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AVALIAÇÃO DO EFEITO DO TIMOL DURANTE A VITRIFICAÇÃO DE FOLÍCULOS PRÉ-ANTRAIS INCLUSOS EM TECIDO OVARIANO BOVINO

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AVALIAÇÃO DO EFEITO TIMOL NA VITRIFICAÇÃO DE FOLÍCULOS PRÉ-ANTRAIS INCLUSOS NO TECIDO OVARIANO BOVINO

Dissertação apresentada ao Programa de Pós-Graduação em Biotecnologia da Universidade Federal do Ceará, como requisito parcial à obtenção do título de Mestre em Biotecnologia. Área de concentração: Biotecnologia Linha de pesquisa: Análise integrativa de Sistemas Biológicos.

Orientador: Prof. Dr. José Roberto Viana Silva.

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2026

“A Deus, meus pais e irmã, pelo empenho e dedicação com os quais me criaram”

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“Aquele que começou a boa obra em vós há de completá-la.” (Filipenses 1:6)

RESUMO

A criopreservação é uma estratégia amplamente utilizada para a preservação da fertilidade; entretanto, a vitrificação ainda apresenta limitações associadas ao estresse mecânico, químico e oxidativo, que podem comprometer a integridade tecidual após o reaquecimento. Nesse contexto, a suplementação com antioxidantes tem sido proposta como uma abordagem promissora para minimizar as crioinjúrias. O presente estudo teve como objetivo avaliar os efeitos da dose–resposta e o potencial antioxidante do timol durante a vitrificação de tecido ovariano bovino. Inicialmente, foi conduzido um ensaio dose-resposta em células do cumulus bovinas, cultivadas in vitro e expostas a diferentes concentrações de timol (2,5; 25 e 250 µg/mL), a fim de avaliar a viabilidade celular e a citotoxicidade por meio de ensaio com calceína-AM e etídio homodímero-1. Em seguida, fragmentos do córtex ovariano bovino foram fixados em paraformaldeído a 10% (controle não vitrificado) ou submetidos à vitrificação em soluções suplementadas com timol nas mesmas concentrações. Após uma semana em nitrogênio líquido, os tecidos foram reaquecidos em soluções com concentrações decrescentes de sacarose; parte das amostras foi analisada imediatamente após o reaquecimento, enquanto as demais foram incubadas in vitro por 24 h. A integridade tecidual foi avaliada por meio da morfologia folicular, da densidade do estroma ovariano e dos componentes da matriz extracelular, incluindo colágeno e glicosaminoglicanos. O estado redox foi analisado pela atividade das enzimas antioxidantes (SOD, CAT e GPX), bem como pelos níveis de tióis e nitrito. Os resultados do ensaio com células do cúmulus mostraram que 25 µg/mL e 250 µg/mL de timol aumentaram a marcação com calceína em relação ao meio controle, o que é um indicativo de viabilidade celular. A vitrificação do tecido ovariano promoveu alterações no estado redox, evidenciadas pela depleção de tióis e pela redução da atividade da GPX. A suplementação com 25 µg/mL timol aumentou a atividade da SOD em tecido vitrificado, o que pode representar uma resposta ao estresse oxidativo. Por outro lado, a presença de 250 µg/mL de timol preservou os níveis de tióis e a atividade da GPX semelhantes aos valores do tecido não vitrificado. Os níveis de nitrito foram reduzidos após a vitrificação em todos os grupos, provavelmente refletindo supressão metabólica. Além disso, o timol, em todas as concentrações, aumentou a proporção de folículos morfologicamente normais, preservou a densidade do estroma ovariano e manteve os componentes da matriz extracelular após o reaquecimento. Conclui-se que a presença de 250 µg/mL de timol na solução de vitrificação de córtex ovariano bovino apresenta efeitos citoprotetores que contribuem para a manutenção do equilíbrio redox após o reaquecimento, e para a preservação da integridade estrutural do tecido.

Palavras-chave: antioxidante; cristais de gelo; fertilidade; reprodução.

ABSTRACT

Cryopreservation is a widely used strategy for preserving fertility; however, vitrification still has limitations associated with mechanical, chemical, and oxidative stress, which can compromise tissue integrity after rewarming. In this context, antioxidant supplementation has been proposed as a promising approach to minimize cryoinjuries. The present study aimed to evaluate the dose-response effects and antioxidant potential of thymol during the vitrification of bovine ovarian tissue. Initially, a dose-response assay was conducted on bovine cumulus cells, cultured in vitro and exposed to different concentrations of thymol (2.5, 25, and 250 µg/mL), in order to evaluate cell viability and cytotoxicity using calcein-AM and ethidium homodimer-1 assays. Next, fragments of bovine ovarian cortex were fixed in 10% paraformaldehyde (non-vitrified control) or subjected to vitrification in solutions supplemented with thymol at the same concentrations. After storage in liquid nitrogen, the tissues were rewarmed in solutions with decreasing concentrations of sucrose; part of the samples was analyzed immediately after rewarming, while the rest were incubated in vitro for 24 h. Tissue integrity was assessed by follicular morphology, ovarian stromal density, and extracellular matrix components, including collagen and glycosaminoglycans. The redox state was analyzed by antioxidant enzyme activity (SOD, CAT, and GPX) and thiol and nitrite levels. The results of the cumulus cell assay showed that 25 µg/mL and 250 µg/mL of thymol increased calcein labeling compared to the control medium, which is indicative of cell viability. Vitrification of ovarian tissue promoted changes in the redox state, evidenced by thiol depletion and reduced GPX activity. Supplementation with 25 µg/mL thymol increased SOD activity in vitrified tissue, which may represent a response to oxidative stress. On the other hand, the presence of 250 µg/mL thymol preserved thiol levels and GPX activity similar to those in non-vitrified tissue. Nitrite levels were reduced after vitrification in all groups, likely reflecting metabolic suppression. In addition, thymol, at all concentrations, increased the proportion of morphologically normal follicles, preserved ovarian stromal density, and maintained extracellular matrix components after rewarming. It is concluded that the presence of 250 µg/mL thymol in the vitrification solution of bovine ovarian cortex has cytoprotective effects that contribute to maintaining redox balance after rewarming, and to preserving the structural integrity of the tissue.

Keywords: antioxidants; fertility; ice crystals; reproduction.

LISTA DE ILUSTRAÇÕES DA DISSERTAÇÃO

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LISTA DE ABREVIATURAS E SIGLAS

EROs	Espécies reativas de oxigênio
DNA	Ácido desoxirribonucleico
RNA	Ácido ribonucleico
RNAm	RNA mensageiro
SOD	Superóxido dismutase
CAT	Catalase
GPX	Glutationa peroxidase
GSH	Glutationa reduzida
GSSG	Glutationa oxidada
GR	Glutationa redutase
MDA	Malonaldeído
BMP15	Proteína morfogenética óssea 15
GDF9	Fator de crescimento e diferenciação 9
KITL	Ligante da tirosina quinase receptora
KIT	Tirosina quinase
mTOR	Alvo mecanístico da rapamicina
FOXO3a	Forkhead box O3a
FSH	Hormônio folículo-estimulante
LH	Hormônio luteinizante
ACPs	Agentes crioprotetores
DMSO	Dimetilsulfóxido
PEG	Polietileno glicol
PBS	Tampão fosfato salino
EG	Etilenoglicol
PCR	Reação em cadeia da polimerase
α-MEM	Meio essencial mínimo alfa
SFB	Soro fetal bovino
t-BHP	Hidroperóxido de tert-butila

cDNA	DNA complementar
ANOVA	Análise de variância
COX-2	Ciclooxigenase-2
EMT	Transição epitelial–mesenquimal
ERK	Quinase regulada por sinal extracelular
GFAP	Proteína ácida fibrilar glial
HO-1	Heme oxigenase-1
IL-6	Interleucina-6
iNOS	Óxido nítrico sintase induzível
JNK	Quinase N-terminal de c-Jun
LPS	Lipopolissacarídeo
MAPK	Quinase ativada por mitógeno
MCP-1	Proteína quimioatraente de monócitos-1
NF-κB	Fator nuclear kappa B
NO	Óxido nítrico
Nrf2	Fator nuclear relacionado ao eritroide 2
PD	Doença de Parkinson
PI3K/Akt	Fosfatidilinositol 3-quinase / Proteína quinase B
PKCα	Proteína quinase C alfa
PSD95	Proteína de densidade pós-sináptica 95
TBARS	Substâncias reativas ao ácido tiobarbitúrico
TLR4	Receptor do tipo Toll 4
TNF-α	Fator de necrose tumoral alfa
COCs	Complexos cúmulus–oócito
CCs	Células do cúmulus
ECM	Matriz extracelular
GAGs	Glicosaminoglicanos
BSA	Albumina sérica bovina
NADPH	Nicotinamida adenina dinucleotídeo fosfato reduzido
DTNB	5,5′-ditiobis(2-nitrobenzoico)
TNB^{2-}	2-nitro-5-tiobenzoato
HEPES	Ácido 4-(2-hidroxietil)-1-piperazinaetanosulfônico
TCM-199	Meio de cultura TCM 199

FBS	Soro fetal bovino
NIH	National Institutes of Health
TM	Timol

LISTA DE SÍMBOLOS

%	Porcentagem
μg	Micrograma
mL	Mililitro
=	Igual a
mg	Miligrama
oC	Graus Celsius
M	Molar
L	Litros
μm	Micrometros
mM	Milimolar
pH	Potencial de hidrogênio
nm	Nanômetro
g	Gramas
±	SEM Erro padrão da média
S	Segundos
μL	Microlitro
$P < 0,05$.	Probabilidade de erro menor que 5%
®	Marca Registrada
μM	Micromolar
kg	Quilograma
β	Beta
κ	Kappa
IC_{50}	Concentração inibitória de 50%
α	Alfa

SUMÁRIO

1 INTRODUÇÃO

O Brasil se destaca como um dos principais produtores mundiais de alimentos, sendo responsável por uma parcela significativa do consumo global de carne bovina, ao mesmo tempo em que precisa atender à crescente demanda populacional, especialmente no que se refere às exportações para a China e a União Europeia (EU COMMISSION, 2019; Aragão, Contini, 2021; Beckman et al., 2022; Buczinski et al., 2023). O aumento da produção bovina é um dos fatores associados ao desmatamento, causando impactos ambientais significativos, assim, o desenvolvimento de métodos sustentáveis para reprodução bovina torna-se essencial (Hoang, Kanemoto, 2021; Sylvester et al., 2024; Sevilla et al 2025). Nesse contexto, as biotécnicas reprodutivas contribuem para a produção de carne bovina de alta qualidade ao otimizarem o melhoramento genético e a eficiência reprodutiva dos rebanhos. A aplicação dessas técnicas permite maior produtividade por animal e por área, reduzindo a necessidade de ampliação das áreas de criação e, consequentemente, os impactos ambientais associados, demonstrando sua relevância econômica e ambiental (Baruselli et al., 2025). Nesse cenário, a criopreservação desempenha papel fundamental ao permitir a conservação e o transporte do material genético de espécies com alto potencial reprodutivo, otimizando as cadeias produtivas de carne e leite (Benedito et al., 2024).

