto, de acordo com Best (2015) os crioprotetores podem ser considerados tóxicos, principalmente quando usados em altas concentrações e em condições não

ideais, o que pode causar ruptura ou dano às membranas celulares, redução da função mitocondrial, dano a proteínas ou outras macromoléculas entre outros efeitos tóxicos. Para superar esses problemas, a vitrificação com baixa concentração de CPA precisa ser explorada para a criopreservação de células e tecidos.

Na tentativa de promover maior proteção em relação aos danos causados pelos procedimentos de criopreservação de células e tecidos, o encapsulamento celular em biomateriais como o hidrogel tem se mostrado capaz de reduzir o uso de altas concentrações de CPAs sem comprometer os resultados na vitrificação (Weng & Beauchesne, 2020). O alginato de sódio é um polissacarídeo aniônico produzido pela alga marrom que, quando em contato com os íons cálcio, é reticulado, formando o hidrogel de alginato. Suas características incluem biodegradabilidade, biocompatibilidade, não toxicidade e capacidade de absorver grande quantidade de água (Leong et al., 2016), além disso esta substância apresenta propriedades antioxidantes. (Król et al., 2017). A reticulação iônica de alginato apresenta vantagens como a baixa quantidade de sal necessária para formar o gel e o controle da reticulação pela concentração de alginato (Devolder & Kong, 2012). A estrutura semelhante a um gel dentro do hidrogel de alginato permite que as células encapsuladas tenham um ambiente semelhante a uma matriz extracelular (ECM) (Gattazzo et al., 2014). O encapsulamento em hidrogel de alginato, mantém a estrutura celular *in vitro* que morfologicamente se assemelha ao ambiente *in vivo*, com melhor porosidade, proliferação celular e resistência mecânica (Kreeger et al., 2006). O hidrogel de alginato é utilizado em engenharia de tecidos (SAROIA et al., 2018), estrutura para cultura de células (WEST et al., 2007) e transplante de células / tecidos (Poels et al., 2016).

Estudos utilizando hidrogel de alginato para criopreservação de células de mamíferos, demonstraram que, a encapsulação em hidrogel de alginato pode reduzir significativamente a concentração e até mesmo eliminar os CPAs. O encapsulamento de células-tronco mesenquimais de camundongo em hidrogel de alginato pode reduzir a concentração de dimetilsulfóxido (Me_2SO) para 1,5 M para vitrificação, resultando em 88,9 ± 2,9% da integridade da membrana (Zhang et al., 2010). Um estudo com vitrificação de células-tronco embrionárias murinas e células-tronco derivadas de tecido adiposo humano encapsuladas em hidrogel de alginato, apresentaram pelo menos 80% de integridade de membrana, utilizando 1,5 M 1,2-propanodiol (PROH) e 0,5 M trealose para microcapilares de quartzo e 2 M PROH e trealose 1,3 M para

palheta de plástico (Huang et al*.,* 2015). Fibroblastos humanos encapsulados em hidrogel de alginato criopreservado por resfriamento lento, na ausência de CPA, exibiram uma atividade metabólica semelhante aos fibroblastos tratados com Me_2SO e trealose (Mohanty et al., 2016). Recentemente, um estudo realizado com vitrificação de tecido ovariano de zebrafish utilizando solução de alginato de sódio (sem encapsular) sem crioprotetores internos, resultou entre 40 e 60% de integridade de membrana do total de oócitos do tecido ovariano (todos os estágios de desenvolvimento) (Gomes, 2020). No entanto, não há estudo utilizando o encapsulamento em hidrogel de alginato de sódio em células de peixes criopreservadas.

Portanto, o objetivo deste estudo foi avaliar a eficiência da técnica de encapsulamento em hidrogel de alginato de sódio na vitrificação de fragmentos de tecido ovariano de zebrafish, visando aprimorar o protocolo de vitrificação.

2. REVISÃO BIBLIOGRÁFICA

2.1 Criopreservação

A criopreservação foi desenvolvida a partir dos conhecimentos da criobiologia, ciência que estuda a vida em baixas temperaturas, que tem como objeto de estudo qualquer material biológico submetido à uma temperatura abaixo de sua variação fisiológica. A criopreservação preserva a viabilidade das células ou dos tecidos armazenados em baixas temperaturas, em estado de quiescência, induzindo à interrupção das reações enzimáticas, possibilitando, após o aquecimento do sistema, a restauração da atividade metabólica (Mazur, 1977). Geralmente, os materiais biológicos são estocados em gelo seco (-80 °C), em ultra freezer (-80 ºC), suspensos em vapor de nitrogênio líquido (abaixo de -130 °C) ou imersos em nitrogênio líquido (-196 °C). Nessas temperaturas os níveis de energia cinética são demasiadamente baixos para permitir o movimento molecular (Grout et al., 1990), e qualquer atividade biológica, incluindo reações bioquímicas que levariam ao envelhecimento e morte celular são efetivamente paralisadas (Mazur, 1984). Uma das primeiras pesquisas que obtiveram sucesso na criopreservação de gametas foi relatada por Polge et al. (1949), com espermatozoides de galo. A primeira criopreservação de sêmen de peixes bem sucedida foi em 1953, utilizando arenque (Blaxter, 1953). Whittingham (1972) e Wilmut (1972) foram os primeiros a criopreservar embriões de camundongos e Trounson (1983) embriões humanos. Desde então, a criopreservação de gametas e tecidos, vem sendo estudada por inúmeros pesquisadores devido ao seu potencial no tratamento da infertilidade humana, estocagem de DNA de animais de alto valor zootécnico, transgênicos ou espécies em perigo de extinção (Taylor et al., 2019). Para a célula ou tecido alcançar a baixas temperaturas, ocorrem reações bioquímicas que podem causar a morte celular, sendo deste modo, importante estudar os mecanismos físicos e biológicos relacionados à lesão celular oriundos do resfriamento. A partir deste contexto, o principal objetivo do processo de criopreservação é minimizar a lesão do tecido por baixas temperaturas abaixo de zero (Shaw & Jones, 2003).

2.2 Crioinjúrias

Os danos causados pelo processo de criopreservação são chamados de crioinjúrias, sendo que sua origem pode ser pela exposição aos crioprotetores e a

redução de temperatura e posterior aquecimento da amostra (Gosden, 2011), causando danos celulares estruturais e metabólicos. Uma outra definição quanto a origem das crioinjúrias, foi definida por Fickel et al., (2007) que relacionam a interação entre as mudanças biofísicas, bioquímicas e ambientais ocorridas durante o processo de criopreservação.

As células e tecidos são ocupadas em grande proporção por água, assim a transição da água no estado líquido para o estado sólido é a preocupação central durante o processo de criopreservação (Bakhach, 2009). Quando a temperatura diminui e atinge o ponto de congelamento, ocorre o processo de nucleação da água e formação de cristais de gelo (John morris & acton, 2013). A formação de cristais de gelo no meio intracelular, é um dos principais causadores de dano no processo de criopreservação, ocasionando rompimento de membrana e morte celular (Kopeika et al,, 2015). O gelo intracelular acomete as cristas e a matriz mitocondrial (Bonetti et al., 2011; Mazoochi et al., 2008), fato que pode prejudicar seriamente o metabolismo celular (Shahedi et al., 2013). Assim, os protocolos de criopreservação visam desidratar o espaço intracelular, consequentemente, minimizando a formação de gelo intracelular, mantendo a concentração de soluto baixa (Smith & Silva, 2004). De acordo com os conceitos clássicos de Criobiologia definidos por Mazur (1977) os cristais de gelo intracelulares podem ser inofensivos se o seu tamanho for controlado. Porém, o mesmo autor observa, que os cristais de gelo são instáveis em um aquecimento lento e podem agregar-se, formando cristais maiores (nucleação). O resultado final, de acordo com Mazur (1977), é o crescimento de cristais de gelo através dos canais aquosos na membrana plasmática, que ocorrem devido aos cristais de gelo extracelulares.

Os cristais de gelo extracelulares são causados devido ao aumento da pressão osmótica no espaço extracelular, criando um gradiente osmótico através da membrana plasmática, o que resulta em água fluindo para fora da célula e congelando externamente (Mazur, 1984). Essa formação de gelo extracelular pode gerar lesões mecânicas nas células e aumenta a concentração do soluto extracelular, resultando em desequilíbrio entre o meio intracelular e a solução extracelular ainda não congelada, com isso, a célula desidrata e não congela (Mazur, 1984). A desidratação celular pode levar a cristalização de sais tampões que resulta em importantes

variações de pH com a consequente desnaturação irreversível de certas proteínas (Karow & Webb, 1965).

As baixas temperaturas também podem produzir danos às células, chamada de lesão por frio, que de acordo com Levitt (1980) é definida como estresse de baixa temperatura na ausência de congelamento. Uma mudança brusca de temperatura (choque térmico) durante o resfriamento pode resultar em lesões nas membranas celulares, mesmo na ausência de formação de cristais de gelo (Farrant & Morris, 1973). O dano também pode ser causado quando as células são mantidas em temperaturas críticas abaixo de sua temperatura fisiológica. De acordo com Watson & Morris (1987) a lesão por frio é um dos fatores limitantes para alcançar a criopreservação ideal, e neste caso produz danos termotrópicos às membranas celulares, produzindo em desequilíbrios metabólicos e mudanças na fluidez da membrana.

Durante o resfriamento, a transição da fase lipídica da fase líquido-cristalina para a fase gel resulta no vazamento de solutos através das membranas (Watson & Morris, 1987), danificando a célula. Este fenómeno pode ser constatado no estudo de Rauen et al. (1999), observando que devido ao rápido resfriamento dos tecidos animais, as mitocôndrias das células tornaram-se mais permeáveis e resultam em danos celulares. Por outro lado, Liu (2000) afirmou que a taxa de resfriamento mais rápida produz tensão aumentada para uma dada redução de temperatura. Após a transição de fase, muitas propriedades biológicas da membrana são alteradas, o que leva à alteração das atividades da membrana, proteínas e enzimas associadas à membrana (Cossins, 1983). Devido a variação de temperatura, ocorre o distúrbio do equilíbrio homeostático, resultante dos diferentes efeitos do frio sobre os processos enzimáticos e não enzimáticos no interior da célula. Esta condição resulta em: desnaturação de proteínas (Lattman et al., 1994) e induz ao estresse oxidativo, levando a produção de espécies reativas de oxigênio (Mckersie et al., 1990; Scandalios, 1993; Tsang et al., 1991). E como resultado final, pode causar a desnaturação de proteínas, peroxidação de lipídeos e apoptose celular (Prasad, 1996; Wood & Youle, 1995).

O processo de criopreservação (resfriamento e aquecimento) aumenta a produção de Espécies reativas a oxigênio (ERO) e causa alterações no metabolismo oxidativo (Dowling & Simmons, 2009). Este processo está relacionado aos estresses

resultantes das mudanças de volume e consequentes alterações nas concentrações de íons e eletrólitos das soluções crioprotetoras intra e extracelulares (Stornelli et al., 2005). As ERO podem causar danos ao DNA, lipídeos, proteínas e açúcares (Evans et al., 2004), que por consequência afetam o desenvolvimento e sobrevivência da célula. Além disso existem os fatores físicos, como a manipulação e mudanças de pressão que podem estar envolvidos na destruição das células (Ashwood-Smith et al., 1988; Schneider & Mazur, 1987).

2.3 Crioprotetores

Para evitar os possíveis danos no processo de criopreservação são utilizados crioprotetores (CPAs), que são compostos químicos naturais ou sintéticos. Entre as funções dos crioprotetores, a principal é remover e/ou substituir o líquido intracelular (Ebertz & Mcgann, 2004) e assim, reduzir a quantidade de gelo formado no processo de criopreservação. O crioprotetores são necessários para aumentar a permeabilidade da membrana e a desidratação parcial da célula, diminuindo o ponto de congelamento, e reduzindo assim a formação de cristais de gelo intracelulares (Rosato & Iaffaldano, 2013). Para este objetivo, existem dois tipos de crioprotetores, os permeáveis e não permeáveis (Borini et al., 2006).

2.3.1 Crioprotetores permeáveis

Os crioprotetores permeáveis são substâncias químicas de alta solubilidade e de baixo peso molecular capazes de se difundir através da membrana plasmática e desempenhar sua ação crioprotetora por todo o citoplasma e organelas (Hubálek, 2003). Os crioprotetores intracelulares mais comumente utilizados são: etileno glicol (EG), Me_2SO, metanol, propileno glicol e o glicerol (Elliott et al., 2017). Esses crioprotetores têm a capacidade de reduzir a concentração de solutos prejudiciais, aumentar a fração descongelada e reduzir a desidratação durante o congelamento e o descongelamento (Mazur, 2004). Os crioprotetores permeáveis entram nas células em resposta às moléculas de água que saem das células devido à formação de gelo extracelular. Isso diminui consideravelmente o ponto de congelamento, resultando na prevenção da formação de gelo intracelular (Shepard et al., 1976).

O Me_2SO é o crioprotetor mais utilizado na criopreservação de ampla variedade de tipos de células, tem peso molecular 78 g/mol e passa rapidamente através das

membranas (Hubálek, 2003), substituindo rapidamente a água que saiu da célula, impedindo a desidratação e formação de gelo intracelular. O etileno glicol e propileno glicol, devido à sua alta viscosidade, promovem pequena cristalização granular e solidificação amorfa em baixas temperaturas, diminuindo a quantidade de água dentro das células e reduzindo a formação de gelo intracelular (Wu & Lee, 1996). O metanol, devido ao seu baixo peso molecular (32 g/mol), tem uma alta taxa de permeabilidade, superando significativamente o Me_2SO (Zhang et al., 2005). No entanto, o uso de álcoois monovalentes é pouco frequente, devido à sua alta toxicidade.