A criopreservação é uma técnica que permite a sobrevivência de células e tecidos em baixas temperaturas com atividade metabólica reduzida ou cessada por longos períodos (Mazur, 1970; Sharafi et al., 2022; Silva, 2022). O crioarmazenamento de tecido ovariano apresenta diversas vantagens para área da reprodução, auxiliando tanto na compreensão da função ovariana quanto na conservação de espécies ameaçadas de extinção e na reprodução de animais de produção (Hildebrant et al., 2021; Lujic et al., 2023). A grande quantidade de folículos inclusos nos tecidos corticais ovarianos torna a criopreservação de tecido ovariano uma técnica promissora para conservar a genética de fêmeas com interesse zootécnico, como os bovinos (Beck et al., 2020). Na maioria das espécies em que a criopreservação do tecido ovariano vem sendo estudada, dois métodos primários de criopreservação têm sido utilizados: o congelamento lento e a vitrificação (Mazur, Leibo, Chu, 1972; Rall, Fahy, 1985; Candelaria, Denicol, 2024; Deligiannis et al., 2024; Schallmoser et al., 2024).

A vitrificação envolve o congelamento rápido do tecido ovariano por meio do uso de altas concentrações de crioprotetores, promovendo a transição das células do estado líquido para o vítreo (Rall, Fahy, 1985; Sharma et al., 2021; Lin et al., 2022). O congelamento lento ocorre por meio do resfriamento em taxas controladas; entretanto, a transição da água intra e extracelular da fase líquida para a sólida durante esse processo favorece a formação de cristais de gelo, os quais podem provocar lesões extracelulares (Mazur, Leibo, Chu, 1972; Behl et al., 2023; Asadi et al., 2024). Embora um protocolo ideal não tenha sido estabelecido, a vitrificação, quando comparada ao congelamento lento, tem se demonstrado uma melhor alternativa, uma vez que proporciona melhor preservação da morfologia e função ovariana, além de apresentar maiores taxas de sobrevivência e desenvolvimento embrionário até o estágio de blastocisto (Labrune et al., 2020; Najafzadeh et al., 2021). Ademais, a vitrificação é uma técnica mais rápida e econômica, por utilizar menores volumes de nitrogênio líquido e dispensar o uso dos freezers programáveis, tornando o processo mais acessível (Kometas, 2021).

Apesar de muitos estudos demonstrarem sucesso no processo de vitrificação, a técnica ainda apresenta algumas limitações, como estresse mecânico e químico ocasionado pela toxicidade dos crioprotetores e formação de cristais de gelo, os quais resultam em danos físicos que afetam as membranas fosfolipídicas das células ovarianas (Abdelhady et al., 2024; Bizarro-Silva et al., 2024). Além disso, o excesso de espécies reativas de oxigênio (EROs), decorrente do estresse osmótico durante o processo de aquecimento, associado à redução das defesas antioxidantes, pode resultar em estresse oxidativo. Esse desequilíbrio redox promove danos ao DNA, comprometimento mitocondrial e peroxidação de proteínas (Gualtieri et al., 2021; Rodrigues et al., 2021; Cao et al., 2022; Cho et al., 2024). Diante dessa realidade, a identificação de substâncias naturais com propriedades antioxidantes capazes de promover efeitos benéficos contra o estresse oxidativo durante protocolos de vitrificação tem se tornado cada vez mais frequente. Nesse contexto, o monoterpeno timol é considerado um composto promissor, em razão de suas propriedades antioxidantes já documentadas.

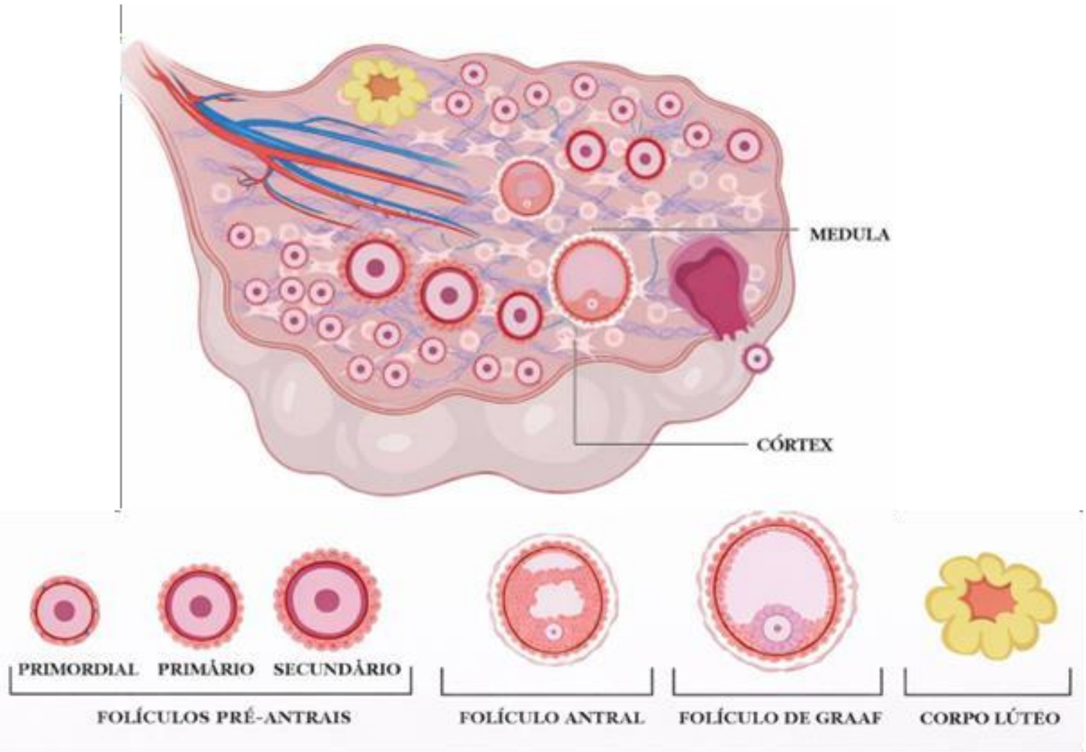
Em modelos experimentais, incluindo células de testículos de ratos, o timol demonstrou reduzir processos inflamatórios e apoptóticos, além de aumentar os níveis de enzimas antioxidantes, como superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPX), estando também associado à diminuição dos níveis de malonaldeído (MDA). Esses achados reforçam o potencial antioxidante do timol e sustentam sua aplicação como estratégia para minimizar danos oxidativos em processos de criopreservação (Tijani et al., 2023). Além disso, efeitos citoprotetores do timol frente ao estresse oxidativo também foram observados em modelos não reprodutivos, como fibroblastos humanos, nos quais o pré-tratamento com timol reduziu significativamente os danos induzidos por agentes pró-oxidantes, evidenciando a amplitude de sua ação antioxidante em diferentes tipos celulares (Dashtaki et al., 2020). Ademais, a administração do timol em ovários de ratas submetidas à radiação gama promoveu a proliferação de células da granulosa, concomitantemente à redução do estresse oxidativo, resultando no resgate da reserva ovariana frente aos efeitos deletérios da radiação (Mahran et al., 2019). Considerando a importância do timol como agente antioxidante e seu potencial impacto na preservação da fertilidade, bem como a relevância da vitrificação de tecido ovariano, a revisão de literatura apresentada a seguir organiza-se em tópicos que abordam os principais fundamentos teóricos que sustentam o presente estudo.

2 REFERENCIAL TEÓRICO

1.1 Características e funções e dos folículos ovarianos

Em mamíferos, a função ovariana é essencial para manutenção da vida reprodutiva adequada, pois assegura a produção dos gametas femininos e a regulação de um ambiente endócrino estável para a fêmea. A estrutura ovariana é bem similar entre os mamíferos, apresentando uma região medular, formada por nervos, vasos sanguíneos (artérias e veias), e uma região cortical contendo folículos ovarianos em diferentes estágios de desenvolvimento, além de corpos lúteos, estruturas temporárias responsáveis pela produção de progesterona após a ovulação, e corpos albicans, que correspondem à forma regressiva e fibrosa do corpo lúteo (Leitão et al., 2009) (Figura 1).

Figura 1. Ovário de mamífero e suas principais estruturas



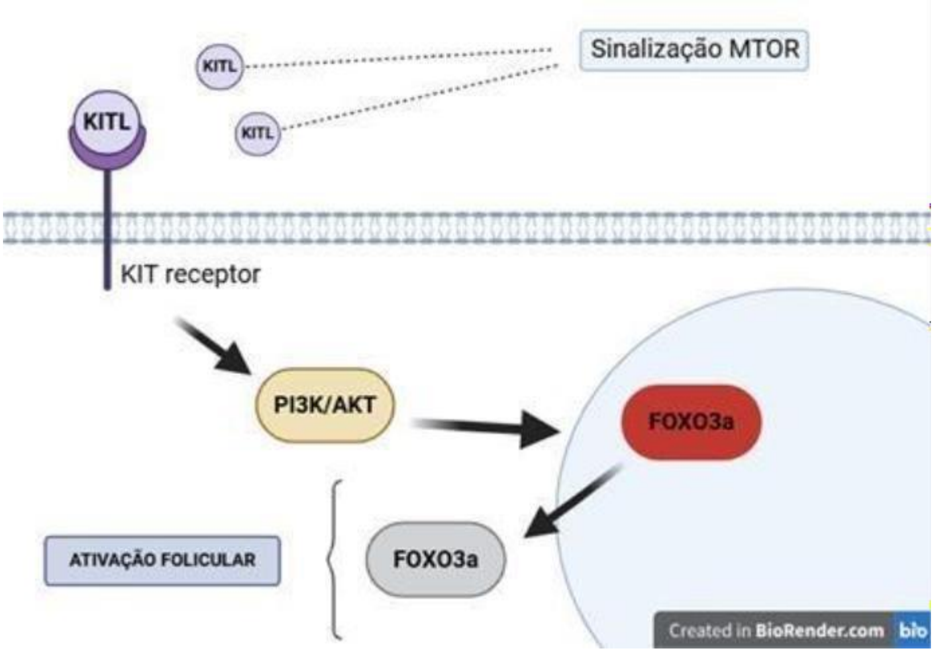
Fonte: Adaptado de Félix et al., 2024.

O ovário contém diversos tipos celulares, incluindo oócitos, células da granulosa, células da teca, células do estroma ovariano e células musculares lisas. Além disso, apresenta células endoteliais, responsáveis pela formação da rede vascular, bem como células do sistema imunológico, como monócitos, linfócitos e células natural killer (Fan et al., 2019; Wagner et al., 2020; Wang et al.,2020; Wu et al., 2024). Esses componentes em conjunto com a matriz extracelular, envolvem os folículos e fornecem suporte físico e biológico, favorecendo a sinalização celular e o desenvolvimento folicular a partir do pool inicial (Hu, Ling, Ren, 2022; Kinnear et al., 2020).

Durante o desenvolvimento embrionário, as células germinativas primordiais são especificadas no epiblasto, passam por um processo de multiplicação e migram para as cristas genitais, onde se diferenciam em oogônias (Tam, Snow, 1981; Hayashi et al., 2002; Saitou, Yamaji, 2010). Posteriormente, formam-se os folículos primordiais, constituídos por um oócito primário, interrompido na prófase da meiose I, circundado por uma única camada de células da granulosa pavimentosa. Esses folículos ficam em estado quiescente, constituindo o pool de reserva ovariana (Zhang et al., 2023; Araújo et al., 2014; Grossman, Shalgi, 2016). Portanto, o estabelecimento do pool de folículos primordiais ocorre durante o período perinatal nas fêmeas de mamíferos, visando fornecer oócitos fertilizáveis ao longo de sua vida reprodutiva (Li et al., 2020). Os folículos primordiais são classificados como folículos pré-antrais e, após sua ativação, iniciam o processo de crescimento folicular, dando origem aos folículos primários, secundários e, posteriormente, aos folículos antrais.

A ativação dos folículos primordiais, por sua vez, é um processo no qual eles deixam seu estado de dormência e iniciam-se o crescimento folicular. Uma vez que o folículo entra no estágio de crescimento, ele não pode retornar ao estado de dormência (Zhang et al., 2015). Esse recrutamento inicial é diferente do recrutamento cíclico que é regulado pelas gonadotrofinas (Zhang, Liu, 2015; Kallen et al., 2018). O recrutamento inicial dos folículos primordiais é regulado por fatores de crescimento secretados pelo oócito, como a proteína morfogenética óssea 15 (BMP-15) e o fator de crescimento e diferenciação 9 (GDF-9), que contribuem para a ativação e o crescimento folicular (Chang, Qiao, Leung, 2017). Além disso, a interação entre o oócito e as células da granulosa desempenha papel central nesse processo. Nessas células, a ativação do alvo mecanístico da rapamicina (mTOR) estimula a expressão do ligante da tirosina quinase receptora (KITL), o qual se liga ao receptor KIT presente no oócito, ativando a via PI3K/Akt e contribuindo para o controle do crescimento oocitário e da ativação do folículo primordial (Zhang et al., 2014; 2015; 2022; 2023). A ativação dessa via resulta na fosforilação da Forkhead Box O3a (FOXO3a) que é transportada para fora do núcleo do oócito, inativando sua função na quiescência levando a ativação dos folículos primordiais (Zhang et al., 2023; Zhang et al., 2024). A partir da ativação, ocorre um aumento do diâmetro do oócito presente no folículo e as células pavimentosas se tornam cúbicas, formando-se folículo primário (Chen et al., 2020).

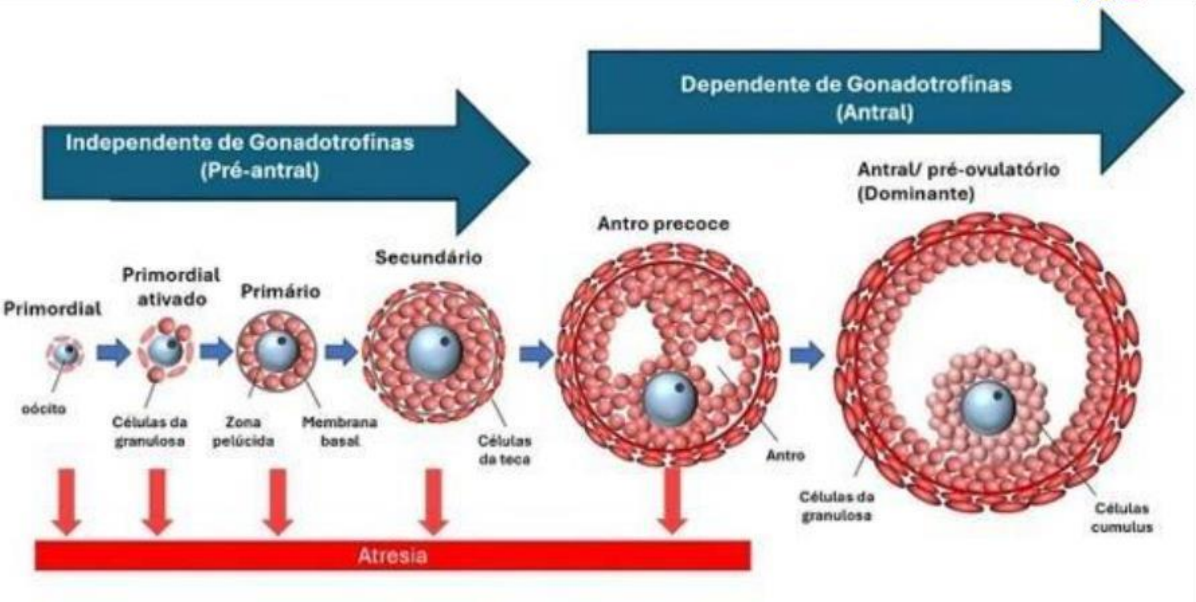
Figura 2. Sinalização MTOR na ativação de folículos primordiais



Fonte: Elaborada pela autora

Os folículos ovarianos são classificados em pré-antrais e antrais. Os folículos pré-antrais incluem os estágios primordial, primário e secundário, caracterizados pela ausência de antro. Embora níveis basais de hormônio folículo-estimulante (FSH) estejam presentes na circulação, os folículos primordiais não são responsivos a esse hormônio, contudo os níveis basais de FSH nessa fase inicial são insuficientes, dependendo predominantemente de sinais intra-ovarianos para sua ativação (Fortune et al., 2003; Jinno, 2025). Durante a ativação folicular, os folículos primordiais iniciam o crescimento e diferenciam-se em folículos primários e, subsequentemente, secundários. Em etapas posteriores do desenvolvimento folicular, ocorre a formação da cavidade antral, seguida pela progressão da maturação oocitária. Esse processo é regulado pelo eixo hipotálamo-hipófise-gônada, após a puberdade (Dhole, Kumar, 2017), que promove a liberação de gonadotrofinas como hormônio folículo estimulante (FSH) e o hormônio luteinizante (LH), sendo fatores importantes para fases de recrutamento, seleção e dominância folicular para ovulação. (Gougeon, 2010; Figueiredo et al., 2018; Adona et al., 2015; Mehlmann, 2005). (Figura 3).

Figura 3. Desenvolvimento folicular: folículos pré-antrais e antrais



Fonte: Adaptado de Lim; Jeremy; Kylie, (2021)

Apesar de diversos folículos iniciarem o processo de crescimento, geralmente apenas um atinge a dominância e completa o processo de ovulação, enquanto os demais são eliminados por atresia folicular. A atresia é um processo fisiológico de degeneração que pode ocorrer em diferentes estágios do desenvolvimento folicular e é caracterizada por alterações morfológicas, como retração do oócito, picnose nuclear e desorganização das células da granulosa (Zhou, Peng, Mei, 2019; Walker et al., 2021). Esse mecanismo de morte celular, ocorre tanto pela ativação da cascata de caspases, levando a fragmentação do DNA, quanto pelo acúmulo de autofagossomos, resultando na morte por autofagia celular (Cacciottola et al., 2023). Essa valiosa fonte de material genético, que frequentemente é perdida, pode ser preservada e otimizada com auxílio das biotécnicas da reprodução, como a criopreservação. Essa técnica não apenas conserva a fertilidade, mas também amplia o aproveitamento da reserva ovariana que seria naturalmente perdida.

1.2 Criopreservação de tecidos e células

A criopreservação de tecido ovariano tem se tornado cada vez mais promissora para otimizar o melhoramento genético, ela consiste na preservação da população gametogênica em baixas temperaturas utilizando nitrogênio líquido (-196ºC) (Sparks, 2015; Gorrincho, 2018;

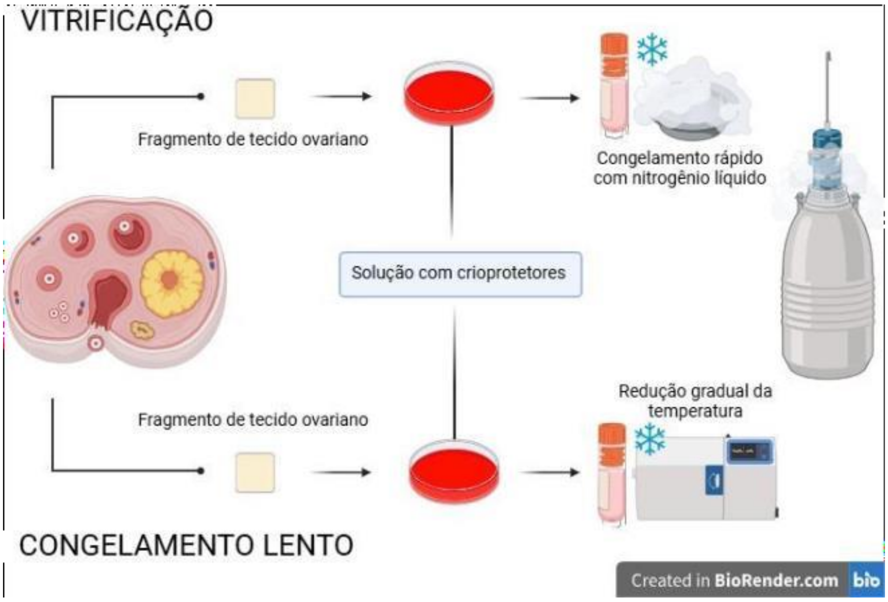
Whaley et al., 2021). Um dos principais objetivos da criopreservação de tecido ovariano é a preservação de folículos pré-antrais ovarianos, que posteriormente transplantados ou submetidos a processos de maturação, apresentam oócitos viáveis para fertilização, aumentando as taxas de reprodução e fecundidade (Zhang et al., 2024). O armazenamento de material biológico apresenta diversas vantagens, inclusive na bovinocultura, ao permitir a conservação e o transporte de material genético de animais com elevado potencial reprodutivo (Benedito et al., 2024). Essa estratégia fornece suporte à aplicação de biotécnicas reprodutivas, como a inseminação artificial, a transferência de embriões e a criopreservação, contribuindo para o aumento da sustentabilidade, da lucratividade e da eficiência produtiva na produção de carne bovina, com reflexos positivos na segurança alimentar global (Baruselli et al., 2025). Além disso, a conservação de material genético de animais de alto valor genético favorece maior eficiência reprodutiva e produtiva dos rebanhos, permitindo a produção da mesma quantidade de alimento com menor área ocupada, o que reduz os impactos ambientais associados à atividade pecuária (Lucy & Pohler, 2024; Brito et al., 2021).

Além dos benefícios relacionados à reprodução animal, a criopreservação também desempenha papel importante para a medicina humana. A criopreservação de tecido ovariano tem sido amplamente utilizada para preservar a fertilidade feminina em casos de doenças malignas ou tratamento de quimioterapias, radioterapia e transplantes (Siegel et al, 2021; Ugai et al., 2022; Di martino et al., 2022; Mercier, Johnson, Kallen, 2024). O tecido ovariano criopreservado e transplantado tem demonstrado resultados significativos com taxas de gravidez bem sucedidas e nascidos vivos (Finkelstein et al., 2024; Wang et al., 2024), além de ser a principal opção para pacientes pré-púberes que não podem se submeter a estimulação ovariana que é necessária para congelamento de oócitos ou embriões (Chung et al., 2023).