2.3.2 Crioprotetores não permeáveis

Os crioprotetores não permeáveis são normalmente açúcares ou polímeros de cadeia longa e de alto peso molecular e, portanto, não conseguem penetrar no interior das células (Hubálek, 2003). A sacarose, glicose e frutose são os açúcares mas utilizados como crioprotetor não permeável. O leite em pó e a gema de ovo também são exemplos de crioprotetores não permeáveis mais utilizados, no qual são fontes ricas em proteínas e lipídeos. Os crioprotetores não permeáveis exercem gradiente osmótico, fazendo com que a água intracelular migre para o ambiente extracelular (Kopeika et al., 2005), resultando na redução da formação de gelo intracelular durante o congelamento. Por serem compostos hidrofílicos, eles se ligam às moléculas de água, aumentando a viscosidade da solução e diminuindo a formação de cristais de gelo extracelular (Fahy, 2007). Além disso, os crioprotetores não permeáveis também melhoram a vitrificação das soluções, estabilizam proteínas e membranas e evitam a formação progressiva de gelo (Fahy et al., 1984; Fahy, 1986; Takahashi et al., 1986).

Os crioprotetores não permeáveis geralmente são utilizados em combinação com crioprotetores permeáveis. Para suportar os crioprotetores permeáveis na vitrificação, açúcares são adicionados para contribuir para a viscosidade e tonicidade do meio de vitrificação, permitindo o uso de concentrações mais baixas de crioprotetores permeáveis e, assim, diminuindo os efeitos de choque osmótico e citotóxico (Best, 2015). Os crioprotetores não permeáveis induzem a desidratação e o encolhimento osmótico da célula, diminuindo o risco de cristalização intracelular do gelo. Além de ajudar a controlar a reidratação celular durante o aquecimento (Yavin et al., 2009).

2.3.3 Toxicidade dos crioprotetores

Embora os crioprotetores protejam as células dos danos causados no processo de criopreservação, eles podem causar danos quando usados em altas concentrações (Fahy, 1986), podendo resultar na ruptura ou danos às membranas celulares, redução da função mitocondrial, danos as proteínas ou outras macromoléculas entre outros efeitos tóxicos (Best, 2015). A adição e remoção dos crioprotetores causam um estresse osmótico inevitável a célula e suas membranas (Armitage, 1987). Os crioprotetores tóxicos podem causar danos nas proteínas por desidratação, ao se ligarem a moléculas de água, impedindo que as mesmas hidratem adequadamente proteínas e outras macromoléculas (Clegg et al., 1982). Talvez este fato explique que cada crioprotetor tem seu próprio mecanismo de toxicidade, apresentam diferentes efeitos físico-químicos e osmóticos (Fahy et al., 2004).

A toxicidade dos diferentes crioprotetores pode ser mais ou menos intensa para diferentes células, tecidos ou organismos. O metanol afeta as membranas celulares, interagindo com fosfolipídios e desestabilizando a bicamada lipídica das membranas (Hoetelmans et al., 2001). No entanto, o metanol é menos tóxico para embriões de zebrafish (Zhang et al., 1993), oócitos (Plachinta et al., 2004), folículos ovarianos isolados (Tsai et al., 2009a; Zampolla et al., 2008) e espermatozoides de várias espécies de peixes (Lahnsteiner et al., 1997). O Me_2SO pode aumentar a concentração de íons cálcio no citoplasma, causando diferentes respostas metabólicas, como despolimerização e montagem do citoesqueleto (Yamamoto, 1989), além de poder causar deformação da membrana e desnaturação de proteínas (Parkes & Smith, 1953). Já o propileno glicol, é caracterizado por uma baixa toxicidade, sendo considerado um composto não-genotóxico ou carcinogênico (Aye et al., 2010).

A toxicidade dos crioprotetores é um dos maiores obstáculos da criopreservação de células e tecidos. Estratégias, como o uso de crioprotetores menos tóxicos e/ou a combinação de dois ou três crioprotetores, tem sido sugeridas nos procedimentos de criopreservação (Vajta & Nagy, 2006). Outra alternativa são as tentativas de otimizar, as taxas de resfriamento e aquecimento ou o tempo de adição de crioprotetores individuais durante o resfriamento (Best, 2015).

2.4 Vitrificação

Os protocolos de criopreservação podem ser classificados em dois grandes grupos: lentos (congelamento lento) e super-rápidos (vitrificação). Se diferem de acordo com a curva de resfriamento e concentração de crioprotetores utilizados (Rosato & Iaffaldano, 2013). Independente do protocolo de congelamento adotado, lento ou super-rápido, ambos, baseiam-se nos mesmos princípios, proteger as células dos danos devido à formação de gelo intracelular, da desidratação celular excessiva e das alterações nas concentrações de solutos. Os protocolos de criopreservação são constituídos basicamente pela exposição à solução crioprotetora; resfriamento e redução gradual da temperatura (congelamento lento) ou redução rápida da temperatura (vitrificação); estocagem em nitrogênio líquido (NL); descongelamento ou aquecimento e remoção da solução crioprotetora (Santos et al*.,* 2008).

A vitrificação surgiu como uma alternativa ao congelamento lento, como a primeira criopreservação “livre de gelo”, utilizando embrião de camundongo (Rall & Fahy, 1985). É uma técnica que possui a taxa de resfriamento extremamente rápida (> 20.000 °C/min ou superior) (Vajta, 2013), no qual utiliza soluções aquosas contendo altas concentrações de crioprotetores (Rall & Fahy, 1985). A alta concentração de crioprotetores aumenta a viscosidade do meio e as células apresentam algumas características semelhantes ao estado líquido e outras próprias de um sólido cristalino (Yamaki et al., 2002). Consequentemente, a água passa do estado líquido para o vítreo, sem exposição ao estágio cristalino (Rall & Fahy, 1985). Assim, as taxas de resfriamento ultrarrápidas reduzem as lesões decorrentes do resfriamento, da porção lipídica das membranas celulares e do citoesqueleto, por passar rapidamente pelas zonas térmicas críticas (Vajta & Kuwayama, 2006).

O método de vitrificação, além de ser um procedimento extremamente rápido, apresenta a vantagem do custo reduzido, já que não requer a utilização por exemplo de equipamentos como freezer de congelamento programável (Yavin et al., 2009). No entanto, o uso de altas concentrações de crioprotetores permeáveis requer atenção, pois aumenta a ocorrência de lesões tóxicas e osmóticas. Portanto, a vitrificação com baixa concentração de crioprotetores precisa ser explorada para a criopreservação de células e tecidos.

2.5 Hidrogel de alginato de sódio

O alginato de sódio é um polissacarídeo aniônico produzido por algas marinhas pardas e portanto, é uma substância biodegradável, biocompatível e não tóxico (Leong et al., 2016). Além disso, esta substância apresenta potencial (propriedades) antioxidantes (Król et al., 2017) e possui capacidade de reter grandes quantidades de líquidos (Josef, 2010). O hidrogel de alginato de sódio é obtido através da reticulação entre o alginato e alguns cátions divalentes ou trivalentes, onde os cátions mais comuns são o Ca^{2+}, Ba^{2+} e Sr^{2+} (Day et al., 2000; Day & Harding, 2008). A reticulação iônica do alginato traz vantagens como a baixa quantidade de sal necessária para a formação do gel, a não toxicidade do sal para as células e o controle da reticulação pela concentração do alginato e do sal (Devolder & Kong, 2012). Soma-se ainda o fato de ser um biomaterial de fácil manipulação, sendo possível a produção de esferas (*beads*), a temperatura ambiente, sem a necessidade de equipamentos complexos, apenas com o uso de béqueres e seringas podendo ser realizado em qualquer laboratório (Leong et al., 2016).

O encapsulamento de células animais em hidrogel de alginato de sódio teve início final dos anos 1980, com as Ilhotas de Langherans e hepatócitos, visando prevenir a rejeição imunológica no transplante dessas células (Bruni & Chang, 1989; Mazaheri et al., 1991; O'shea & Sun, 1986). Nesses estudos foi descoberto que o hidrogel de alginato permite a difusão de pequenas moléculas e metabólitos para dentro e para fora das células transplantadas. Com isso, o encapsulamento das células e tecidos em hidrogel de alginato tem sido muito aplicado em engenharia de tecidos, como uma estrutura 3D para células em suspensão, permitindo a passagem dos nutrientes do meio de cultura para as células. O hidrogel de alginato foi utilizado como estrutura para apoiar o crescimento *in vitro* de folículos isolados de tecido ovariano humano (Yin et al., 2016), e mais recentemente na criopreservação de células de mamíferos (Benson *et al.*,2018). Na tentativa de promover maior proteção em relação aos danos causados pelos procedimentos de criopreservação de células e tecidos, o encapsulamento celular em biomateriais de hidrogel tem se mostrado capaz de reduzir o uso de altas concentrações de crioprotetores sem comprometer os resultados de vitrificação (Mohanty et al., 2016; Weng & Beauchesne, 2020). Soma-se ainda, vantagens para a manipulação das células no processo de criopreservação (Camboni et al., 2013).

Estudos utilizando hidrogel de alginato para criopreservação de células de mamíferos, demonstraram que o encapsulamento em hidrogel pode reduzir significativamente a concentração e até mesmo eliminar os crioprotetores tradicionais. O encapsulamento de células-tronco mesenquimais de camundongo em hidrogel de alginato usando concentração de Me_2SO 1,5 M para vitrificação resultou em 88% da integridade da membrana, enquanto as células não encapsuladas, com a mesma concentração de crioprotetores, resultaram em apenas 42% (Zhang et al., 2010). Um estudo com vitrificação de células-tronco embrionárias murinas encapsuladas e células-tronco humanas derivadas do tecido adiposo em hidrogel de alginato resultaram em 80% de integridade de membrana utilizando uma concentração 4 vezes menor de crioprotetor, onde o autor notou que as células encapsuladas tiveram um grande impacto na inibição da formação de gelo intracelular durante o aquecimento (Huang *et al.,* 2015). O congelamento lento de fibroblastos humanos encapsulados em hidrogel de alginato, na ausência de crioprotetores, resultou em uma atividade metabólica semelhante à dos fibroblastos criopreservados com Me_2SO e trealose (Mohanty et al., 2016).

O encapsulamento em hidrogel de alginato de folículos ovarianos humanos permitiu, além de uma estrutura para os folículos em suspensão, auxiliar como crioprotetor em conjunto com outros crioprotetores convencionais. Um estudo conduzido por Camboni et al. (2013) com congelamento lento de folículos primordiais humanos encapsulado em hidrogel de alginato junto com 1,4 M Me_2SO + 2% Albumina sérica humana (HSA), os autores obtiveram 93,2% de integridade de membrana (avaliada com sondas fluorescentes). Folículos pré-antrais humanos encapsulados em hidrogel de alginato por Bian et al. (2013) foram vitrificados com 40% Etileno Glicol (EG), 0,6 mol/l sacarose, e 20% HSA e apresentaram 81,25% de integridade de membrana quando avaliados por marcadores “live” (calceína-AM) e “dead“ (homodímero de etídio-1). No entanto, folículos pré-antrais bovinos encapsulados e vitrificados juntamente com 15% Me_2SO, 15% EG, 0,5 M sacarose, apresentou menor integridade de membrana (45,9%) quando comparado com folículos não encapsulados e vitrificados com a mesma solução de vitrificação (87,5%), em que o autor cita que a vantagem do uso do alginato seria para auxiliar na manipulação dos folículos (Bus et al., 2018).

Os resultados descritos evidenciam que o encapsulamento celular em hidrogel de alginato de sódio não apenas pode substituir ou reduzir os crioprotetores

tradicionais, como também pode aprimorar protocolos junto com os crioprotetores e dar suporte para as células em suspensão no processo de criopreservação. No entanto, não há estudos de células de peixes encapsuladas em hidrogel de alginato sódio e criopreservadas até o momento.

2.6 Estágios de desenvolvimento dos oócitos de peixes teleósteos

Devido a grande variedade de espécies de peixes e estudos sobre estes, diversas terminologias para descrever a fase reprodutiva e estágios de desenvolvimento dos gametas foram descritas. Isso tornou difícil para cientistas e gestores de recursos se comunicarem e fazer comparações entre os estudos, e com isso, Brown-Peterson et al. (2011) padronizou as terminologias sobre o desenvolvimento reprodutivo de peixes.

A oogênese é iniciada a partir da oogônia, uma vez que iniciam a meiose, entram em estágio de desenvolvimento do nucléolo da cromatina, são considerados oócitos (Lowerre-Barbieri et al., 2011). Os desenvolvimento dos oócitos são descritos em Crescimento primário (CP ou PG em inglês) e Crescimento secundário (CS ou SG em inglês). Quando a meiose é interrompida e há o desenvolvimento do corpúsculo de Balbani os oócitos são caracterizados como oócitos em crescimento primário (CP) (Gier et al., 2009). O crescimento secundário é composto pelos estágios alvéolo cortical (CA) e vitelogênicos (Vtg) (Brown-Peterson et al., 2011). A oogênese temina na maturação e ovulação.

Os oócitos CA estão em estágio previtelogênico em que os oócitos aumentam de tamanho e, muitas vezes, adquirem gotículas de óleo (Lowerre-Barbieri et al., 2011) (Fig. 1), sua formação depende de gonadotropina (Lubzens et al., 2010). Já o estágio vitelogênico é dividido em três fases, vitelogênico primário (Vtg1), vitelogênico secundário (Vtg2) e vitelogênio terciário (Vtg3) (Fig.1). Os oócitos Vtg1 apresentam pequenos grânulos de vitelo na periferia do oócito e núcleo, e possuem gotículas de lipídeo. Os oócitos Vtg2 possuem grânulos de vitelo maiores por todo o citoplasma e possui gotículas de lipídeos. E no oócito Vtg3 o acúmulo de vitelo está basicamente completo, com numerosos glóbulos de vitelo e grandes preenchendo todo o citoplasma com gotículas de óleo envolvendo o núcleo (Matsuyama et al. 1990; Murua et al. 1998).

A maturação dos oócitos é dividido em quatro estágios (Fig. 2): Migração de vesícula germinal (GVM); Coalescência do vitelo (YC); Quebra de vesícula germinal (GVBD); Hidratação. Após a ovulação há a formação do folículo pós ovulatório (POF).