A técnica envolve a extração e preparação da camada cortical do ovário para o processo de criopreservação, a qual, além de possibilitar a restauração da função reprodutiva, também contribui para a preservação da função endócrina ovariana (Dolmans, 2018). A manutenção dessa função é particularmente relevante, uma vez que a menopausa, caracterizada pela interrupção da produção de estrógeno, afeta mulheres por aproximadamente 30 a 40% de suas vidas e está associada ao aumento do risco de diversas complicações, como doenças cardiovasculares e osteoporose (Milicevic, Balaban, 2017; Shin et al., 2022; Bijelic). Um estudo conduzido com camundongos avaliou a reposição hormonal em tecido ovariano na redução da perda de tecido ósseo pós-menopausa de ovários de camundongos criopreservados e autotransplantados. Os autores reportaram que não apenas a estrutura óssea foi restaurada como também a saúde uterina com a retomada da foliculogênese e produção dos hormônios no grupo experimental (Yoo et al., 2022). A criopreservação de tecido ovariano com transplante é uma inovação promissora para medicina reprodutiva, com potencial na preservação da fertilidade e como método natural para o retardo da menopausa (Sacinti et al., 2024).

A criopreservação pode ser realizada a partir de dois métodos principais, a vitrificação e o congelamento lento (Marques et al., 2019) (Figura 4). No processo de vitrificação as células são submetidas a temperaturas baixas rapidamente, se convertendo do estado líquido para o vítreo, amenizando a formação dos cristais de gelo (Sharma et al., 2021; Deligiannis et al, 2024). Como estratégia de conservação de espécies ameaçadas, um estudo realizou a vitrificação de oogônias de duas espécies de esturjões, grupo de peixes em elevado risco de extinção. Após o aquecimento, as células vitrificadas mantiveram a capacidade de colonizar as cristas gonadais dos esturjões receptores, com desempenho semelhante ao controle fresco, demonstrando o potencial da criopreservação celular para a preservação da fertilidade (Lujic et al., 2023). O congelamento lento é dependente de um equipamento responsável pelo controle da temperatura, como um freezer programável, as células passam do estado líquido para sólido, esse processo pode durar horas para ser finalizado, o que contribui para formação dos cristais de gelo (Zhang et al., 2016; Esmeryan et al.,2024).

Figura 4. Técnicas de criopreservação: vitrificação e congelamento lento



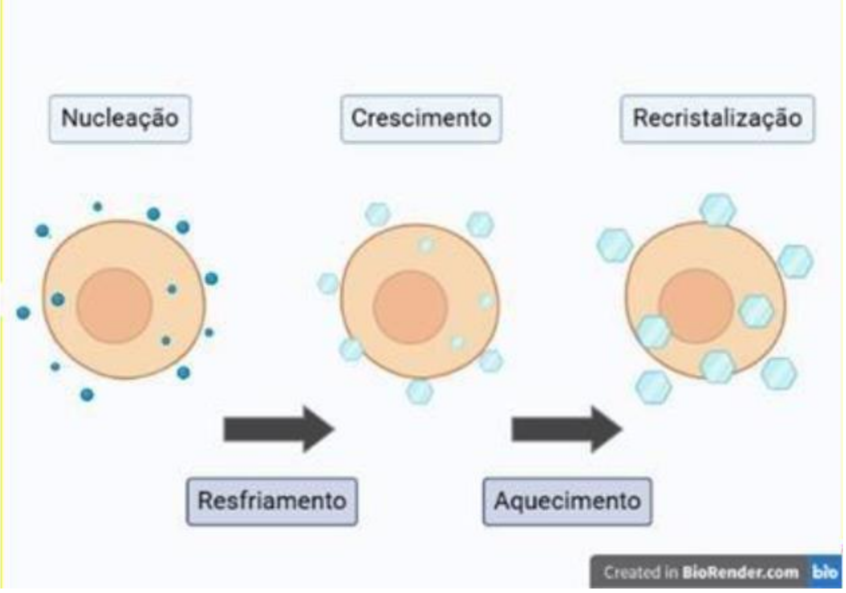
Fonte: Elaborado pela autora.

Nesse sentido, a vitrificação tem mostrado resultados promissores em relação ao congelamento lento. A vitrificação manteve a viabilidade de células reprodutivas de animais em extinção (Lujic et al., 2023), como também obteve melhor preservação da morfologia folicular e expressão de genes relacionados à esteroidogênese nas células da granulosa, como CYP11A e STAR, bem como à preservação da expressão de genes oocitários (GDF9 e ZP3) e de genes envolvidos na regulação do ciclo celular (CCND2 e CDKN1A) (Labrune et al., 2020). A técnica demonstrou ser a alternativa mais eficaz para a criopreservação de blastocistos bovinos, com influência para melhoria genética de rebanhos (Najafzadeh et al., 2021). Demonstrou ser mais eficiente, econômica e rápida, reduzindo tempo para congelar tecido ovariano tornando mais acessível ao público alvo (Kometas et al., 2021), sendo reconhecida com destaque em relação ao congelamento lento com base em relatos de sucesso no nascimento de bebês após a vitrificação de tecido ovariano (Schallmoser et al., 2024). Contudo, apesar de obter potenciais resultados, a criopreservação de tecido ovariano ainda apresenta alguns desafios como o estresse oxidativo, o qual induz a fragmentação do DNA, e reduz o potencial mitocondrial devido a produção excessiva de radicais livres (Gualtieri et al., 2021; Cao et al., 2022; Luo, Trao, 2023). A compreensão mais aprofundada sobre o estresse oxidativo é essencial para amenizar seus efeitos durante a vitrificação e assim, aprimorar a viabilidade e a qualidade das amostras.

1.3 Estresse oxidativo durante a criopreservação

Uma das principais dificuldades no processo de criopreservação ocorre em função do congelamento da água intracelular. Esse processo promove a formação de cristais de gelo, que comprometem a viabilidade das células ocasionando estresse mecânico (Chang, Zhao, 2021; Lee et al., 2021; Abdelhady et al., 2024). A formação de cristais de gelo durante a criopreservação ocorre principalmente ao longo dos ciclos de resfriamento e aquecimento. Durante o resfriamento, a diminuição da temperatura leva ao super-resfriamento da água intracelular, tornando-a instável e favorecendo o início do processo de nucleação. Já na fase de aquecimento, pode ocorrer a recristalização, na qual pequenos cristais de gelo se fundem e aumentam de tamanho, causando danos físicos às células e comprometendo sua viabilidade (Wilkins et al., 2019; Fowler & Toner, 2006; Huang, He & Yarmush, 2021). De forma geral, a formação dos cristais de gelo envolve três etapas principais: nucleação, crescimento e recristalização. A nucleação corresponde à etapa inicial, na qual as moléculas de água se organizam em um arranjo cristalino estável, formando um núcleo sólido. Em seguida, ocorre a fase de crescimento, caracterizada pela deposição contínua de moléculas de água sobre esse núcleo, resultando na formação de cristais de maior tamanho. A recristalização ocorre predominantemente durante o aquecimento, quando cristais menores se unem a cristais maiores, um fenômeno conhecido como maturação de Ostwald (Figura 5), intensificando os danos celulares (Zhu, Zhou & Sun, 2019; Lin, Cao & Li, 2023; Markov, 2016; Bogdanova, Fureby & Kocherbitov, 2022).

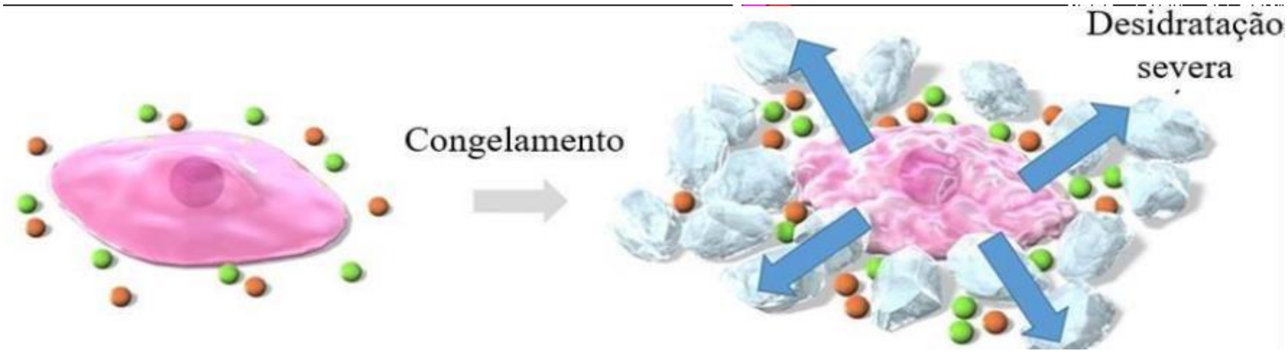
Figura 5. Formação dos cristais de gelo durante a criopreservação



Fonte: Elaborado pela autora.

Os cristais de gelo formados intracelular e extracelular durante a técnica, causam crioinjúrias e desestabilizam os gradientes osmóticos, aumentando a concentração de solutos levando a desidratação celular e estresse osmótico (Chang, Zhao, 2021; Murray, Gibson, 2022; Gowthami et al., 2024). Após o congelamento a água se solidifica deixando a solução de solutos mais concentrada, forçando a saída de água do interior da célula para equilibrar as concentrações. Esse estresse osmótico pode danificar proteínas e outros componentes celulares importantes devido a desidratação intensa (Murray, Gibson, 2022; Góngora, Holt, Gosálvez, 2024; Cho et al., 2024). Um estudo investigou os efeitos citotóxicos do estresse osmótico durante a criopreservação e demonstrou redução na contagem celular associada a lesões osmóticas. Essas lesões ocorrem principalmente durante a reexpansão celular após o encolhimento induzido pelas variações nas concentrações de solutos intra e extracelulares. Tanto células viáveis quanto não viáveis podem sofrer ruptura de membrana, comprometendo a integridade e a viabilidade celular, sendo as células de menor tamanho as mais suscetíveis à lise por expansão (Traversari et al., 2022; Traversari et al., 2024).

Figura 6. Estresse osmótico na criopreservação



Fonte: Adaptado de Matsumura et al., 2021.

Os danos mecânicos causados pelos cristais de gelo e as alterações osmóticas favorecem o desequilíbrio redox celular, resultando em estresse oxidativo e comprometimento da viabilidade celular (Gualteri et al., 2021; Góngora, Holt, Gosálvez, 2024). Diante disso, tornou-se fundamental a utilização de agentes crioprotetores (ACPs) nos protocolos de criopreservação para atenuar esses efeitos (Ben-Amar, Allel, Bouamama-Gzara, 2024; El Cury-Silva et al., 2021). Os ACPs podem ser organizados em dois grupos distintos, de acordo com sua capacidade de atravessar a membrana celular. Os ACPs com características penetrantes são compostos químicos de baixo peso molecular, eles atuam aumentando a viscosidade das moléculas de água e diminuindo seu ponto de congelamento (Whaley et al., 2021; Verheijen et al., 2019). Dessa forma a interação entre as moléculas diminuem, mitigando a formação dos cristais de gelo, entre os crioprotetores permeáveis mais comuns nos protocolos de criopreservação estão o dimetilsulfóxido (DMSO) e o etilenoglicol (EG) (Ishizaki et al., 2023).