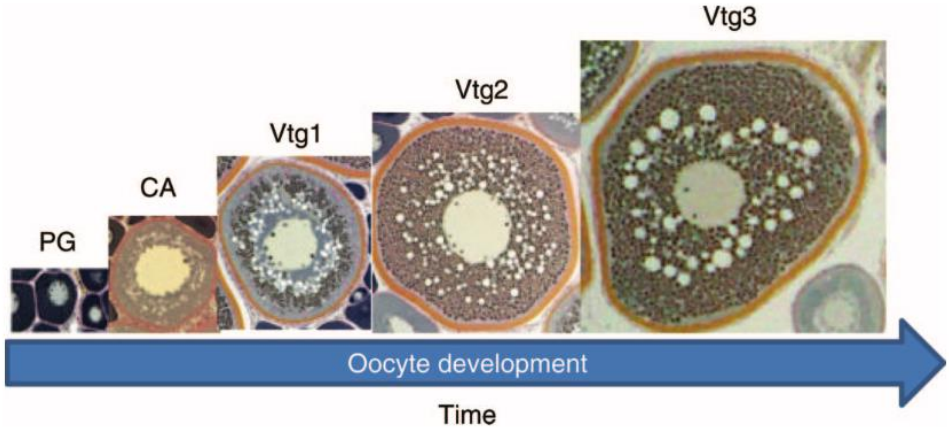

Figura 1. Estágios de desenvolvimento dos oócitos de truta marinha pintada *Cynoscion nebulosus.* Adaptado de Lowerre-Barbieri et al., 2011.

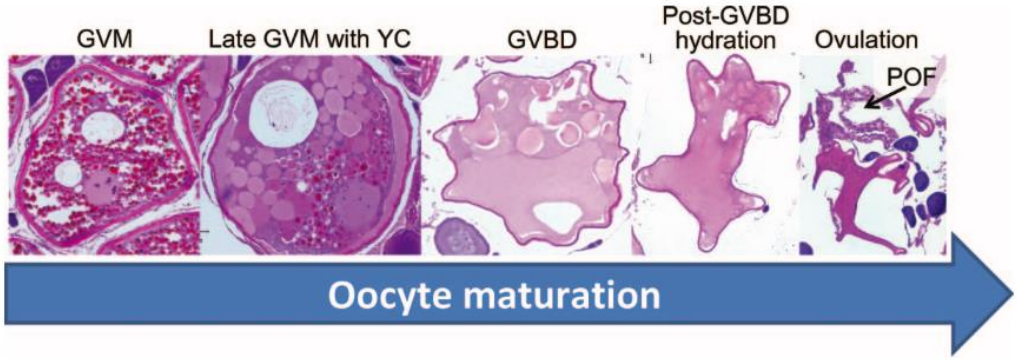

Figura 2. Fases da maturação dos oócitos de truta marinha pintada *Cynoscion nebulosus.* Adaptado de Lowerre-Barbieri et al., 2011

2.7 Criopreservação de oócitos de peixe

Protocolos de criopreservação de espermatozoides foram desenvolvidos em mais de 200 espécies de peixes até ano 2000, das quais aproximadamente 40 são espécies marinhas (Tiersch & Mazik, 2000). Recentemente Asturiano et al. (2017) relacionou estudos sobre criopreservação de espermatozoides de mais 44 espécies de peixes publicados desde o ano 2000. A criopreservação dos gametas paternos e maternos forneceria uma fonte confiável de material genético de peixes para fins científicos e para a aquicultura, bem como para a conservação da biodiversidade (Tsai

et al., 2009a). A criopreservação do genoma materno é importante, pois preserva o DNA mitocondrial e os mRNAs que determinam os estágios iniciais do desenvolvimento embrionário (Tsai et al., 2010). No entanto, a criopreservação de oócitos e embriões de peixes ainda não está bem estabelecida. Estudos mostraram que existem vários obstáculos à criopreservação de embriões de peixes: (a) o grande tamanho dos embriões de peixes, resultando em uma baixa área de superfície em relação ao volume, o que reduz a taxa de movimentação de água e crioprotetores durante a criopreservação; (b) o complexo sistema de membrana e a baixa permeabilidade dos embriões de peixes não permitem a desidratação celular suficiente nem a penetração de crioprotetores, resultando na formação de gelo intracelular que é letal para as células; e (c) os embriões de peixes têm um alto conteúdo de vitelo associado à sua alta sensibilidade ao frio, tornando-os mais suscetíveis a lesões durante a exposição a temperaturas abaixo de zero (Zhang et al., 2008).

Em comparação aos embriões, os oócitos são menores em tamanho, oferecendo uma área de superfície mais vantajosa em relação ao volume e possuem uma estrutura muito mais simples, sua tolerância à toxicidade dos crioprotetores é muito maior (Plachinta et al., 2004), e as membranas de oócitos de peixes são mais permeáveis à água e aos crioprotetores (Zhang et al., 2005). Porém, inúmeros estudos foram realizados sobre a criopreservação de folículos ovarianos isolados de peixes em diferentes estágios, e todos levam a uma viabilidade baixa e comprometedora. Estudos demonstrando a criopreservação oócitos de zebrafish em estágio vitelogênico primário (Vtg1) com o método de congelamento lento e obtiveram viabilidade, utilizando a coloração de azul de tripan, entre 14,9 e 46,2% (Zhang et al., 2008; Guan et al., 2008a; Zampolla et al., 2012) . Por outro lado, Tsai et al. (2009a) criopreservaram oócitos em estágio de crescimento primário (CP) e alvéolo cortical (CA) de zebrafish e obtiveram resultados de viabilidade por coloração de fluorescência com diacetato de fluoresceína combinados com iodeto de propídio de 50,7%. Por outro lado, por apresentarem um menor tamanho CP e CA em relação ao Vtg1, 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.

O método de criopreservação utilizado na maioria das pesquisas foi o de congelamento lento controlado. Nos primeiros estudos que investigaram a eficiência da vitrificação na criopreservação de oócitos de estágio Vtg1 de peixes, os autores relataram perda da viabilidade folicular após o aquecimento (Guan et al., 2010; Godoy et al., 2013). Um provável motivo para a perda de viabilidade, é explicado por Gosden (2011) que relaciona a exposição dos oócitos aos crioprotetores, a redução de temperatura e o posterior aquecimento da amostra criopreservada, que podem ocasionar danos celulares estruturais e metabólicos. No estudo conduzido por Guan et al*.,* (2010) a viabilidade (coloração de azul de tripan) dos folículos ovarianos logo após o descongelamento foi superior a 80%. Os fragmentos de tecido ovariano contendo oócitos em estágio Vtg1 vitrificado por Godoy et al. (2013), apresentaram integridade da membrana de 59,9%. Porém os autores observaram que a viabilidade diminuiu após duas horas de incubação à temperatura ambiente e o nível de ATP nos folículos ovarianos diminuiu significativamente imediatamente após o descongelamento.

A criopreservação de fragmentos de tecido ovariano é uma alternativa promissora para a preservação de oócitos, pois apresentam como principais vantagens para a criopreservação a presença de múltiplos folículos imaturos. Dentre as vantagens, possuem tamanho reduzido e, portanto, alta taxa de relação de superfície/volume, maior permeabilidade de membrana do que embriões, e ausência de um córion completamente formado (Zhang et al., 2005; Tsai et al., 2009a). Essas características garantem a redução no índice de danos que seriam causados durante o processo de criopreservação, mesmo havendo formação de cristais de gelo, não haveria lesões de estruturas complexas, como no caso de oócitos maduros. A criopreservação do tecido ovariano foi proposta pela primeira vez em zebrafish por Zampolla et al*.*, (2011), em que foi utilizada a técnica de congelamento lento, após: e subsequente avaliação da viabilidade utilizando azul de tripan e sondas fluorescentes. Os autores concluíram, que os fragmentos ovarianos contendo oócitos nos estágios CP e CA (imaturos) eram menos sensíveis à criopreservação do que o oócito ovariano de estágio Vtg1 (vitelogênico). Estudos posteriores, seguindo esta linha de pesquisa tem obtido resultados positivos para as criopreservações de tecido ovariano de peixes (Marques et al*.,* 2015; Lujić et al., 2017; Marques et al*.*, 2018; Marques et al*.*, 2019). Um elevado percentual de oócitos imaturos com membranas íntegras foi obtido por

Marques et al. (2015), pós-vitrificação de fragmentos de tecido ovariano de zebrafish (76% no estágio CP e 43% no estágio CA). Em outro estudo conduzido por Marques et al. (2018), foi utilizado o mesmo protocolo de vitrificação de oócitos de zebrafish em *Piaractus mesopotamicus* e os folículos imaturos mostraram 70% de integridade de membrana. No estudo de Lujić et al. (2017) os autores demonstraram uma vitrificação bem sucedida de tecido ovariano de salmonídeos, sendo obtido\descrito 40,34% de integridade de membrana. A comparação das técnicas de criopreservação utilizando tecido ovariano de Zebrafish foi o objetivo de Marques et al. (2019) que concluíram ser a vitrificação mais eficiente que o congelamento lento, onde o método de vitrificação foi o mais eficiente, pois obteve menor valor de Espécies reativas a oxigênio (ERO), capacidade antioxidante (FRAP) e maior atividade mitocondrial pela análise de MTT, quando comparado ao método de congelamento lento.

Com a criopreservação de folículos ovarianos em estágio inicial, são necessários procedimentos de maturação *in vitro* para posterior fertilização. Alguns protocolos vêm sendo desenvolvidos para a maturação de oócitos de zebrafish, porém poucos estudos registram testes em oócitos criopreservados. Neste caso, podemos citar o estudo de Tsai et al. (2010) que relataram diferenciação de oócitos em estágio CP em estágio CA e oócitos em estágio CA em estágio Vtg1 em folículos não criopreservados de Zebrafish e a cultura *in vitro* de oócitos em fragmentos de tecido ovariano de zebrafish e folículos em estágio CP e estágio CA, por 24 h a 28° C por Anil et al. (2018). Cabe ressaltar que os autores não apresentaram crescimento quando os oócitos foram criopreservados.

2.8 Métodos de avaliação da qualidade dos oócitos pós criopreservação

A avaliação da qualidade dos oócitos pós criopreservação é uma questão importante, pois determinará se o protocolo de criopreservação foi bem sucedido e possibilitará identificar os danos estruturais causados. 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, 2006; Gosden, 2011). Existem diferentes tipos de avaliações para determinar a qualidade do oócito, dependendo do seu modo de ação: avaliação de

integridade da membrana plasmática, atividade mitocondrial, estresse oxidativo e dano de DNA.

A ruptura da membrana da célula é uma das principais causas de morte celular no processo de criopreservação, que ocorre em grande parte devido a formação de cristais de gelo. Para aferir a extensão dos danos celulares provocados pelos cristais de gelo após o processo de criopreservação, uma variedade de colorações tem sido utilizada. A coloração com azul de tripan é amplamente utilizada para avaliar a integridade da membrana das células (Zampolla et al., 2011). O azul de tripan cora as células de azul com membrana rompida e as células que não são coradas, são consideradas com membrana íntegra. No entanto, Hudson & Hay (1980) ponderou que a exposição prolongada à coloração de azul de tripan pode ser imprecisa, pois o número de células coradas de azul aumenta com o passar do tempo. Marcadores fluorescentes, como o diacetato de fluoresceína (FDA) e o iodeto de propídio (PI), mensuram as atividades metabólicas e a integridade da membrana (Tsai et al., 2009a; Zampolla et al., 2009). Neste caso, o FDA é um corante de inclusão e PI é um corante de exclusão. O FDA passa pelas membranas plasmáticas e é hidrolisado por esterases intracelulares que produzem fluoresceína livre. Em contraste, o PI passa pelas membranas celulares danificadas e intercala-se com DNA e RNA para formar um complexo fluorescente (Edidin, 1970; Krishan, 1975). Assim, as células da membrana intactas são coradas em verde brilhante com FDA e as células danificadas na membrana são coradas em vermelho brilhante com PI. Um estudo com oócitos de zebrafish, relatou que a coloração FDA+PI era mais sensível que a coloração tripan blue (Zampolla et al., 2008). No entanto, a presença de esterases no meio extracelular e/ou altas concentrações de FDA podem fornecer uma coloração de fundo elevado, podendo gerar resultados falso positivo (Boyd et al., 2008). Outra sonda fluorescente que permite avaliar a integridade da membrana é o SYBR-14®, um corante lipossolúvel, que ao entrar na célula perde o grupo acil e se liga à fita dupla de DNA das células vivas, e emite luz verde fluorescente (Garner & Johnson, 1995). Podendo ser também, associado aos corantes Brometo de Etídeo ou Iodeto de Propídio (fluorescência vermelha) para indicar as células com membranas danificadas.

A avaliação morfológica por meio da histologia permite observar e quantificar danos estruturais causados pela criopreservação, podendo ser avaliados qualitativamente e em alguns casos quantitativamente, dependendo do tamanho do fragmento e do número de oócitos observados. É possível identificar danos na

membrana, alterações na superfície celular (Borrelli et al., 1986), volume celular (Laiho et al., 1971) e também permite identificar danos causados ao núcleo, com queda da densidade do núcleo (Fotakis & Timbrell, 2006) e picnose nuclear, indicando apoptose celular. Para uma avaliação mais detalhada dos danos e alterações nas organelas, é utilizado a microscopia eletrônica de transmissão (MET), que tem um poder de resolução mil vezes superior ao da microscopia de luz (Salehnia et al., 2002). Em um estudo recente de Marques et al. (2018) com vitrificação de tecido ovariano imaturo de *P. mesopotamicus*, foi possível observar o núcleo esférico e centralizado, os nucléolos na periferia do núcleo, as organelas distribuídas uniformemente por todo o citoplasma e as mitocôndrias.

As mitocôndrias são organelas responsáveis por produzir maior parte da energia celular através da respiração celular, produzindo energia na forma de trifosfato de adenosina (ATP). Desse modo, o ATP é aceito como marcador de células viáveis (Knoll-Gellida & BABIN, 2007). O ensaio de ATP é um método que utiliza luciferase e a reação que gera fótons de luz. Este ensaio de ATP foi utilizado por *Zampolla et al.*, (2011) e relataram que a criopreservação causou decréscimo do nível de ATP de fragmentos de tecido ovariano contendo oócitos em estágios CP, CA e Vtg1. Outra forma de determinar a atividade mitocondrial é o ensaio de MTT (3-(4,5-dimetiltiazol-2yl)-2,5-difenil brometo de tetrazolina). Sendo este um teste de alta precisão e confiável, que tem como principio o fato da mitocôndria reduzir o MTT em cristais de formazan na cor roxa, através da enzima desidrogenase succínica (Shokrgozar et al., 2007). Dessa forma, quanto mais células viáveis mais cristais de formazan são produzidos e esses cristais são dissolvidos e medidos em espectrofotômetro.

O estresse oxidativo é um fator importante a ser avaliado com relação aos danos causados pela criopreservação (Tatone et al., 2010), no qual, aumenta a produção de ERO e causa alterações no metabolismo oxidativo (Dowling & Simmons, 2009). O estresse oxidativo ocorre devido ao desbalanço de ERO e outras espécies pró-oxidantes e as defesas antioxidantes enzimáticas e não-enzimáticas. Existem inúmeros ensaios para a determinação das ERO, podendo ser classificados em métodos diretos e indiretos. Os métodos indiretos são medidos através da formação de produtos finais específicos de oxidação das reações das ERO com lipídeos, proteínas ou ácidos nucleicos (Mrakic-Sposta et al., 2017). Diacetato de diclorodihidrofluoresceína (DCFH-DA), é um exemplo de método direto para detecção de espécies reativas, sendo a sonda mais amplamente usada (Kalyanaraman et al.,

2012). Um método indireto de avaliação do status antioxidante, relativamente simples e muito utilizado, é o ensaio do poder antioxidante redutor de ferro (FRAP). Neste caso a atividade antioxidante é mensurada quanto ao poder de redução do Ferro (Benzie & Strain, 1996). O ensaio de produtos reativos ao ácido tiobarbitúrico (TBARS) se baseia na mensuração por espectrofotometria da concentração dos produtos oriundos da peroxidação dos lipídios, como aldeídos (malondialdeido - MDA) (Sanocka & Kurpisz, 2004), no qual é considerado um marcador de dano oxidativo a lipídios. Dentre os métodos indiretos para determinação da ERO mais utilizados estão: a avaliação da atividade enzimática da superóxido dismutase (SOD); catalase; glutationa peroxidase (GSH-Px); glutationa redutase (GSH- Rd) e glutationa reduzida (GSH) (França et al., 2013).

O processo de criopreservação pode resultar em fragmentação do DNA, mesmo na ausência de deformação da morfologia celular. Recentemente um estudo com criopreservação de tecido ovariano de zebrafish, mostrou que a criopreservação resultou em fragmentação do DNA (Marques et al., 2019). A fragmentação do DNA em altos níveis é a principal característica da apoptose celular, sendo utilizada como marcador de apoptose (Majtnerová & Roušar, 2018). No entanto, se a fragmentação do DNA for baixa, ela pode ser reparada pelos mecanismos de reparação celular e do organismo. Os principais métodos de avaliação da fragmentação de DNA são: ensaio de escada de DNA, Ensaio TUNEL e ensaio cometa. Esses ensaios permitem avaliação da célula individualmente.

Além desses métodos, uma forma importante para a determinação da qualidade dos oócitos seria a fertilização. Porém, como é possível apenas criopreservar oócitos imaturos seria necessário realizar maturação *in vitro* ou *in vivo* (transplante) desses oócitos, para posterior fertilização. A maturação de oócitos ainda é um campo pouco explorado em peixes e quando criopreservados não foi possível observar crescimento e maturação dos oócitos (Anil et al., 2018; Tsai et al., 2010).

O uso de vários métodos é sugerido para validar com precisão a qualidade dos oócitos, especialmente para procedimentos de criopreservação e cultura de células (Fauque et al., 2007; Kortner & Arukwe, 2007; Guan et al., 2008b).

2.9 Zebrafish (*Danio rerio*)

O zebrafish (*Danio rerio*) é um teleósteo tropical de água doce, pertencente à família Cyprinidae e é caracterizado pelo pequeno tamanho, podendo atingir em torno de 4-5 cm de comprimento (Spence et al., 2007). Possui dimorfismo sexual, sendo que os machos são delgados e em forma de torpedo, com brilho dourado na barriga, nadadeira ventral, nadadeiras pélvicas e nadadeiras peitorais. E as fêmeas são mais prateadas e arredondadas na região ventral (Figura1), o que é mais evidente no período próximo da desova. Tem ovários em formato de cacho de uva e assíncronos (Figura 2), contendo folículos em todos os estágios de desenvolvimento e por isso, procriam durante todo o ano. A maturidade sexual é atingida por volta de 10-12 semanas de vida e as fêmeas podem desovar a cada 2-3 dias, sendo que cada desova pode conter centenas de oócitos (Lawrence, 2007). No entanto, visando a viabilidade máxima de embriões e não esgotar os animais é recomendado a procriação em intervalos de 10 dias (Niimi & Laham, 1974; Selman et al., 1993). O zebrafish apresenta diversas vantagens em relação a outros modelos de peixes, como pequeno porte, fácil manutenção, baixo custo de criação, além de completar sua embriogênese em 72 h e possuir intervalo curto entre gerações.

O zebrafish é considerado um organismo modelo para outras espécies de peixes, pois são pequenos, podem desenvolver-se rapidamente e ser facilmente criado em laboratório (Rugh, 1948). Também é um importante modelo experimental para estudos de biologia do desenvolvimento, fisiologia e genética (Squire et al., 2008). A semelhança genética entre humanos e zebrafish é de 70%, apresentando 12.719 genes em comum (Howe et al., 2013), sendo um modelo importante para a compreensão do desenvolvimento humano, doenças e toxicologia (Menke et al., 2011). O zebrafish é utilizado para a identificação e determinação de funções de genes identificados no genoma humano, devido a facilidade em realizar modificações em seu gene, criando inúmeros modelos de zebrafish para o estudo de doenças humanas como câncer, doenças cardíacas, Alzheimer, Parkinson e distrofia muscular (Whitfield, 2002; Zon &Peterson, 2005; Lieschke & Currie, 2007). Além disso, são considerados um excelente modelo para estudar a genética do comportamento social, pois são animais naturalmente sociais que demonstram preferência pela presença de membros da mesma espécie (Norton & Bally-Cuif, 2010).

Figura 3. Fêmea (acima) e macho (abaixo) de zebrafish (*Danio rerio*) (Figura adaptada de: https://pascalaquariumsnaturels.com/reproduction-danio-rerio/)

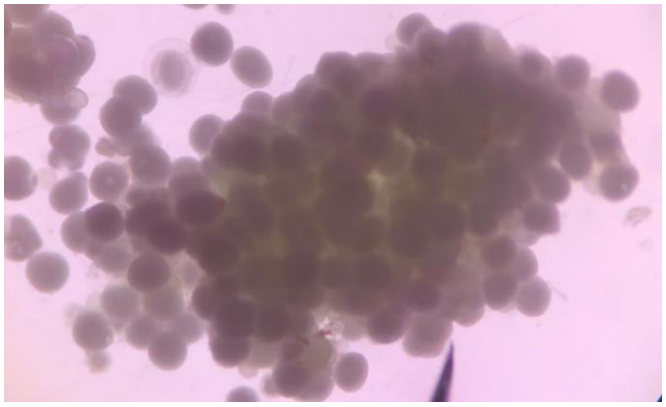

Figura 4. Ovário de zebrafish (*Danio rerio*).

3 HIPÓTESES E OBJETIVOS

3.1 Hipótese

- O encapsulamento de fragmentos de tecido ovariano de zebrafish (*Danio rerio*) em hidrogel de alginato de sódio aumenta a proteção contra as crioinjúrias causadas no processo de vitrificação;
- O encapsulamento do tecido ovariano de zebrafish (*Danio rerio*) em hidrogel de alginato de sódio permite a redução da concentração ou a remoção dos crioprotetores tradicionais no processo de vitrificação.

3.2 Objetivos

3.2.1 Objetivo geral

Avaliar a eficiência da técnica de encapsulamento em hidrogel de alginato de sódio na vitrificação de fragmentos de tecido ovariano de zebrafish (*Danio rerio*), visando aprimorar o protocolo de vitrificação.

3.1.2 Objetivos específicos

- Avaliar o efeito da forma de encapsulamento do tecido ovariano em hidrogel de alginato sódio, do momento da exposição da solução de equilíbrio e vitrificação (antes ou após o encapsulamento) e da temperatura de aquecimento, sobre a integridade de membrana, por meio da coloração de azul de tripan;
- Avaliar se a técnica de encapsulamento do tecido ovariano em hidrogel de alginato de sódio reduz os danos causados pela vitrificação, sobre a integridade de membrana, morfologia celular, peroxidação lipídica e atividade mitocondrial;
- Avaliar se a técnica de encapsulamento do tecido ovariano em hidrogel de alginato de sódio permite a redução/remoção da concentração dos crioprotetores.

CAPÍTULO II[1]

[1] Artigo elaborado conforme as normas do periódico *Cryobiology*

SODIUM ALGINATE HYDROGEL ENCAPSULATION AS A HANDLING AND VITRIFICATION TOOL FOR ZEBRAFISH (*Danio rerio*) OVARIAN TISSUE

ABSTRACT

The aim was to evaluate the efficiency of the sodium alginate hydrogel encapsulation technique during the zebrafish ovarian tissue vitrification procedure. In Experiment 1, were evaluated the encapsulation form (immersion or 30 µL alginate bead), the moment of exposure to cryoprotectants (before or after encapsulation) and the warming temperature (28, 37 and 50ºC). The treatment encapsulated by immersion, exposed to cryoprotectants after encapsulation and heated to 28ºC showed greater membrane integrity in all oocyte stages (PG: 37.71 ± 3.86 %; CA: 29.93 ± 4.18 %; Vtg1: 18.61 ± 4.69 %), so it was chosen to be used in Experiment 2.In Experiment 2 were evaluated four vitrified groups: non-encapsulated (VS); encapsulated with cryoprotectants (VS1-A); encapsulated with half the concentration of cryoprotectants (VS2-A); encapsulated without cryoprotectants (VA). The variables evaluated were membrane integrity, morphology, mitochondrial activity and lipid peroxidation. VA treatment showed a lower membrane integrity percentage, while VS demonstrated a higher membrane integrity in which it did not differ from the VS1-A. Mitochondrial activity was greater in the non-encapsulated treatment (VS) and the encapsulated treatments had the lowest values. VA treatment obtained the highest lipid peroxidation level, while VS1-A and VS obtained the lowest values in which VS was not different from the VS2-A treatment. These results showed that, alginate hydrogel did not have a cryoprotective action on membrane integrity and did not promote a greater mitochondrial activity. However, the encapsulation allowed oocytes support, which helped in ovarian tissue handling and reduced the loss of oocytes during vitrification process.

1.Introduction

Zebrafish (*Danio rerio*) is an important model organism in different research fields, like biomedicine and biology [16] since its genome is approximately 70% similar to the human genome [15]. Several wild-type, mutant, and transgenic strains of

zebrafish are available in laboratories around the world for research [13]. The preservation of these valuable genotypes is particularly important and presents significant and urgent challenges, for this, tissues and gametes cryopreservation has proved to be an efficient tool capable of supply this demand. Previous studies have shown that the cryopreservation of ovarian tissue fragments is a promising alternative for the conservation of fish oocytes [24–27]. The main advantage for ovarian tissue cryopreservation is the greater permeability of oocyte membrane when compared to embryo that has a fully formed chorion [42,47]. Authors showed that vitrification was more efficient than slow freezing in preventing mitochondrial damage and avoid oxidative stress on zebrafish ovarian tissue [27]. Vitrification is an ultra-fast cooling cryopreservation that prevents the ice crystals formation using high concentration of cryoprotective agents (CPAs) [17]. However, cryoprotectants can be considered toxic, especially when used in high concentrations, which can cause disruption or damage to cell membranes, reduced mitochondrial function, damage to proteins or other macromolecules among other toxic effects [2]. To overcome these problems, low-CPA vitrification needs to be explored for cell and tissue cryopreservation.

In an attempt to promote greater protection in relation to the damages caused by the cryopreservation procedures to cells and tissues, cell encapsulation in hydrogel biomaterials has been shown to be able to reduce the usage of high CPAs concentration without compromising the vitrification outcomes [29,45]. Sodium alginate is an anionic polysaccharide produced by brown seaweed, when it is in contact with calcium ions it is crosslinked, forming the alginate hydrogel. The main advantages of alginate hydrogel are biodegradability, biocompatibility, non-toxicity and capacity of absorbing large amount of water [22], in addition this substance has antioxidant properties [21]. Alginate ionic crosslinking has advantages such as the low amount of salt required to form the gel and the control of crosslinking by the concentration of alginate [7]. The gel-like structure within the alginate hydrogel allows encapsulated cells to have an environment similar to an extracellular matrix (ECM) [9]. Alginate hydrogel encapsulation, maintains cell structure *in vitro* that morphologically resembles *in vivo* ambient, with improved porosity, cell proliferation and mechanical strength [19].

Alginate hydrogel is usually used in tissue engineering [38], cell culture structure [46] and cell/tissue transplantation [33].