Os ACPs não permeáveis, por sua vez, são compostos com alto peso molecular, logo, não ultrapassam a membrana plasmática, mas atuam no meio extracelular (Jia et al., 2021). Estes compostos promovem a retirada da água livre causando desidratação intracelular devido seu efeito osmótico amenizando a formação dos cristais de gelo. Dentre os mais utilizados estão o polietileno glicol (PEG) e açúcares como a sacarose e trealose (Megoura, Ispas-szabo, Mateescu, 2023; Jia et al., 2024). Assim, percebe-se que o uso de ACPs é fundamental na redução da formação dos cristais de gelo, destacando sua importância e recomendação para as técnicas de criopreservação (Yuan, et al., 2024; Aramli; Sarvi, Pourahad, 2024; Tahmasebi et al., 2024; Abdelhady et al., 2024). Contudo, embora ACPs apresentem efeitos positivos, também são identificadas algumas limitações relacionadas às concentrações utilizadas, sendo a principal delas a citotoxicidade, levando ao estresse oxidativo e danos prejudiciais às células (Montoya et al., 2024).

Um estudo sobre a criopreservação com tecido ovariano bovino relatou a importância do DMSO proteger contra EROs quando utilizado em baixas concentrações, contudo, o mesmo estudo observou quando em altas concentrações ele pode ocasionar efeitos negativos promovendo o dano oxidativo (Bizarro-Silva et al., 2024). Além disso, o DMSO também pode ocasionar mudanças epigenéticas na metilação do DNA, causando alterações em oócitos, células embrionárias e espermáticas (Depincé et al., 2020; El Kamouh et al., 2023; Sciorio et al., 2024). Do mesmo modo com a sacarose, apesar de demonstrar efeitos protetores na criopreservação de esperma, em concentrações elevadas pode causar alterações sobre as células e tecidos, prejudicando a competência embrionária (Markowski et al., 2024; Tahmasebi et al., 2024). Esses efeitos adversos estão frequentemente associados ao aumento do estresse celular, incluindo a produção de EROs, onde diversos fatores contribuem para a sua produção durante a criopreservação, sendo os principais a formação de cristais de gelo, estresse osmótico e a utilização de altas concentrações de ACPs. O acúmulo de EROs leva ao estresse oxidativo, o qual afeta de forma significativa as células e tecidos a serem preservados. Portanto, o controle dessas espécies desempenha um papel importante no sucesso da criopreservação (Gongora, Holt, Gosálvez, 2024; Gualtieri et al., 2021; Rodrigues et al., 2021).

As EROs referem-se a um conjunto de moléculas derivadas do oxigênio com alto potencial reativo. Em níveis equilibrados, as EROs contribuem para processos celulares importantes como a homeostase (Sies, Jones, 2020; Sies, 2017). Os radicais livres tem origem em processos endógenos e exógenos, sendo a mitocôndria uma importante fonte para sua produção. Durante a fosforilação oxidativa na cadeia transportadora de elétrons mitocondrial, o radical superóxido (O2•¯) é formado, gerando demais radicais como peróxido de hidrogênio (H2O2) e o radical hidroxila (OH•), especialmente em condições de estresse intenso (Sachdev et al., 2021; Martemucci et al., 2022). As EROs são geradas continuamente nos sistemas fisiológicos, e mantidas em equilíbrio por diversas defesas antioxidantes, incluindo o sistema enzimático e não enzimático. O sistema de defesas enzimática incluem a ação de enzimas como superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPX) (Averill-Bates, 2023; Wang et al., 2019; Dvorak et al., 2021; Ma et al., 2019; Furtado et al., 2021). O sistema de defesa não enzimático é formado por substâncias exógenas que contribuem para neutralização dos radicais como ácido ascórbico, glutationa e os fenólicos. A glutationa em especial, demonstra efeitos positivos na criopreservação, melhorando a performance reprodutiva (Chirinos et al., 2023; Yang et al., 2025). No entanto, quando os níveis de radicais livres aumentam, ocorre um desequilíbrio entre a produção dessas espécies e a capacidade antioxidante, resultando no estresse oxidativo prejudicando a função celular, gerando peroxidação de lipídeos e proteínas, além de danos ao DNA que levam à morte celular (Van der Pol et al., 2019; Martemucci et al., 2022). Os danos causados pelas EROS ao DNA podem resultar em mutações ou alterações epigenéticas, características de doenças malignas. Os danos oxidativos afetam principalmente as bases de nucleotídeos e a espinha dorsal dos ácidos nucleicos, ocasionando a quebra das fitas desencadeando processos de apoptose e/ou necrose (Cadet et al., 2017; Caliri, Tommasi, Besaratina, 2021; Dizdaroglu, 2015; Averill-Bates, 2023). Outro efeito adverso das EROS é a peroxidação lipídica, na qual os oxidantes reagem com lipídios, adicionando oxigênio e formando radicais lipídicos peroxil, que ao sofrerem decomposição geram produtos como malonaldeído ocasionando dano oxidativo. Esse processo ocorre por meio de vias não enzimáticas e enzimáticas, induzindo a apoptose pelo estresse no retículo endoplasmático e com o aumento na permeabilidade da membrana externa mitocondrial, do citocromo c é liberado para o citoplasma ativando as caspases-3 e iniciando processo de apoptose (Campos Petean et al., 2008; Wang et al., 2023; Bock, Tait, 2020; Bou-teen et al., 2021). Todos esses processos contribuem para a degeneração celular e tecidual, demonstrando como o estresse oxidativo afeta a viabilidade e qualidade das amostras criopreservadas. Portanto, é essencial buscar compostos antioxidantes que possam atuar em conjunto com os ACPs, a fim de reduzir esses efeitos.

1.4 Importância dos antioxidantes na vitrificação de tecido ovariano

A vitrificação por ser um processo de congelamento mais rápido, transforma a água em um estado vítreo semelhante ao vidro, amenizando a formação dos cristais de gelo. Contudo, apesar dessa vantagem, a técnica não está isenta de lesões celulares, devido a necessidade de altas concentrações de crioprotetores e ao processo de recristalização durante a etapa do aquecimento (Dou, Lu, Rao, 2022; Murray, Gibson, 2022; Powell-palm et al., 2023; Guo et al., 2024). Embora os crioprotetores sejam utilizados contra as crioinjúrias, em altas concentrações contribuem com o estresse oxidativo (Bizarro-Silva et al., 2024; Whaley et al., 2021; Abdelhady et al., 2024). Dessa forma, a suplementação das soluções de vitrificação e aquecimento com antioxidantes, surge como uma opção eficaz para aumento da sobrevivência de tecidos ovariano criopreservados (Afzali et al., 2023).

A vitrificação de tecido ovariano na presença de compostos como anetol, rubina, resveratrol e romã demonstraram resultados significativos, com ações anti-inflamatórias e antioxidantes, contribuindo para preservação de folículos pré-antrais e células do estroma (Dos Santos Morais et al., 2019; Wang et al, 2021; Martins et al., 2026). Além disso, a presença de antioxidantes na vitrificação de embriões apresentou efeitos positivos reduzindo o stress oxidativo resultando no aumento do número de células e redução de células apoptóticas com benefícios para desenvolvimento fetal (Truong, Gardner, 2020). Estudos recentes apresentaram o potencial da melatonina sobre o estresse oxidativo na criopreservação de tecido e células reprodutivas, com mecanismos protetores contra danos da vitrificação em células embrionárias (Sun et al., 2020; Najafi et al., 2018; Mazoochi et al., 2018; Feng et al., 2020; Ji et al., 2023). Na vitrificação de tecido ovariano bovino, a adição de Aloe vera resultou em maior proporção de folículos ovarianos morfologicamente normais após o reaquecimento, quando comparada ao grupo vitrificado sem suplementação, com base em avaliação histológica. Além disso, contribuiu para o aumento dos níveis de RNAm para SOD, GPX1 e PRDX6, bem como para a manutenção dos níveis de colágeno, indicando o potencial promissor da adição de antioxidantes naturais aos protocolos de vitrificação (Costa et al., 2021).

Esses processos destacam a importância no uso de compostos antioxidantes na vitrificação, apresentando resultados promissores na redução do estresse oxidativo e preservação folicular. Assim, a exploração de substâncias de origem vegetal com potencial antioxidante, normalmente encontradas em óleos essenciais ou extratos de plantas, surge como uma alternativa promissora para a investigação em pesquisas primárias (Costa et al., 2022). Dentre essas substâncias, destaca-se o timol, um monoterpeno fenólico presente em diversos óleos essenciais e reconhecido por suas propriedades antioxidantes e citoprotetoras. Dessa forma, o capítulo a seguir apresenta uma revisão da literatura sobre a bioatividade do timol, abordando seus principais mecanismos de ação, alvos terapêuticos e propriedades químicas.

1.5 Bioatividade do timol: mecanismos, alvos terapêuticos e propriedades químicas

ARTIGO DE REVISÃO

Thymol's Bioactivity: A Review on Mechanisms, Therapeutic Targets, and Chemical Properties

Thymol's Bioactivity: A Review on Mechanisms, Therapeutic Targets , and Chemical Properties

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ABSTRACT

Thymol is a naturally occurring monoterpenoid phenol widely distributed in the essential oils of several aromatic plants and has attracted increasing scientific interest due to its broad spectrum of biological activities. Owing to its low molecular weight, high lipophilicity, and phenolic structure, thymol readily penetrates biological membranes and modulates multiple cellular pathways. This review provides a comprehensive overview of thymol's bioactivity, emphasizing its physicochemical properties and molecular mechanisms of action. We summarize current evidence from in vitro and in vivo studies demonstrating thymol's antioxidant, anti-inflammatory, cytoprotective, and neuroprotective effects across different cell types and tissues. Particular attention is given to the signaling pathways involved in these effects, including NF-κB, MAPK, Nrf2, PI3K/Akt, and mitochondria-associated pathways related to redox balance and cell survival. Importantly, thymol's biological effects are highly dependent on dose and cellular context, reflecting its capacity to modulate redox homeostasis rather than exert indiscriminate cytotoxicity. By integrating chemical, biological, and mechanistic insights, this review highlights thymol's potential relevance in conditions associated with oxidative stress and inflammation and underscores the importance of dose-dependent effects, bioavailability, and pharmacokinetic considerations. Further studies combining silico, in vitro, and in vivo approaches are essential to better define its safety profile and optimize its application as a bioactive natural compound.

Keywords: Monoterpenoid phenols, Oxidative stress, Redox modulation, Cytoprotection.

INTRODUCTION

Plants produce a wide variety of secondary metabolites with significant therapeutic potential for the treatment of human diseases. The medicinal use of plant-derived products dates back to ancient civilizations and remains highly relevant in modern pharmacology (Hoffmann, 2020). In recent decades, growing scientific interest in natural products has led to extensive investigations into bioactive plant compounds, including alkaloids, neolignans, flavonoids, glucosides, phenolic acids, and terpenoids (Hou et al., 2022; Masyita et al., 2022). Among these classes, monoterpenes represent a large group of volatile plant metabolites characterized by low molecular weight and high lipophilicity. These physicochemical properties favor membrane permeability and bioavailability, thereby enhancing their pharmacological potential and biological activity (Zielińska-Blajet & Feder-Kubis, 2020; De Alvarenga et al., 2023).