Studies using alginate hydrogel for cryopreservation of mammalian cells, have demonstrated that, encapsulation in hydrogel can significantly reduce the concentration and even eliminate the CPAs. Encapsulation of mouse mesenchymal stem cells into alginate hydrogel using 1.5 M Me_2SO concentration for vitrification, resulted in 88.9 ± 2.9% of membrane integrity, while non-encapsulated cells, with the same CPA concentration, resulted in 42.0 ± 4.4% of membrane integrity [23]. A study with vitrification of encapsulated murine embryonic stem cells and human adipose-derived stem cells in alginate hydrogel showed 80% membrane integrity using 1.5 M 1,2-propanediol (PROH) and 0.5 M trehalose (approximately 4-times lower concentration) and encapsulated cells had a major impact on inhibiting intracellular ice formation during warming [14]. The slow freezing of human fibroblasts encapsulated in alginate hydrogel in the absence of CPAs showed a metabolic activity similar to that of cryopreserved fibroblasts with Me_2SO and trehalose CPAs. [29]. Thus, this results evidence that cell encapsulation in alginate hydrogel can be an alternative to replace or reduce the CPAs in cryopreservation process. Recently a study carried out with zebrafish ovarian tissue vitrification using non-crosslinked sodium alginate without CPAs, showed similar membrane integrity results to vitrified ovarian tissue with 1.5 M methanol + 5.5 M Me_2SO + 0.5 M sucrose [10]. However, there is no study using the crosslinked alginate encapsulation in cryopreserved fish cells.

Therefore, the aim of this study was to evaluate the sodium alginate hydrogel encapsulation technique efficiency on vitrification of zebrafish ovarian tissue fragments.

2. Materials and methods

2.1 Fish care and sample collection

Mature female zebrafish (1 - 2 years old) were maintained in filtered and aerated 40 L aquariums (stock density 7 fish / L) at 27°C ± 2°C, under a photoperiod 14/10 h

(light/dark), pH between 7.2± 2, zero levels of toxic ammonia and nitrite. Fish were fed with TetraMin® dry flake fish food (Tetra, Germany) three times a day.

The females (average weight 1.490 ± 0.390 g) were euthanized with a lethal dose of tricaine methane sulfonate (0.6 mg/mL, pH 7.4) [28], followed by decapitation. The ovaries (average weight 0.249 ± 0.103 g) were collected and placed in 90% Leibovitz L-15 medium (pH 9.0). The average gonadosomatic index (GSI = [ovaries weight/body weight] × 100) was 0.1566 ± 0.0577. Fragments were dissected from the ovaries and cut into slices (3x3 mm) using syringe needles. Ten to 12 fragments were collected from each female and randomly distributed among treatments.

The 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: 38864

2.2 Experimental design

The objective of Experiment 1 was to define the encapsulation technique for the zebrafish ovarian tissue in alginate hydrogel. Two groups were tested, in group 1 the fragments were encapsulated in 30 µL of sodium alginate hydrogel (Fig. 1a), in which was the sufficient volume to envelop the fragment of ovarian tissue. In group 2 the fragments were encapsulated by immersion in alginate (Fig. 1b). The moment of exposure to cryoprotectants (CPA) (before or after encapsulation) and the warming temperature (28, 37 or 50ºC) were also tested (Figure 2). The warming temperature of 28ºC was according to vitrification protocol of zebrafish ovarian tissue [27]; 37ºC warming temperature was according to cryopreservation protocols with alginate hydrogel encapsulated mammalian cells [3,6]; and 50ºC warming temperature was obtained in a previous test, which we evaluated the temperature that defrosts 30µl alginate bead in 60 s.

Ovarian tissue fragments from 9 females were randomly distributed into thirteen experimental groups; One fresh control and twelve treatment groups combining the parameters described above (VA1-VA12) (Fig.2). Eight replicates were performed for each group (vitrified treatments and control/fresh ovarian tissue) and membrane

integrity was analyzed by trypan blue staining. The encapsulation form, the moment of exposure to CPA and warming temperature, which presented the highest percentage of membrane integrity, were used in Experiment 2.

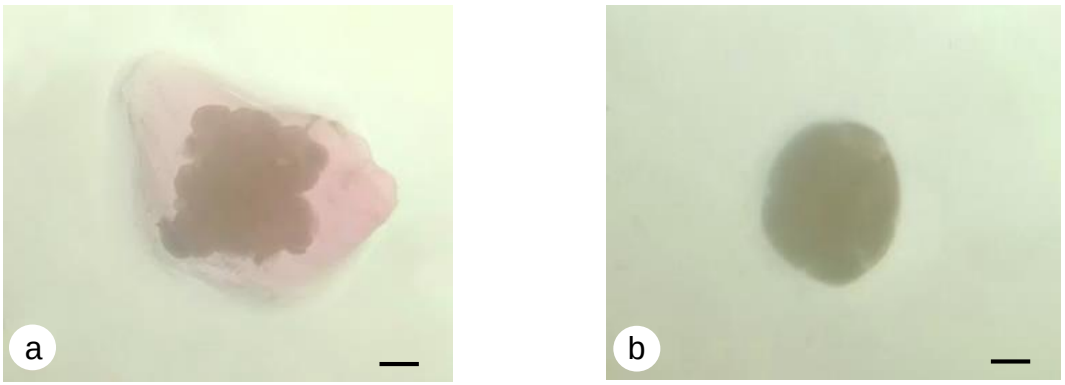

Figure 1. Sodium Alginate encapsulation form. (a) Zebrafish ovarian tissue fragment encapsulated in 30 µL of sodium alginate hydrogel (b) Zebrafish ovarian tissue fragment encapsulated by immersion in sodium alginate hydrogel. Scale Bars: 1 mm

The aim of Experiment 2 was to evaluate the alginate hydrogel as a cryoprotectant or as auxiliary cryoprotectant along with traditional cryoprotectants. Ovarian tissue fragments from 19 females were randomly distributed into five experimental groups; One fresh control and 4 treatment groups (VS: 1.5M Methanol + 5.5M Me_2SO + 0.5M sucrose; VS1-A: 1.5M Methanol + 5.5M Me_2SO + 0.5M sucrose – encapsulated in alginate; VS2-A: 0.75M Methanol + 2.75M Me_2SO + 0.25M sucrose – encapsulated in alginate; VA: encapsulated in alginate) (Fig.2). Membrane integrity were analyzed by SYBR-14 and propidium iodide (SYBR-14/PI), histology by hematoxylin and eosin (HE) stain, lipid peroxidation by thiobarbituric acid reactive substances (TBARS) and mitochondrial activity by MTT assay, that was analyzed immediately after warming and 120 min after in 90% Leibovitz L-15 medium. Eight replicates were performed for each analysis.

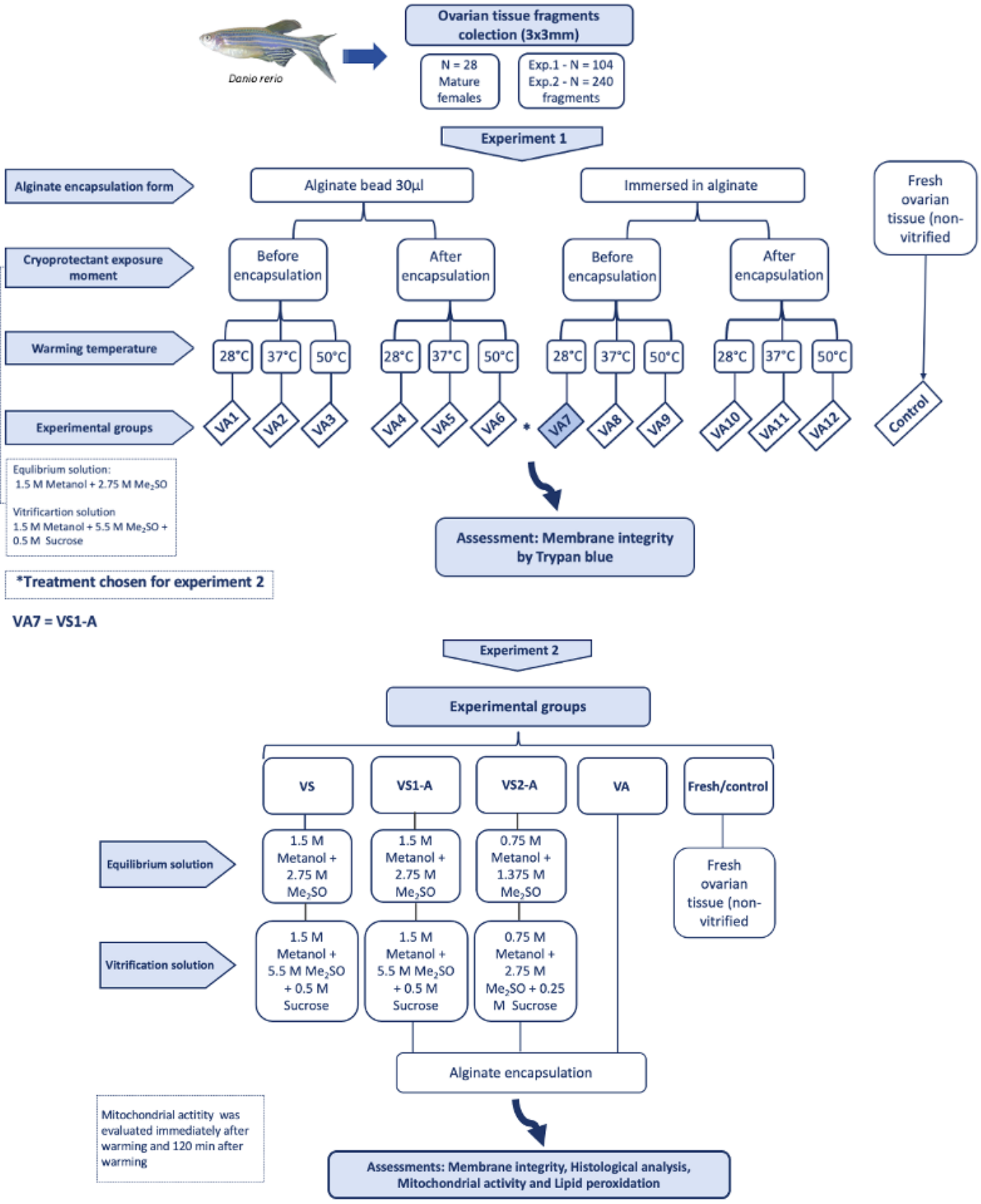

Figure 2. Experimental design illustrating the two experiments conducted with the zebrafish (*Danio rerio*) experimental model. Experiment 1: VA1 (30 µL alginate

bead; CPA before encapsulation; 28ºC warming temperature); VA2 (30 µL alginate bead; CPA before encapsulation; 37ºC warming temperature); VA3 (30 µL alginate bead; CPA before encapsulation; 50ºC warming temperature); VA4 (30 µL alginate bead; CPA after encapsulation; 28ºC warming temperature); VA5 (30 µL alginate bead; CPA after encapsulation; 37ºC warming temperature); VA6 (30 µL alginate bead; CPA after encapsulation; 50ºC warming temperature); VA7 (Immersed in alginate; CPA before encapsulation; 28ºC warming temperature); VA8 (Immersed in alginate; CPA before encapsulation; 37ºC warming temperature); VA9 (Immersed in alginate; CPA before encapsulation; 50ºC warming temperature); VA10 (Immersed in alginate; CPA after encapsulation; 28ºC warming temperature); VA11 (Immersed in alginate; CPA after encapsulation; 37ºC warming temperature); VA12 (Immersed in alginate; CPA after encapsulation; 50ºC warming temperature); Fresh/control = fresh ovarian tissue fragments; (*) Treatment chosen for experiment 2. Experiment 2: VS (1.5 M Methanol + 5.5 M Me_2SO + 0.5 M sucrose); VS1-A (1.5 M Methanol + 5.5 M Me_2SO + 0.5 M sucrose – encapsulated in alginate); VS2-A (0.75 M Methanol + 2.75 M Me_2SO + 0.25 M sucrose – encapsulated in alginate); VA (encapsulated in alginate); Fresh/control = fresh ovarian tissue fragments.

2.3 Ovarian tissue encapsulation in alginate hydrogel

The sodium alginate solution was prepared at 2% concentration in 90% Leibovitz L-15 medium (pH 9), the solutions were kept under agitation until complete solubilization at room temperature (22ºC). For ovarian tissue encapsulation in 30 µL sodium alginate (VA1, VA2, VA3, VA4, VA5 and VA6 groups), the ovarian tissue fragment was gently placed in a 1/8 plastic teaspoon (Fig. 3a), was added 30 µL of alginate solution and then, for crosslinking, the spoon with the fragment and alginate solution were immersed in 4% calcium chloride solution for 60 s (22ºC), forming a bead (Fig. 3b). For the experimental groups encapsulated immersed in alginate (VA7, VA8, VA9, VA10, VA11 and VA12 groups), the fragment was immersed in sodium alginate solution with the aid of a 1ml syringe with curved needle (Fig. 3c) and then immersed in 4% calcium chloride solution (22ºC), forming a thin film of sodium alginate hydrogel around the ovarian tissue fragment (Fig. 3d).