Thymol is a crystalline, colorless monoterpenoid phenol with a characteristic aromatic odor. Chemically known as 5-methyl-2-isopropylphenol (or 2-isopropyl-5-methylphenol), thymol is a major active constituent of the essential oils obtained from several aromatic plant species, including Origanum vulgare, Thymus broussonetii, Thymus capitatus, and Thymus vulgaris (Sivaraman et al., 2022; Tagnaout et al., 2022; Peng et al., 2024). Its phenolic structure contributes to its redox activity and underlies many of its biological effects. Due to its abundance in essential oils and favorable chemical characteristics, thymol has been widely studied as a bioactive natural compound.

Experimental evidence from in vitro and in vivo studies has demonstrated that thymol exhibits a broad range of biological activities, including antioxidant, anti-inflammatory, antibacterial, antifungal, and immunomodulatory effects (Escobar et al., 2020; Rathod et al., 2021; Liu et al., 2022; Santos Filho et al., 2023). Previous studies have shown that thymol can reduce inflammation and apoptosis, improve sperm quality in animal models, and inhibit the formation of toxic byproducts derived from heme degradation and oxidative processes (Tijani et al., 2023; Fakharian et al., 2024). In addition, thymol has attracted attention for its potential neuroprotective effects in experimental models of neurological disorders, including Alzheimer’s disease, Parkinson’s disease, memory impairment, and depression (Deng et al., 2015; Guo et al., 2022; Nourmohammadi et al., 2022; Timalsina et al., 2023; Peng et al., 2024;). Despite the growing body of evidence supporting thymol’s therapeutic potential, important gaps remain regarding its mechanisms of action. In particular, there is limited integration of data linking thymol’s chemical properties to its molecular targets, signaling pathways, and dose-dependent effects across different biological systems. Moreover, the lack of consensus on safe and effective dosage ranges, as well as insufficient information on bioavailability and pharmacokinetics, hinders the translation of preclinical findings into clinical applications. Therefore, a clearer understanding of thymol’s mechanisms of action is essential for elucidating its biological relevance and supporting the rational development of thymol- based approaches. In this context, the present review aims to summarize and critically discuss:

(1)the effects of thymol on different cell types and tissues, (2) the molecular mechanisms underlying its biological activities based on in vitro and in vivo studies, and (3) its chemical and physicochemical characteristics.

THYMOL: PHYSICOCHEMICAL CHARACTERISTICS

Terpenoids constitute the largest and most extensively studied class of plant secondary metabolites due to their diverse biological properties. Among them, thymol is a monoterpenoid phenol with an aromatic structure and an isomer of carvacrol, differing only in the position of the hydroxyl group on the phenolic ring, as shown in Figure 1 (Panigrahy et al., 2021; Costa et al., 2024).

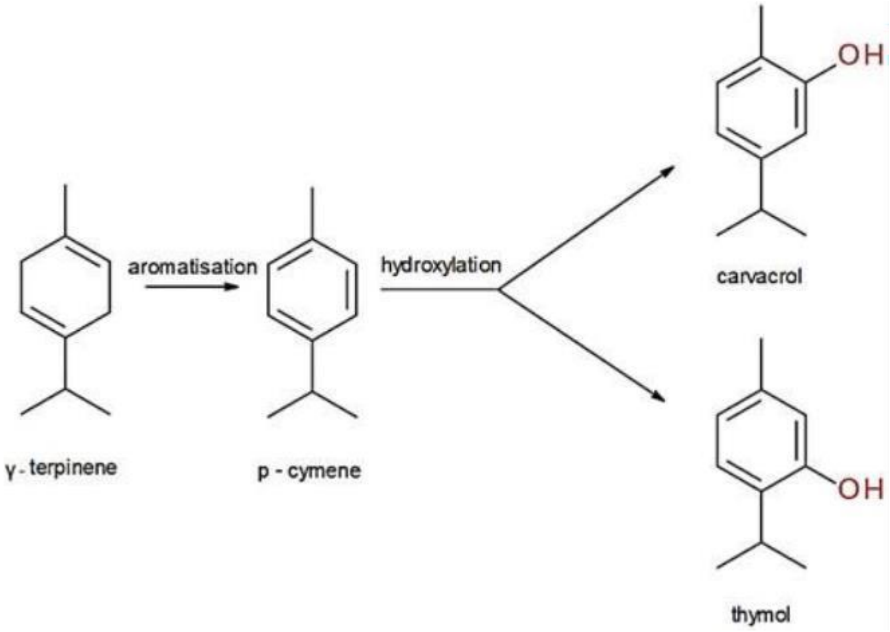

Figure 1. Biosynthetic pathway and chemical structures of thymol and its carvacrol isomer.

Thymol is derived from the hydroxylation of p-cymene. During its biosynthesis, the aromatization of γ-terpinene leads to the formation of p-cymene, which is subsequently converted into thymol and carvacrol (Kainat & Mushtaq, 2019). Thymol can be obtained from the essential oils of several aromatic plant species (De Sousa et al., 2022). Its chemical formula is $C_{10}H_{14}O$, and it is classified as a meta-cresol characterized by a trisubstituted aromatic ring with a 1,2,4 substitution pattern, containing a phenolic hydroxyl group in addition to methyl and isopropyl substituents (Reddy & Domb, 2023; Boye et al., 2020).

Thymol is a white crystalline compound with a pleasant odor and is sensitive to light and heat. It remains liquid at lower temperatures but crystallizes at room temperature (Sun et al., 2021). Thymol's low molecular weight and high lipophilicity are key physicochemical features that likely contribute to its biological activity by favoring passive diffusion across biological membranes, a behavior commonly described for monoterpenes and other lipophilic plant-derived compounds (Zielińska-Blajet & Feder-Kubis, 2020; De Alvarenga et al., 2023). These properties facilitate its interaction with lipid bilayers, enhancing cellular uptake and intracellular distribution and potentially allowing access to subcellular compartments, including mitochondrial and endoplasmic reticulum membranes. As a lipophilic phenolic monoterpene, thymol can interact with membrane phospholipids, leading to changes in membrane fluidity and redox balance, which is consistent with the antioxidant, anti- inflammatory, and pro-apoptotic effects reported in different cellular models (Boye et al., 2020; Nourmohammadi et al., 2022; Batool et al., 2022). Such effects are highly dependent on concentration and cellular context, reflecting thymol's dual redox behavior described in experimental studies (Nourmohammadi et al., 2022).

From a pharmacokinetic perspective, these physicochemical characteristics may favor rapid absorption and broad tissue distribution; however, thymol's low aqueous solubility represents an important limitation for oral bioavailability and systemic exposure (De Alvarenga et al., 2023). Thymol is sparingly soluble in water but readily soluble in organic solvents such as ethanol, methanol, and oils, which poses challenges for formulation and clinical application (Sun et al., 2021; Reddy, Domb, 2023). Consequently, formulation strategies, including nanoencapsulation, polymeric nanoparticles, lipid-based carriers, and inclusion complexes, have been explored to enhance thymol's solubility, stability, controlled release, and overall bioavailability, aiming to optimize its therapeutic potential (De Sousa et al., 2022; Reddy, Domb, 2023).

CYTOPROTECTIVE EFFECTS OF THYMOL AND ITS MECHANISMS OF ACTION

Cytoprotective effects refer to the ability of a compound to preserve cellular integrity and function by preventing or attenuating damage induced by oxidative stress, inflammation, toxic insults, and apoptotic signaling. These effects are particularly relevant in pathological conditions in which excessive production of reactive oxygen species (ROS), mitochondrial dysfunction, and dysregulation of cell death pathways contribute to tissue injury and disease progression (Tubbs & Nussenzweig, 2017). In this context, natural compounds with antioxidant and redox-modulating properties have gained increasing attention as potential cytoprotective agents.

Thymol has been widely reported to exert cytoprotective and anti-inflammatory effects across different cell types and tissues in both in vitro and in vivo models. Its protective activity is largely associated with its ability to modulate oxidative stress, maintain mitochondrial integrity, and regulate apoptosis-related pathways (Nourmohammadi et al., 2022; Batool et al., 2022). At low to moderate concentrations, thymol acts predominantly as an antioxidant by scavenging ROS and enhancing endogenous defense systems, including the activation of the Nrf2/Keap1 pathway and the upregulation of antioxidant enzymes such as heme oxygenase-1 (HO-1), superoxide dismutase (SOD), and catalase. This antioxidant response contributes to the preservation of mitochondrial membrane potential, reduction of lipid peroxidation, and maintenance of cellular homeostasis.

Importantly, thymol exhibits a dose-dependent behavior. While low concentrations promote cytoprotection and cell survival by attenuating oxidative stress, higher concentrations may induce pro-oxidant effects, leading to increased ROS generation, mitochondrial dysfunction, and activation of apoptotic pathways (Dashtaki et al., 2020; Nourmohammadi et al., 2022; Balan et al., 2021). These effects involve alterations in the balance between anti- and pro-apoptotic proteins, such as Bcl-2 and Bax, followed by caspase activation and loss of mitochondrial membrane potential, as reported in different cellular models treated with thymol (Jamali et al., 2018; Seraj et al., 2022). Such dual behavior highlights the critical role of dose and cellular context in determining thymol's biological outcome.

The following subsections summarize and discuss the cytoprotective effects of thymol in different cellular systems, with emphasis on fibroblasts, adipose-derived cells, and neural cells. Particular attention is given to the molecular mechanisms underlying these effects, including redox regulation, mitochondrial integrity, and apoptosis-related signaling pathways (Nourmohammadi et al., 2022; Batool et al., 2022).

Protective effects of thymol on fibroblasts and fat cells

Dashtaki et al. (2020) reported that thymol exerts protective effects on fibroblast cells against tert-butyl hydroperoxide (t-BHP)-induced oxidative damage. In this study, fibroblasts were pretreated with thymol at different concentrations (0, 50, 100, 200, 400, and 800 μg/mL). A significant cytoprotective effect was observed at 50 μg/mL, characterized by reduced oxidative stress and preservation of cell viability. However, thymol exhibited cytotoxic effects at higher concentrations (200–800 μg/mL), resulting in decreased fibroblast viability compared with untreated controls. These findings indicate that thymol's antioxidant and cytoprotective actions in fibroblasts are strongly dose-dependent.

Consistent with these observations, Günes-Bayir et al. (2020) demonstrated that low concentrations of thymol promoted the proliferation of human fibroblasts. This proliferative effect occurred without significant changes in intracellular ROS levels but was accompanied by a reduction in glutathione (GSH) content, suggesting a complex redox-modulating effect rather than a purely antioxidant action. In another experimental model, human foreskin fibroblasts pretreated with thymol for 24 h showed marked protection against oxidative damage induced by tert-butyl hydroperoxide (50 μM), further supporting thymol's cytoprotective potential under controlled oxidative stress conditions (Dashtaki et al., 2020).

In addition to fibroblasts, thymol has demonstrated beneficial effects in adipose-related cellular and animal models. In obese rats fed a hyperlipidic diet, thymol administration reduced serum lipid peroxidation and enhanced antioxidant defenses, leading to decreased visceral fat accumulation, hypolipidemic effects, and improved insulin and leptin sensitivity (Haque et al., 2014). At the cellular level, Brito et al. (2018) reported that supplementation of culture media with thymol-rich essential oil from Lippia origanoides (88.2% thymol) increased the proliferation of human adipose-derived stem cells, an effect potentially linked to thymol's cytoprotective properties. Moreover, Dinsmore et al. (2016) observed that specific concentrations of thymol promoted the differentiation of preadipocytes into adipocytes, indicating a possible role in adipose tissue development and function.

Despite these promising findings, it is important to note that the evidence discussed in this subsection is derived exclusively from in vitro experiments and animal models. To date, clinical studies evaluating thymol's cytoprotective effects in humans are lacking. Furthermore, the concentrations of thymol employed in in vitro studies may not be directly achievable in vivo due to limitations related to absorption, bioavailability, metabolism, and distribution. Therefore, caution is required when extrapolating these results to clinical settings, highlighting the need for pharmacokinetic studies and well-designed clinical trials to assess the translational relevance and safety of thymol-based interventions.

Neuroprotective effects of thymol

Neurodegenerative and neurodevelopmental disorders are commonly associated with oxidative stress, neuroinflammation, mitochondrial dysfunction, and dysregulation of apoptotic pathways. These mechanisms contribute to neuronal loss, synaptic impairment, and cognitive or motor deficits observed in disorders such as Parkinson's disease, Alzheimer's disease, autism spectrum disorder, and chemically induced neurotoxicity. Experimental evidence indicates that thymol exerts neuroprotective effects across different disease models by modulating redox homeostasis, inflammatory signaling, mitochondrial integrity, and cell survival pathways.

Parkinson disease models

In experimental models of parkinson disease, thymol has demonstrated significant neuroprotective effects primarily associated with attenuation of oxidative stress and preservation of dopaminergic neurons. Javed et al. (2019) reported that oral administration of thymol (50 mg/kg) to rats with chemically induced parkinson disease increased endogenous antioxidant defenses, including superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH), while reducing malondialdehyde (MDA) levels, a marker of lipid peroxidation. These biochemical changes were accompanied by recovery of dopaminergic neuron fibers, indicating protection of the nigrostriatal pathway.

Consistent with these findings, Nourmohammadi et al. (2022) demonstrated that thymol exerted protective effects in both in vivo and in vitro parkinson's disease models. In rats treated with thymol (20–30 mg/kg) and in PC12 cells exposed to thymol (50–100 µM), the compound reduced ROS generation, preserved mitochondrial function, and improved motor performance. Together, these results suggest that thymol-mediated neuroprotection in parkinson's disease involves redox regulation, maintenance of mitochondrial integrity, and prevention of neuronal degeneration.

Alzheimer's disease models

In alzheimer's disease models, thymol has been shown to modulate signaling pathways related to synaptic function and neuronal survival. Azizi et al. (2022) reported that thymol administration at a low dose (2 mg/kg) increased the expression of protein kinase C alpha (PKCα) in the hippocampus of alzheimer's disease-induced rats. PKCα plays a key role in synaptic plasticity and neuroprotection against amyloid-associated toxicity. Thymol treatment preserved hippocampal neuronal integrity and was associated with improved cognitive performance, indicating that modulation of PKC-dependent signaling pathways contributes to its neuroprotective effects in alzheimer's disease.

Autism spectrum disorder models

Autism spectrum disorder is characterized by neuroinflammation, synaptic dysfunction, and altered neuronal signaling. In an autism spectrum disorder-induced rat model, Xiong et al. (2023) demonstrated that thymol treatment (30 mg/kg, oral administration) significantly reduced the phosphorylation of Pin1 and p38 MAPK in the prefrontal cortex. These signaling molecules are closely associated with inflammatory responses and neuronal stress. Suppression of Pin1 and p38 MAPK phosphorylation was accompanied by increased expression of synaptophysin and PSD95, key proteins involved in synaptic structure and plasticity, suggesting that thymol improves synaptic integrity and neuronal communication in autism spectrum disorder models.

Chemically induced neurotoxicity models

Thymol has also shown neuroprotective effects in models of chemically induced neurotoxicity. In a monosodium glutamate induced neurotoxicity model, Mostafa et al. (2021) reported that thymol administration (800 mg/kg for 15 days) activated the nuclear erythroid 2–related factor 2 (Nrf2) signaling pathway. Activation of Nrf2 led to upregulation of antioxidant genes, enhancing endogenous cellular defense mechanisms. This response was associated with reduced oxidative stress and decreased expression of glial fibrillary acidic protein in the hippocampus, indicating attenuation of astrocyte activation and neuroinflammation.

Mechanistic integration of thymol-induced neuroprotection

Collectively, the studies discussed above indicate that thymol's neuroprotective effects involve multiple interconnected mechanisms, including activation of antioxidant pathways such as Nrf2/HO-1, modulation of kinase signaling pathways (PKCα and MAPK), reduction of reactive oxygen species (ROS) levels, preservation of mitochondrial function and membrane potential, and regulation of apoptosis-related pathways. Evidence from Parkinson's disease models demonstrates that thymol enhances endogenous antioxidant defenses and reduces oxidative damage, contributing to dopaminergic neuron preservation (Javed et al., 2019; Nourmohammadi et al., 2022). In Alzheimer's disease models, thymol-mediated neuroprotection has been linked to modulation of PKCα signaling and improved synaptic and cognitive function (Azizi et al., 2022).

Furthermore, studies in autism spectrum disorder models indicate that thymol attenuates neuroinflammatory signaling through inhibition of Pin1 and p38 MAPK phosphorylation, while enhancing expression of synaptic plasticity–related proteins such as synaptophysin and PSD95 (Xiong et al., 2023). In chemically induced neurotoxicity models, activation of the Nrf2 pathway and upregulation of antioxidant defenses have been reported as key mechanisms underlying thymol's protective effects in the hippocampus (Mostafa et al., 2021). Although the relative contribution of each pathway may vary depending on disease model, dose, and experimental conditions, convergence of these mechanisms supports thymol's role as a multitarget neuroprotective agent acting on redox balance, mitochondrial integrity, inflammatory signaling, and neuronal survival.

Anti-inflammatory effects of thymol

In vivo models of inflammation

In animal models, thymol has demonstrated pronounced anti-inflammatory and antioxidant effects through the modulation of redox balance and inflammatory mediators. Al-Khrashi et al. (2021) investigated the protective effects of thymol in a mouse model of 5-fluorouracil (5-FU)-induced intestinal mucositis. Oral administration of thymol at doses of 60 and 120 mg/kg significantly restored antioxidant defenses and reduced oxidative damage. Specifically, thymol increased glutathione (GSH) levels, normalized superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities, and reduced lipid peroxidation, as indicated by decreased thiobarbituric acid reactive substances. These findings highlight a clear dose-dependent antioxidant and anti-inflammatory effect, with the higher dose (120 mg/kg) showing superior efficacy.

Similarly, Zhang et al. (2017) demonstrated that thymol administration (20 mg/kg in vivo and 40 μg/mL in vitro) alleviated uterine inflammation induced by leptospira infection. This effect was associated with suppression of NF-κB signaling, evidenced by reduced phosphorylation of p65, as well as inhibition of MAPK pathway components, including p38, extracellular signal-regulated kinase (ERK), and c-Jun N-terminal kinase (JNK). These molecular changes were accompanied by tissue-protective effects, suggesting that thymol contributes to the restoration of inflammatory homeostasis in infected uterine tissue.

In vitro models of inflammation

In cellular models, thymol has been shown to directly modulate inflammatory mediator production and enzyme activity. Braga et al. (2006) reported that thymol inhibited elastase release in human neutrophils in a concentration-dependent manner (2.5–20 μg/mL), indicating suppression of neutrophil-mediated inflammatory responses. In the same cellular context, thymol reduced intracellular ROS production and inhibited myeloperoxidase activity, further supporting its anti-inflammatory potential (Perez-Roses et al., 2016).

Wang et al. (2018) investigated the effects of thymol on lipopolysaccharide (LPS)-induced inflammation in human peritoneal mesothelial cells (HMrSV5). Thymol treatment significantly reduced the production of pro-inflammatory cytokines, including tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1), as well as the expression of α-smooth muscle actin (α-SMA). Mechanistically, these effects were linked to inhibition of Toll-like receptor 4 (TLR4)-mediated activation of the RhoA/NF-κB signaling pathway, suggesting a direct role of thymol in suppressing transcriptional regulation of inflammatory genes.

In macrophage-based models, thymol also demonstrated significant anti-inflammatory activity. In LPS-stimulated RAW264.7 cells, thymol inhibited the production of key pro- inflammatory mediators, including NO, TNF-α, and IL-6, and reduced the expression of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). Notably, thymol's anti- inflammatory effects were enhanced when combined with silibinin (40 μM silibinin and 120 μM thymol), resulting in a more pronounced reduction of ROS levels and inflammatory enzyme expression (Chen et al., 2020). These findings indicate that thymol can act synergistically with other bioactive compounds to suppress NF-κB and MAPK signaling pathways and downstream inflammatory mediators.

Mechanistic integration and critical considerations

Collectively, the evidence indicates that thymol exerts anti-inflammatory effects through coordinated modulation of multiple molecular pathways, including inhibition of NF-κB and MAPK signaling, suppression of pro-inflammatory enzymes such as COX-2 and iNOS, reduction of cytokine and chemokine production, and enhancement of antioxidant defenses potentially mediated by Nrf2 signaling. These mechanisms converge to limit oxidative stress, inflammatory cell activation, and tissue damage.

However, it is important to note that most of the evidence supporting thymol's anti-inflammatory activity derives from in vitro studies and animal models. Differences in experimental conditions, doses, and routes of administration complicate direct comparisons between studies. Moreover, the concentrations of thymol used in cellular assays may exceed those achievable in vivo, highlighting the need for pharmacokinetic studies and clinical investigations to establish safe and effective dosing regimens. Despite these limitations, the available data support thymol as a multitarget anti-inflammatory agent, and a schematic diagram summarizing its molecular targets and signaling pathways would aid in visualizing the integration of these mechanisms.

CONCLUSION

In summary, thymol exhibits a broad spectrum of biological activities, including antioxidant, neuroprotective and anti-inflammatory effects. These actions are closely associated with its chemical structure, which favors membrane permeability and redox modulation, enabling interaction with multiple intracellular targets. Accumulating evidence indicates that thymol modulates key molecular pathways involved in oxidative stress, inflammation, apoptosis, and cell survival, including NF-κB, MAPK, Nrf2, and PI3K/Akt signaling cascades. Importantly, thymol's biological effects are strongly dose- and context- dependent, reflecting its capacity to modulate redox homeostasis rather than inducing nonspecific cytotoxicity.

Despite these promising findings, the current body of evidence is largely derived from in vitro experiments and preclinical animal models, with a notable lack of clinical studies evaluating thymol's safety, efficacy, and pharmacokinetic profile in humans. Variability in experimental models, doses, routes of administration, and outcome measures limits direct comparison between studies and hinders the establishment of standardized therapeutic parameters.