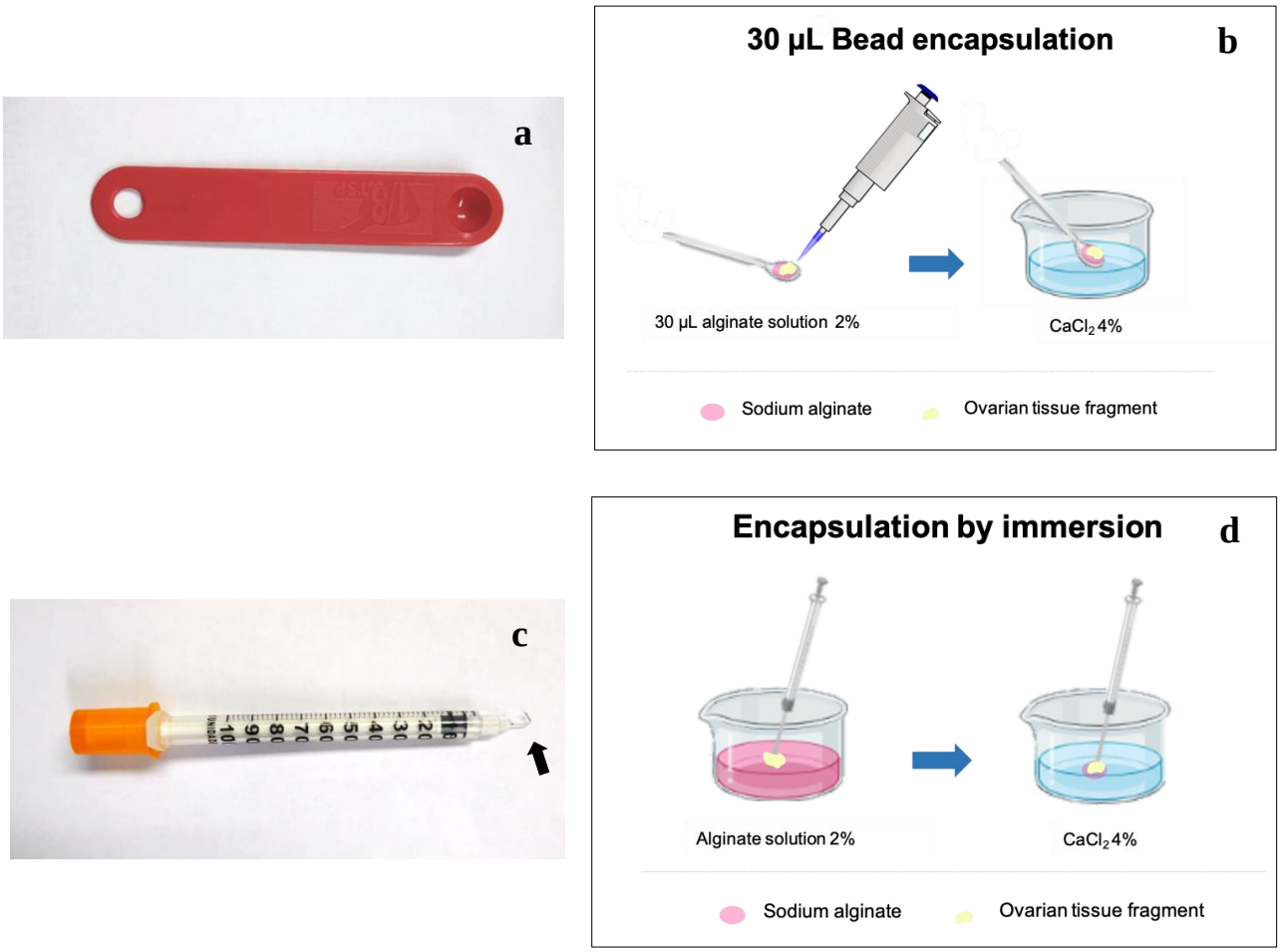

Figure 3. Ovarian tissue encapsulation in sodium alginate hydrogel. (a) 1/8 plastic teaspoon used for 30 µL bead ovarian tissue encapsulation; (b) Ovarian tissue encapsulation in 30 µL alginate bead; (c) Syringe used for encapsulation by immersion in alginate (arrow) curved needle; (d) Ovarian tissue encapsulation by immersion in alginate.

2.4 Vitrification procedure

Ovarian tissue fragments were transferred to 2 mL cryotubes containing 300 µL of equilibrium solution (ES1: 1.5 M methanol + 2.75 M Me_2SO - vitrified treatments of Experiment 1 and VS, VS1-A of Experiment 2; or ES2: 0.75 M methanol + 1.375 M Me_2SO - VS2-A treatment) for 7 min (4°C). Then, the samples were exposed to 300 µL of vitrification solution for 90 s (1.5 M methanol + 5.5 M Me_2SO - vitrified treatments of Experiment 1 and VS, VS1-A of Experiment 2; or 0.75 M methanol + 2.75 M Me_2SO

- VS2-A treatment). After that time, the vitrification solution was removed, the samples with alginate were encapsulated and the cryotubes was directly plunged at the liquid nitrogen. The protocol and solutions used on experiment 1, VS and VS1-A treatments were according to Marques et al. [25], with modifications. After 7 days, the cryotubes were warmed in a water bath for 60 s at 28, 37 or 50ºC in Experiment 1 and for 60 s at 28ºC in Experiment 2, while the samples were exposed to the first warming solution containing 1 M sucrose, then to a second solution containing 0.5 M sucrose for 3 min, and finally to 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) and then primary growth (PG), cortical alveolar (CA) and primary vitellogenic (Vtg1) oocytes - according to Brown-Peterson et al. [4] classification - were analyzed using the following methodologies. Secondary vitellogenic (Vtg2) and tertiary vitellogenic (Vtg3) were not evaluated because they were all with damaged membranes.

2.5 Membrane integrity assay

Two membrane integrity staining protocols were used after warming of vitrified and fresh/control oocytes: Trypan blue in experiment 1; SYBR-14 and propidium iodide (SYBR-14/PI) in experiment 2. Trypan blue is a dye exclusion method based on the principle that cells with damaged membranes are dead, in which they are stained in blue. The oocytes were gently separated using syringe needles in 90% Leibovitz L-15 medium, were incubated in 0.4% trypan blue solution for 3 min and then washed three times in 90% L-15 medium. The fragments were observed under light microscope (Nikon Eclipse E200, Tokyo, Japan 40x objective lens). SYBR-14/PI staining is a fluorometric method to assess membrane integrity, which SYBR-14 is an inclusion dye and PI is an exclusion dye. SYBR-14/PI is a more sensitive method for determining membrane integrity than trypan blue staining. SYBR-14 passes through plasma membranes and loses the acyl group and binds to the double stranded DNA of living cells, and emits green fluorescent light. In contrast, PI pass through cell damage membrane. Thus, intact membrane cells are bright green stained with SYBR-14 and membrane damage cells are bright red stained with PI. The fragments were incubated with 100 µL of PBS with 4 µL of SYBR-14 (0.02mM) for 4 min and 1.5 µL of PI (4.8mM) for 1 min in the dark at room temperature (22°C) and analyzed under fluorescence

microscope (Carl Zeiss, AxioVert, Germany, 20x objective lens). The percentage of membrane integrity each oocyte phase (PG, CA and Vtg1) within the fragments was calculated as follows: (Membrane integrity (%) = [Number membrane damage oocytes/Total number of oocytes] × 100).

2.6 Histological analysis

Ovarian tissue fragments were fixed in Karnovsky's solution (2% paraformaldehyde + 2.5% glutaraldehyde + phosphate buffer solution, pH 7.5 – 7.8) for 24 h, then were dehydrated in an increasing series of alcohol (70%, 80% and 95%) embedded in Leica historesin (methacrylate glycol) and 3 µm sections were obtained on a Leica RM2245 microtome with glass knives and stained with Hematoxylin and Eosin (HE). Morphological integrity of oocytes (phase PG, CA, Vtg1, Vtg2 and Vtg3) were evaluated with a light microscope (Nikon Eclipse E200, Tokyo, Japan 10x and 40x objective lens). The evaluation of the fresh/control group and the vitrified ovarian tissue was descriptive.

2.7 Mitochondrial activity

Mitochondrial activity was assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) assay [23], based on the action of the mitochondrial enzyme succinyl dehydrogenase, that promotes the reduction of MTT to formazan crystals, which is active only in living cells. MTT was evaluated immediately after ovarian tissue fragments warming and 120 min later - incubated in 90% L-15 medium pH 9.0 at 28ºC. The ovarian tissue fragments were incubated in 400 µL of MTT (5 mg/mL) for 120 min at 28°C. The supernatant was carefully removed and then, dimethyl sulfoxide (400 µL) was added to solubilize the formazan crystals, resulting in a purple solution. Next, 100 µL of the colored solution was transferred in 96 well microplate and the absorbance were read at 570nm on a SpectraMax® 250 Microplate Spectrophotometer. Each sample was analyzed in triplicate.

2.8 Lipid peroxidation

The thiobarbituric acid reactive substances (TBARS) levels are indicative of lipid peroxidation, which starts in the presence of reactive oxygen species (ROS). The ovarian tissue fragment was homogenized in cold (4ºC) 150µL of phosphate buffered saline solution (PBS), pH 7.4 and centrifuged for 10 min at 3000 x g [35]. The low-speed supernatant was used to determine the proteins quantification and the sample volume that corresponds 50 µg of proteins was calculated [36]. Then, the samples were incubated with Trichloroacetic acid (TCA) 20% + Thiobarbituric acid (TBA) 0.5% (150µL) and water (50µL) at 100ºC for 30 minutes. The species reactive to thiobarbituric acid (TBARS) were determined by spectrophotometry at 532 nm as described by Sachett et al. [37]. Lipid peroxidation was expressed by nmol MDA / mg of protein.

3. Statistical analysis

All data are presented as mean values ± standard error (mean ± SD). Homogeneity (Levene's test) and normality (Shapiro-Wilk, Kolmogorov-Smirnov and D'Augostino & Pearson) tests were performed. When necessary, the data were transformed (LOG) and the outliers were excluded. For Experiment 1, in the evaluation of membrane integrity, the data were analyzed using three-way analysis of variance (Three-Way ANOVA), considering the effect of the alginate encapsulation, the moment of exposure to CPAs, the heating temperature of the samples, and the interaction between these effects. When a significant effect was observed for one of the factors, the groups were compared using the Tukey or Bonferroni test. For Experiment 2, the membrane integrity data (SYBR-14/PI) and TBARS, were analyzed using Kruskal-Wallis analysis, followed by Dunn's test. The MTT test was analyzed by means of two-way analysis of variance (Two-Way ANOVA), considering the experimental groups (cryopreservation protocols), the timing of the MTT test (immediately and 120 minutes later) , and the interaction between these factors. When a significant effect was observed for one of the factors, the groups were compared using the Tukey or Bonferroni test. The data analyzed by ANOVA are presented in bar graphs (mean and standard deviation) and the data analyzed by Kruskal-Wallis analysis are presented in Box and Whiskers type graphs (Median, maximum and minimum). Analyzes were

performed using Statistical Analysis System v.9.4 (SAS) software and GraphPad Prism 7.0.

4. Results

4.1 Experiment 1

All parameters (Encapsulation form; Moment of exposure to CPA; Warming temperature) had a significant effect on PG, CA and Vtg oocytes membrane integrity after warming on experiment 1 (Fig.4). The exposure of ovarian tissue encapsulated in 30 µL bead to the CPA after encapsulation in alginate hydrogel, resulted in greater damage to PG, CA, and Vtg1 oocytes membranes. Ovarian tissue fragments encapsulated immersed in alginate hydrogel before or after cryoprotectant exposure, showed higher PG, CA and Vtg1 oocyte membrane integrity when warmed at 28ºC. Vtg1 oocyte membrane integrity was best preserved when the fragments were encapsulated by immersion in alginate, exposed to CPA before encapsulation and warmed at 28ºC (VA7: 18.61 ± 4.69 %) and it was numerically greater for PG (VA7: 37.71 ± 3.86 %) and CA (VA7: 29.93 ± 4.18 %). So, the encapsulation form by immersion in alginate, the exposure to CPA before encapsulation and the 28ºC warming temperature (VA7) were chosen for Experiment 2.

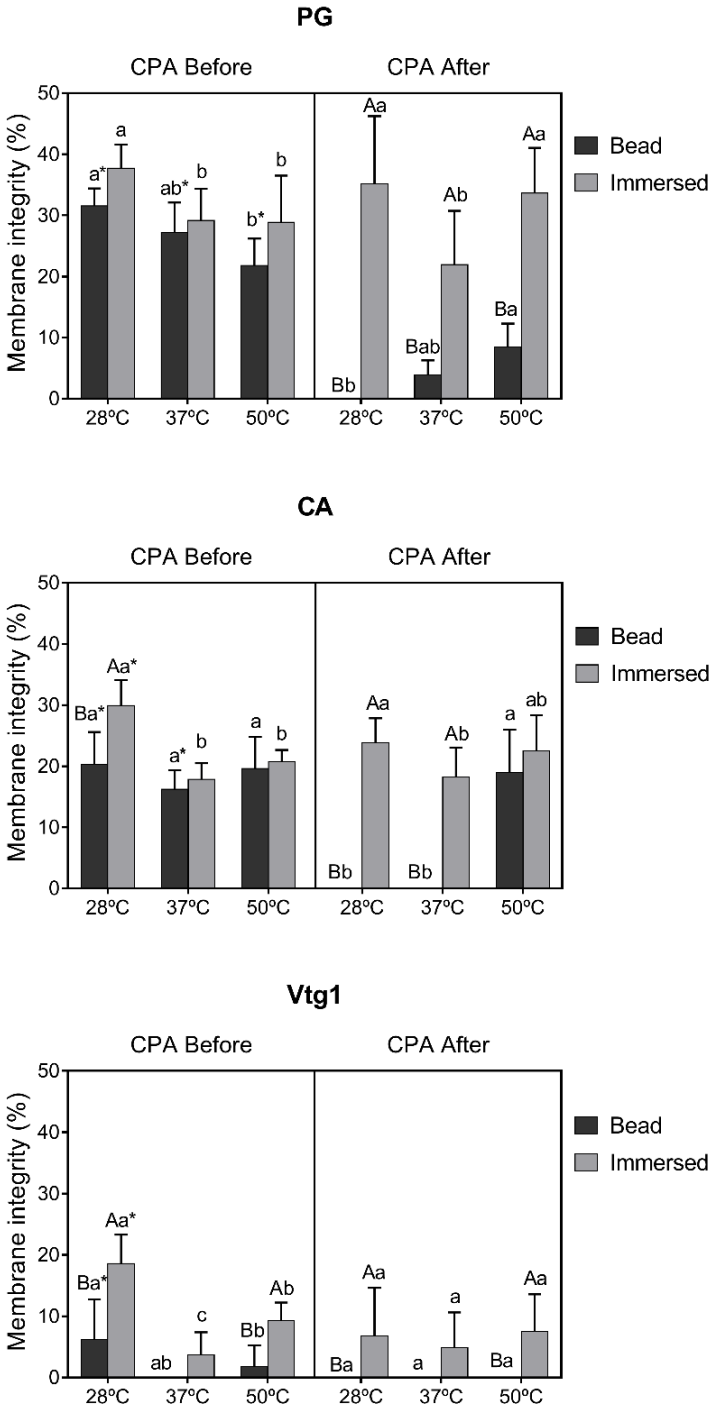

Figure 4. Membrane integrity by trypan blue. PG oocytes: Temperature effect (p = 0.0016), CPA exposure effect (p <0.0001), encapsulation effect (p <0.0001), Temperature x CPA exposure effect (p <0.0001), Temperature x Encapsulation effect (p = 0.0026), CPA Exposure x Encapsulation effect (p <0.0001) and Temperature x CPA Exposure x Encapsulation effect (p = 0.0733); CA oocytes: Temperature effect (p

<0.0001), CPA exposure effect (p <0.0001), encapsulation effect (p <0.0001), Temperature x CPA exposure effect (p <0 , 0001), Temperature x Encapsulation effect (p <0.0001), CPA Exposure x Encapsulation effect (p <0.0001) and Temperature x CPA Exposure x Encapsulation effect (p = 0.0026); Vtg1 oocytes: Effect of temperature (p <0.0001), effect of exposure to CPA (p = 0.0002), effect of encapsulation (p <0.0001), effect Temperature x Exposure to CPA (p <0.0001), Temperature x Encapsulation effect (p = 0.0558), CPA Exposure x Encapsulation effect (p = 0.4110) and Temperature x CPA Exposure x Encapsulation effect (p = 0.2581); Different capital letters indicate a difference in the form of encapsulation (bead and immersed), within the same warming temperature and the form of exposure to CPA. Lower case letters indicate difference between warming temperatures (28, 37 and 50 ºC), within the same form of encapsulation and exposure to CPA. Asterisk (*) indicates when there is a difference between the moment of exposure to CPA (before or after), within the same warming temperature and in the same encapsulation form. Data presented as mean ± SD.

4.2 Experiment 2

The membrane integrity measured by SYBR-14/PI stain for PG and CA oocytes was significantly higher in the VS experimental group (PG: 54.57 ± 12.28 %; CA: 23.66 ± 9.95%), compared to the VS2-A (PG: 16.35 ± 5.53 %; CA: 5.44 ± 4.19%) and VA groups (PG: 1,21 ± 1.40 %; CA: 0.33 ± 0.62 %) (Fig. 5). The VS1-A experimental group (PG: 33.01 ± 5.47 %; CA: 17.69 ± 8.31%) differed only from the VA group. For oocytes in Vtg1 stage, the highest membrane integrity was observed in the VS (12.65 ± 5.06 %) and VS1-A groups (12.85 ± 7.87 %), which differed statistically from the other groups.

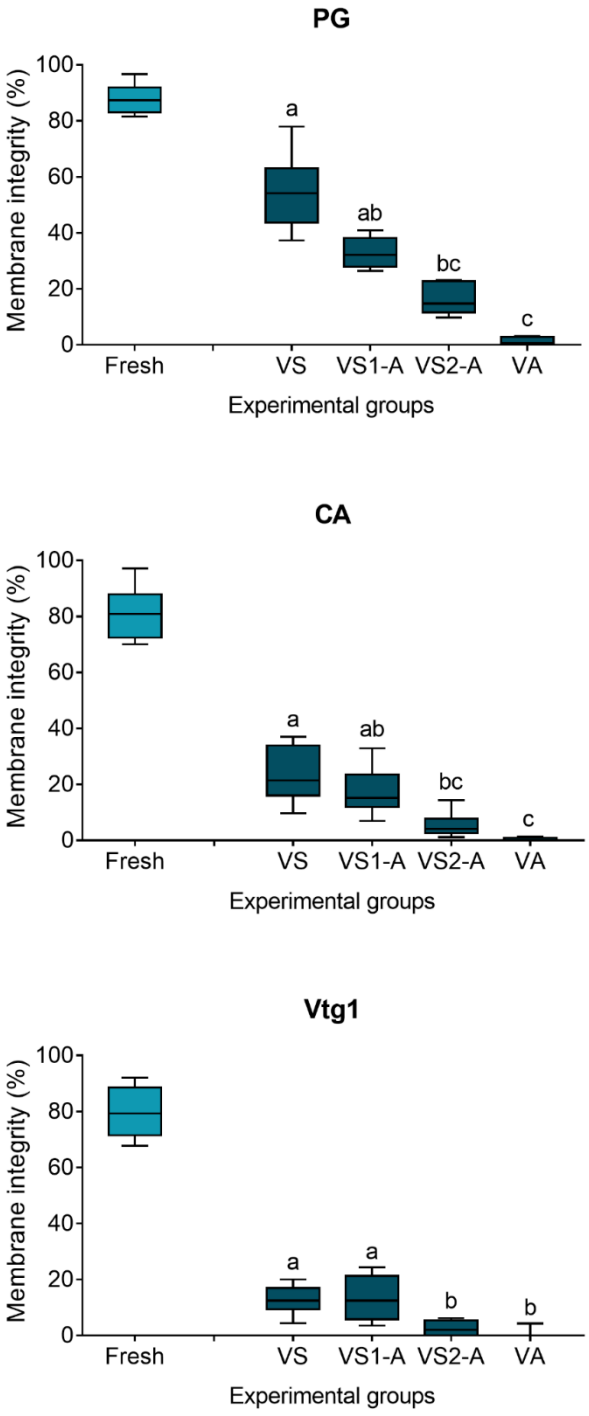

Figure 5. Membrane integrity after vitrification by SYBR-14 / PI. VS (1.5 M Methanol + 5.5 M Me_2SO + 0.5 M sucrose); VS1-A (1.5 M Methanol + 5.5 M Me_2SO + 0.5 M sucrose – encapsulated in alginate); VS2-A (0.75 M Methanol + 2.75 M Me_2SO + 0.25 M sucrose – encapsulated in alginate); VA (encapsulated in alginate); Fresh = fresh ovarian tissue fragments. PG ($p < 0.0001$); CA ($p < 0.0001$) and Vtg1 ($p < 0.0001$).

Data presented as mean ± SD. Different letters indicate a difference between the experimental groups.

The histological analysis showed in all vitrified treatments (VS, VS1-A, VS2-A and VA) that CA and vitellogenics oocytes (Vtg1, Vtg2 and Vtg3) presented cytoplasmic alterations, with apparent rupture of the cortical alveoli and nucleus degeneration (Fig. 6 n, k, h, e). However, no membrane damage was in vitellogenics oocytes. On the other hand, in PG vitrified oocytes membrane damage, nucleus fragmentation and the formation of vacuums in the cytoplasm were frequently observed (Fig. 6 o, l, I, f).

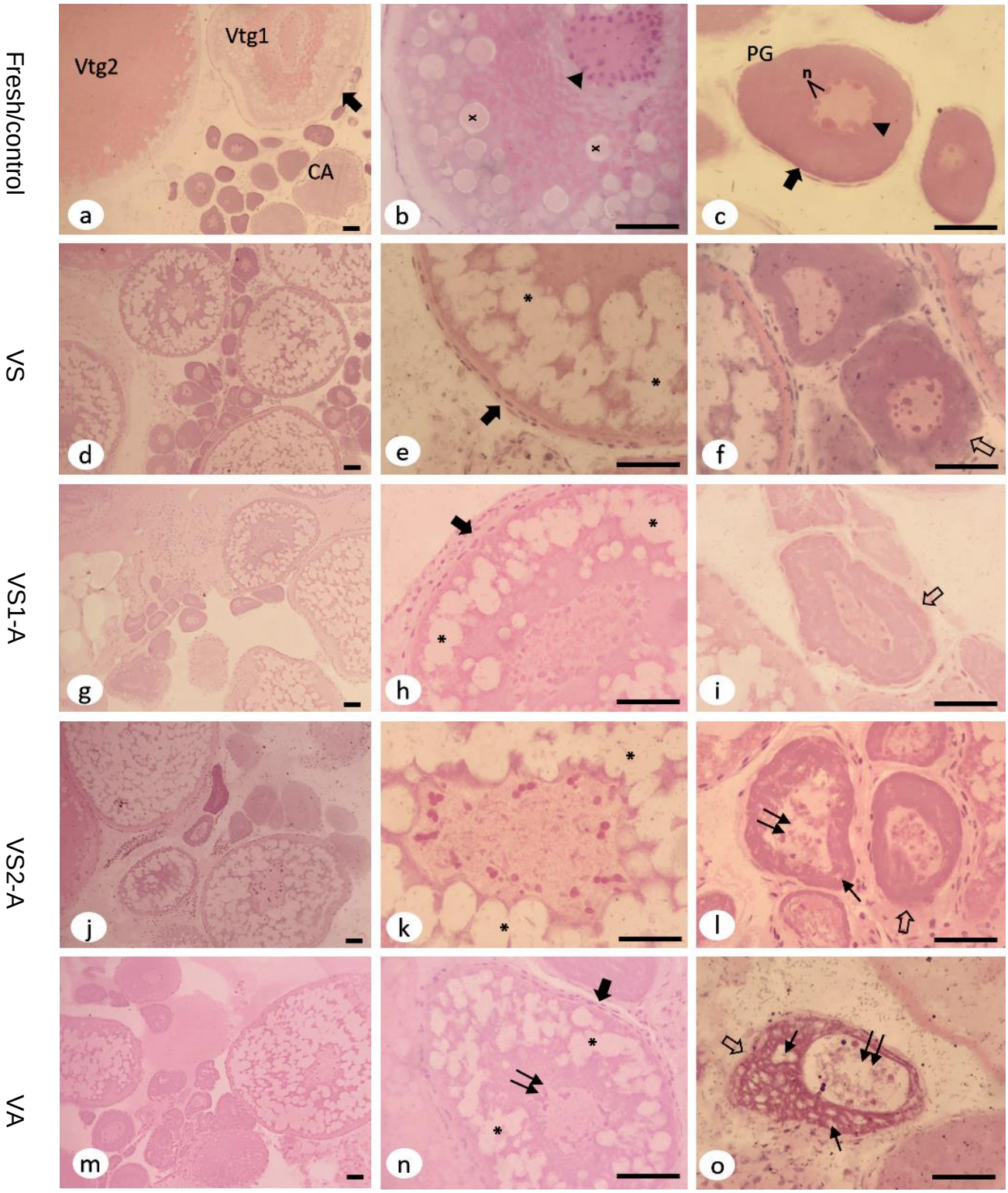

Figure 6. Oocytes morphology after vitrification. a-c) Fresh/control group; d-f) VS group; g-i) VS1-A group; j-l) VS2-A group; m-o) VA group. Primary growth stage (PG). Cortical alveolus stage (CA). Primary vitellogenic stage (Vtg1). Secondary vitellogenic stage (Vtg2). Nucleoli (n). Rupture of the cortical alveoli (asterisks). Intact cortical alveoli (x symbol). Nucleus fragmentation (double arrow). Intact nucleus (arrowhead).

Cytoplasm with vacuole formation (black arrow). Follicular membrane rupture (hollow arrow) intact follicular membrane layer (thick arrow). Scale Bars: 30 µm; Stain: H.E.

Mitochondrial activity by MTT showed no effect alteration for fragments analyzed immediately or 120 min after warming (p = 0.2262) (Fig. 7). Samples analyzed immediately after warming, showed greater mitochondrial activity in the VA (43.37 ± 17.91) and VS groups (36.41 ± 15.48), which differed statistically from the VS1-A group (14.26 ± 5.06). For the analyzed samples 120 min after warming, we observed greater mitochondrial activity in the VS groups (62.24 ± 28.62), which differed from the other groups.

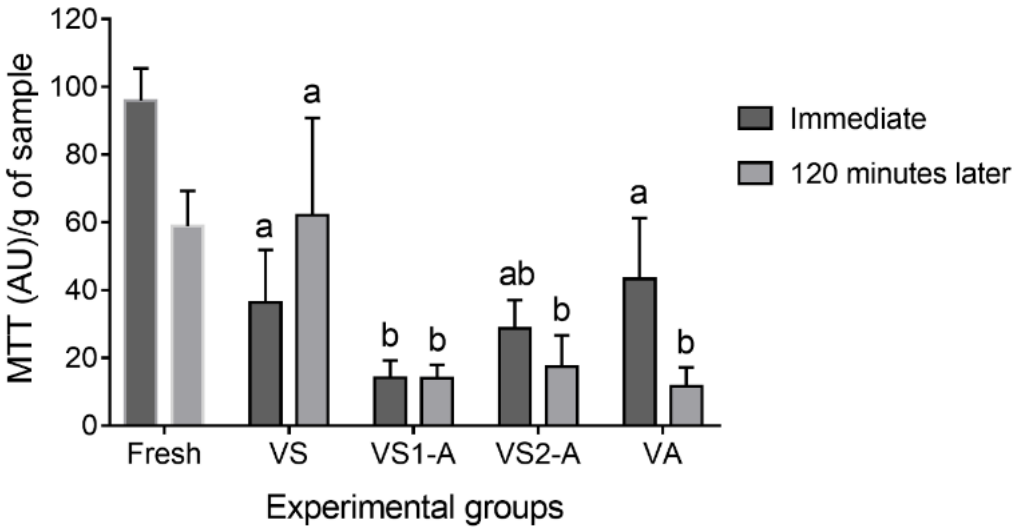

Figure 7. Mitochondrial activity (AU / g) by thiazolyl blue tetrazolium bromide (MTT). Effect of experimental groups (p <0.0001), effect of the moment of analysis (p=0.2262), effect of interaction (p <0.0001). VS (1.5 M methanol + 5.5 M Me_2SO + 0.5 M sucrose); VS1-A (1.5 M methanol + 5.5 M Me_2SO + 0.5 M sucrose; encapsulated in alginate hydrogel); VS2-A (0.75 M methanol + 2.75 M Me_2SO + 0.5 M sucrose; encapsulated in alginate hydrogel); VA (Encapsulated in alginate hydrogel); Fresh = fresh ovarian tissue fragments. Data presented as mean ± SD. Different lower-case letters indicate difference between the experimental groups at the same moment of the analysis.

Lipid peroxidation by TBARS assay values was higher in VA group (124.7 ± 7.86) and did not differ from VS2-A group (63.58 ± 3.13). The lowest lipid peroxidation value was in VS1-A group (39.39 ± 4.36) that did not differ from VS group (40.5 ± 3.31) (Fig. 8).

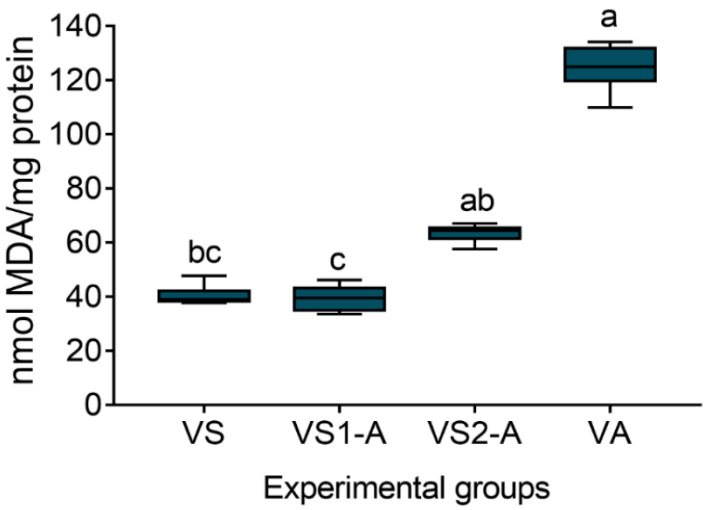

Figure 8. Thiobarbituric acid reactive substances (TBARS) analyzed after vitrification. VS (1.5 M Methanol + 5.5 M Me_2SO + 0.5 M sucrose); VS1-A (1.5 M Methanol + 5.5 M Me_2SO + 0.5 M sucrose – encapsulated in alginate); VS2-A (0.75 M Methanol + 2.75 M Me_2SO + 0.25 M sucrose – encapsulated in alginate); VA (encapsulated in alginate); Fresh = fresh ovarian tissue fragments. Significant difference ($p < 0.0001$) by the Kruskal-Wallis analysis. Data presented as mean ± SD. Different letters indicate difference between treatments by Dunn's test.

5. Discussion

One of the main damages that occur in the cryopreservation process is the intracellular ice formation, which causes membrane damage and, consequently, cell death [18]. In Experiment 1, the exposure to cryoprotectants after ovarian tissue encapsulation in 30 µL alginate hydrogel bead resulted in low membrane integrity of all developmental oocyte stage, this indicates that 30 µL alginate hydrogel bead can hinder or at least slow down the penetration of the CPA in the cell, because of its

greater thickness. Insufficient permeation of cryoprotectants may affect oocyte survival [30,34]. Previous studies have reported that alginate hydrogel granules with a larger diameter resulted in less membrane integrity of Leydig testicular cells [32]. According to these authors, larger granules have a small proportion between surface and volume and difficult temperature transfer with liquid nitrogen [32]. In the present study, when the ovarian tissue was exposed to cryoprotectants before being encapsulated by immersion in alginate hydrogel and warmed to 28ºC, the membrane damage of the Vtg1 and CA oocytes was significantly lower. On the other hand, the integrity of the PG oocyte membrane did not differ in both moments of exposure to cryoprotectants. During maturation, fish oocytes become less permeable [39] and the exposition to cryoprotectant after encapsulation may affect more CPA penetration in CA and Vtg1 oocytes. The temperature around 28ºC is considered ideal for zebrafish maintenance [43] and it is the recommended temperature for warming fragments of zebrafish ovarian tissue following vitrification[25,27].

In Experiment 2, membrane integrity of all oocytes stage assessed by SYBR-14/PI stain showed no difference between vitrified ovarian tissue encapsulated with cryoprotectants (VS1-A) and the vitrified ovarian tissue not encapsulated with the same cryoprotectants (VS). This result suggests that the encapsulation in alginate hydrogel did not increase oocytes membrane damage. Bian et al. [3] without reducing the concentration of cryoprotectants, obtained 81.25% membrane integrity after vitrification alginate-encapsulated human preantral follicles. However, bovine pre-antral follicles encapsulated and vitrified with cryoprotectants (15% Me_2O, 15% EG, 0.5 M sucrose) showed less membrane integrity (45.9%) when compared to non-encapsulated and vitrified follicles with same cryoprotectants concentration (87.5%) [5]. Our results showed a lower integrity of the oocyte membrane after the ovarian tissue was vitrified encapsulated in alginate hydrogel with half CPA (VS2-A) and without CPA (VA). Therefore, the reduction and removal of CPA in encapsulated ovarian tissue increased damage to oocytes. This result is not in agreement with Mohanty et al. [29], which in human fibroblasts encapsulated in alginate hydrogel, was observed that 2% alginate concentration reduces/eliminates the need of CPAs of the encapsulated cells during freezing using Mr. Frosty. The alginate bead structure may

not prevent the encapsulated cells from cryopreservation damages at non-optimal conditions [12]. These results illustrate that the protocol for the vitrification of encapsulated ovarian tissue in alginate hydrogel needs to be adjusted, with regard to the concentration of sodium alginate. Furthermore, the size of the fragments may have interfered negatively, considering that, most of the studies with cells encapsulated in sodium alginate hydrogel and cryopreserved, were carried out using a cell suspension [3,6,29], where the entire cell was involved with the hydrogel. Thus, it would be interesting to use smaller fragments of ovarian tissue.

During cell cryopreservation, cryoprotectants have both protective and toxic effects [49]. Methanol has been considered to be the least toxic cryoprotectant to zebrafish oocytes compared to Me_2SO [49]. Me_2SO is one of CPA most utilized for cryopreservation, but its toxicity limits its usage. It was observed that Me_2SO have toxicity effects on zebrafish oocytes [50] and can increase the concentration of calcium ions in cytoplasm, causing a variety of metabolic responses [51]. The RNA splicing may also be affect by Me_2SO [52]. Although the cryoprotective response observed in oocyte membrane integrity of the ovarian tissue fragments encapsulated in alginate hydrogel with the half of CPA concentration (VS2-A), was significantly lower when compared to the treatment with VS (16.35 and 54.57% for PG, respectively), probably these cells suffered less exposure to CPA and its toxic effects, especially Me_2SO. In further studies, it would be interesting to evaluate, if the decrease of CPAs concentration in ovarian tissue fragments encapsulated in sodium alginate hydrogel allows a better development of oocytes and less molecular damage.

The damages observed in vitellogenics and CA vitrified oocytes (VS, VS1-A, VS2-A and VA) on histology analysis are in agreement with other study that vitrified zebrafish ovarian tissue [25], which were also observed the coalescence of cortical alveoli and may have been caused by the formation of intracellular ice crystals. Pyknosis (reduction of cellular volume and chromatin condensation) and nuclear fragmentation (karyorrhexis), were some characteristics of cell apoptosis [20]. In the present study, nucleus fragmentation in all developmental stages of vitrified ovarian were observed, indicating the occurrence of cellular apoptosis. Autophagic cell death is morphologically characterized by an accumulation of cytoplasmatic autophagic vacuoles [11]. In PG oocytes vitrified were possible to observe presence of vacuoles

in cytoplasm, which may represent the dissolution of the organelles and autophagic cell death. Follicular membrane rupture of vitrified PG oocyte was observed, and is one of the main damages caused by the cryopreservation process [18]. However, no membrane damage was observed on vitellogenics oocytes. Mature female zebrafish have asynchronous ovaries, containing follicles of all stages of development [31], and in histology sections of small size ovarian tissue was not possible to observe large number of each developmental oocyte stage. Then, some characteristics may not have been possible to observe.

Membrane integrity alone are not as informative as the number of cells that have survived the cryopreservation and warming procedures, followed by a functional test, which evaluates metabolic activity [12]. In this study were evaluated the mitochondrial activity immediately and after 120 min in L-15 culture medium (28ºC). The results showed no effect for moment of the analysis, suggesting that there are no changes in mitochondrial activity after 120 min on culture medium. However, the encapsulated ovarian tissue in alginate hydrogel treatments (VS1-A, VS2-A and VA) analyzed 120 min after warming, showed lowest mitochondrial activity when compared to non-encapsulated and vitrified ovarian tissue (VS). The concentration of alginate used in this study may have affected the passage of nutrients from the culture medium. High alginate concentration reduce the pore size and porosity, and can prevent the transport of oxygen and nutrients to the cells and, consequently, contributed to reduced metabolic activity of cells [29].

Oxidative stress is caused when the physiological balance between reactive oxygen species (ROS) production and antioxidant defenses is not efficient [46], it can cause damage to DNA and induce lipid peroxidation which adversely affects membrane structure, fluidity and function [8]. Cryopreservation and freeze–thaw stress, can damage the antioxidant enzymes which protect against lipid peroxidation [48]. Sodium alginate is a rich source of antioxidant compounds [21] and it has recently been reported to prevent lipid peroxidation in cryopreserved buffalo sperm by slow freezing [31]. The vitrified ovarian tissue encapsulated in alginate hydrogel without CPA (VA) showed the highest levels of lipid peroxidation and membrane damage (SYBR-14/PI), while the ovarian tissue encapsulated with CPA (VS1-A) had the lowest

levels and membrane integrity similar to non-encapsulated vitrified tissue (VS). The freeze-thaw process is known to promote lipid peroxidation of the membrane so that cell membranes lose their permeability barriers at a faster rate than cell membranes not treated with CPA, thus shortening cell life [1]. In this sense, the addition of cryoprotectants has a peroxidative protective action. In our study, one of the cryoprotectants used was Me_2SO which is described as being able to reduce the rigidity of the membrane, contributing to the reduction of mechanical and osmotic stress during cell swelling and shrinkage [44]. Therefore, although CPAs have toxic effects, they have important benefits during the cryopreservation process. The non-encapsulated vitrified ovarian tissue, but containing CPA (VS), did not differ from the encapsulated vitrified ovarian tissue with half the CPA concentration (VS2-A). This information suggests that the decrease in CPA in encapsulated and vitrified samples did not result in an increase in lipid peroxidation. During the cryopreservation process, the high content of lipids tends to increase the production of free radicals that induce lipid peroxidation, which leads to cell death [8,41]. Considering the high lipid content of CA and vitellogenic oocytes, it is particularly important that there is no increase in the levels of lipid peroxidation.

In the present study, the sodium alginate hydrogel did not have a cryoprotective action on the integrity of the oocyte membrane and did not promote a greater mitochondrial activity of the oocytes obtained from vitrified and encapsulated zebrafish ovarian tissue. In contrast, the levels of lipid peroxidation of the encapsulated and vitrified samples with half the concentration of CPA (VS2-A) did not differ from the non-encapsulated vitrified ovarian tissue (VS). An important observation made in the study was that the use of the alginate hydrogel encapsulation technique reduced the loss of oocytes during the manipulation of fragments of ovarian tissue. Zebrafish ovaries have a morphology like bunches of grapes in which the oocytes are easily detached from the tissue. Therefore, alginate hydrogel promoted greater support for oocytes, consequently, encapsulation in alginate hydrogel limited the consequences of mechanical stress. Given these results, an interesting alternative would be the oocyte maturation of ovarian tissue encapsulated in alginate hydrogel both *in vitro* and *in vivo* by transplantation, promoting oocytes support. However, it is important to note that this

is the first study reporting the cryopreservation of fish ovarian tissue encapsulated in sodium alginate hydrogel, thus providing the first information of this technique.

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CAPÍTULO III

CONSIDERAÇÕES FINAIS

Os dados obtidos após vitrificação dos fragmentos de tecido ovariano de zebrafish encapsulados em hidrogel de alginato de sódio sugerem que a técnica de encapsulamento não foi capaz de fornecer ação crioprotetora para as membranas dos oócitos ou de reduzir a concentração de crioprotetores. No entanto, é importante ressaltar que este é o primeiro trabalho relatando a criopreservação de tecido ovariano de peixe encapsulado em hidrogel de alginato de sódio, fornecendo assim, as primeiras informações sobre essa técnica. Portanto, mais estudos são necessários para avaliar os efeitos da técnica de encapsulamento de tecido ovariano de zebrafish em hidrogel de alginato na criopreservação, como a utilização de diferentes concentrações de alginato, outros crioprotetores e método de criopreservação (congelamento lento).

A anatomia do ovário de zebrafish tem formato de cacho de uva, no qual os oócitos se desprendem facilmente, e isso se intensifica nos fragmentos de tecido. Durante a execução dos experimentos observou-se que o encapsulamento em hidrogel de alginato de sódio funcionou como um suporte para as células, evitando perda de oócitos durante a adição e remoção de soluções. Portanto, a técnica de encapsulamento de tecido ovariano de zebrafish em hidrogel de alginato de sódio, se mostrou vantajosa nesse sentido, e pode ser utilizada para o cultivo de tecido ovariano de zebrafish, pois proporcionou maior segurança durante a manipulação da amostra. Considerando que a vitrificação de tecido ovariano de zebrafish é capaz apenas de preservar oócitos imaturos, é necessário o desenvolvimento de um protocolo efetivo de maturação *in vitro* ou *in vivo* para que possa ser feito a fertilização desses oócitos. Sendo assim, a utilização do encapsulamento do fragmento de tecido ovariano em hidrogel de alginato pode ser uma alternativa para auxiliar e aprimorar os protocolos de maturação dos oócitos de zebrafish, que até o momento são precários e necessitam de aprimoramentos.

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APÊNDICE

Apêndice 1 – Normas do periódico *Cryobiology*

Guide for Authors

Introduction

The Official Journal of the Society for Cryobiology

Types of article

•Regular Papers
•Brief Communications
•Reviews
•Letters to the Editor

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

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