Future research should focus on addressing these limitations through well-designed pharmacokinetic and toxicological studies, followed by controlled in vivo investigations and clinical trials. The development of optimized delivery systems, such as nanoencapsulation, lipid-based carriers, and other advanced formulations, may enhance thymol's bioavailability, stability, and tissue targeting. Additionally, integrative approaches combining in silico modeling with experimental validation may facilitate dose optimization, identification of novel molecular target, and prediction of therapeutic windows. Collectively, these efforts will be essential to support the further development of thymol as a promising bioactive natural compound for the management of inflammatory and neurodegenerative conditions.

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3 JUSTIFICATIVA

A preservação da fertilidade em pacientes oncológicos encontra na criopreservação uma alternativa eficaz, com taxas satisfatórias de nascidos vivos relatadas na literatura (Wang et al., 2024). Além disso, a criopreservação contribui para a conservação do material genético de espécies animais ameaçadas de extinção e constitui uma ferramenta estratégica para o melhoramento genético de bovinos de corte, atendendo à crescente demanda populacional e ao consumo global de carne bovina (EU Commission, 2019; Beckman et al., 2022; Lujic et al., 2023; Baruselli et al., 2025). Apesar dos avanços obtidos, essa biotécnica ainda apresenta limitações associadas, principalmente, ao estresse oxidativo, o qual compromete de forma significativa a qualidade das amostras criopreservadas (Gualtieri et al., 2021; Cao et al., 2022; Luo et al., 2023). Diante disso, o aprimoramento de biotécnicas como a criopreservação de tecido ovariano torna-se fundamental para promover novos avanços no campo da reprodução animal e humana, contribuindo para a geração de conhecimento científico e para a superação de lacunas ainda existentes na literatura. Nesse contexto, pesquisas voltadas ao aperfeiçoamento da vitrificação de tecido ovariano são essenciais, uma vez que podem beneficiar tanto pacientes oncológicas quanto a produção animal, ao viabilizar a conservação de recursos genéticos não apenas de bovinos, mas também de espécies em risco de extinção. Entre as estratégias investigadas para minimizar os efeitos deletérios do estresse oxidativo, destaca-se o uso de substâncias de origem vegetal com propriedades antioxidantes (Costa et al., 2022). Dentre essas substâncias, o timol surge como uma alternativa segura e natural, amplamente estudada por sua capacidade de neutralizar radicais livres e reduzir o estresse oxidativo celular (Tijani et al., 2023; Mahran et al., 2024). Evidências recentes demonstram que o timol apresenta elevada capacidade antioxidante, incluindo maior eficiência na remoção de íons de ferro quando comparado a antioxidantes clássicos, como a vitamina C (Sun et al., 2025). Além disso, essa atividade antioxidante tem se refletido em efeitos biológicos positivos em sistemas reprodutivos, uma vez que, no cultivo de tecido ovariano bovino, o timol promoveu a ativação de folículos primordiais e preservou a sobrevivência folicular, a densidade do estroma ovariano e as fibras de colágeno, mantendo o equilíbrio redox tecidual (Caetano Filho et al., 2024). Dessa forma, a suplementação da solução de vitrificação com timol configura-se como uma estratégia promissora para reduzir os danos oxidativos, contribuindo para o aprimoramento dos protocolos de vitrificação de folículos pré-antrais inclusos em tecido ovariano bovino e preenchendo uma lacuna relevante na literatura. Destaca-se ainda o caráter inédito deste estudo, uma vez que, até o momento, não há relatos na literatura que avaliem de forma integrada os efeitos dose–resposta do timol e sua adição como antioxidante em soluções de vitrificação de tecido ovariano bovino.

4 HIPÓTESES CIENTÍFICAS

- O timol em diferentes concentrações (2.5, 25 e 250 µg/mL) não apresenta efeitos tóxicos durante o cultivo in vitro de células do cumulus bovinas.

- A adição do timol em diferentes concentrações (2,5; 25 e 250 µg/mL) à solução de vitrificação de tecido ovariano bovino preserva a morfologia folicular e promove a ativação folicular após o reaquecimento, além de preservar a matriz extracelular e as células do estroma.

A adição de timol à solução de vitrificação reduz o estresse oxidativo e mantém a atividade das enzimas antioxidantes SOD, CAT e GPX, bem como o conteúdo de tióis no tecido ovariano bovino após o reaquecimento, contribuindo para a manutenção do equilíbrio redox e da integridade folicular.

5 OBJETIVOS

5.1 Objetivo geral

Avaliar os efeitos da suplementação da solução de vitrificação com Timol sobre a manutenção da integridade de folículos, células do estroma e matriz extracelular de córtex ovariano bovino.

5.2 Objetivos específicos

Avaliar o efeito de diferentes concentrações de timol (2,5; 25 e 250 µg/mL) durante a vitrificação de tecido ovariano bovino sobre a ativação e a morfologia folicular, bem como sobre a integridade da matriz extracelular e densidade do estroma.

Analisar a atividade das enzimas SOD, CAT e GPX após o reaquecimento de tecido ovariano bovino vitrificado na presença de timol.

Avaliar os efeitos dose-dependentes do timol sobre a viabilidade celular de células do cúmulus bovinas cultivadas in vitro.

6 CAPITULO 2

Thymol improves redox balance in bovine ovarian cortex and contributes for maintenance of follicular and extracellular matrix integrity during vitrification

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ABSTRACT

The present study aimed to evaluate the dose-response effects and antioxidant potential of thymol during the vitrification of bovine ovarian tissue. Initially, a dose-response assay was conducted on bovine cumulus cells cultured in vitro and exposed to different concentrations of thymol (2.5, 25, and 250 μg/mL), in order to evaluate cell viability and cytotoxicity using calcein-AM and ethidium homodimer-1 assays. Subsequently, fragments of bovine ovarian cortex were exposed to vitrification solutions supplemented with thymol at the same concentrations. After one week of storage in liquid nitrogen, the tissues were warmed. Part of the samples was analyzed immediately, while the remainder were incubated in vitro at 38.5oC for 24h. Tissue integrity was assessed by follicular morphology, ovarian stromal cell density, and extracellular matrix components, including collagen and glycosaminoglycans. The redox status was analyzed by antioxidant enzyme activity (SOD, CAT, and GPX) and thiol and nitrite levels. The results of the cumulus cell assay demonstrated that 25 and 250 μg/mL of thymol increased calcein labeling compared to the control medium, which is indicative of cell viability. Vitrification of ovarian tissue promoted changes in the redox status, evidenced by thiol depletion and reduced GPX activity. Vitrification solution using 25 μg/mL of thymol increased SOD activity, which may represent a response to oxidative stress. On the other hand, in the vitrified ovarian tissue, the presence of 250 μg/mL thymol preserved thiol levels and GPX activity similar to those in non-vitrified tissue. Nitrite levels were reduced after vitrification in all groups, likely reflecting metabolic suppression. In addition, thymol preserved the proportion of morphologically normal follicles, preserved ovarian stromal density, and maintained extracellular matrix components after warming. It is concluded that the presence of 250 μg/mL thymol in the vitrification solution of bovine ovarian cortex contributes to maintaining redox balance after warming, which helps to preserve the follicular morphology in vitrified tissue.

Keywords: Cryoprotectant, Oxidative Stress, Monoterpene.

INTRODUCTION

In recent years, ovarian tissue cryopreservation has emerged as a promising strategy for preserving fertility and endocrine function in patients undergoing gonadotoxic treatments, such as chemotherapy and radiotherapy (Rodrigues et al., 2021). This technique involves the surgical removal, processing, and freezing of ovarian tissue fragments containing follicles, which may later restore reproductive and hormonal function through orthotopic or heterotopic transplantation (Khattak et al., 2022; Dolmans., 2018). Beyond fertility preservation, ovarian tissue cryopreservation has also gained attention as a potential approach to delay menopause and reduce associated long-term health risks, which represent a growing concern for women worldwide (Johnson et al., 2024).

The ovarian tissue cryopreservation is currently the only available fertility preservation option for prepubescent girls requiring urgent oncological treatment and for women who are unable or unwilling to undergo ovarian stimulation (Shapira et al., 2020; Dolmans et al., 2021; Chung et al., 2023). Cryopreservation can be performed mainly by two techniques: slow freezing and vitrification. Slow freezing consists of a controlled and gradual decrease in temperature, allowing progressive cellular dehydration and reducing the formation of intracellular ice crystals (Rienzi et al., 2017). In contrast, vitrification involves ultra-rapid cooling in the presence of high concentrations of cryoprotectants, which prevents ice crystal formation by solidifying the solution into a glass-like state (Jaiswal et al., 2022). Although vitrification reduces ice crystal formation, high cryoprotectant concentrations still induce cryoinjuries that compromise tissue viability by disrupting redox homeostasis. (Gook, 2017; Nafaji, Asadi, Benson, 2023; Abdelhady et al., 2024). Oxidative stress affects not only follicular cells but also the ovarian stromal compartment, compromising antioxidant defenses, mitochondrial function, enzymatic activity, and ultimately follicular survival after warming (Alfradique et al., 2026; Cao et al., 2022; Gualtieri et al., 2021).

To mitigate these deleterious effects, plant-derived compounds with antioxidant potential have been investigated, including thymol as a promising candidate for ovarian tissue protection. Thymol, a crystalline monoterpenoid phenol, with distinct odor with chemical names (5-methyl-2-isopropyl phenol and 2-isopropyl-5-methyl phenol), it can be found in some thyme species including Origanum vulgare, Thymus broussoneti, Thymus capitatus and Thymus vulgaris (Peng et al., 2024; Sivaram et al., 2022; Tagnaout et al., 2022). Some in vitro and in vivo studies have demonstrated Its properties such as antioxidant, anti- inflammatory, antibacterial, antifungal, antiviral and immune-regulation functions of thymol (Escobar et al., 2020; Rathod et al., 2021; Tagnaout et al., 2022; Filho et al., 2023).

Given that vitrification induces profound disturbances in ovarian redox homeostasis, thymol may represent a promising supplement for cryoprotective solutions. However, despite its well-documented biological properties, the effects of thymol during vitrification of ovarian tissue remain unexplored. Therefore, the present study aimed to investigate the dose– response effects of thymol addition in the vitrification solution of bovine ovarian tissue, focusing on follicular morphology, stromal cells preservation, and extracellular matrix organization.

MATERIALS AND METHODS

Chemicals

Unless otherwise specified, all chemicals and culture media were obtained from Sigma- Aldrich (USA).

Ovaries source and ethical approval

All bovine ovaries were obtained from adult females at a local slaughterhouse. A total of 24 ovaries were used in Experiment 1, whereas 10 ovaries were used in Experiment 2. After collection, each pair of ovary was directly washed in ethanol (70%) for 10 seconds, in and twice in 0,9% saline solution at 4°C, combined with penicillin (100 μg/ml) and streptomycin (100 μg/ml). Then, all ovaries were individually transported to the laboratory in conical tubes including α-MEM supplemented with penicillin (100 μg/ml) and streptomycin (100 μg/ml) for 1 h. This study was approved and performed in accordance with the rule and guidelines of the Ethics and Animal Welfare Committee of Federal University of Ceará (N◦P06/25).

Experiment 1: Dose-dependent effects of thymol on bovine cumulus cell viability

Cumulus–oocyte complexes (COCs) were obtained by aspiration of antral follicles (3– 6 mm in diameter) using an 18-gauge needle attached to a syringe. The recovered COCs were selected in HEPES-buffered TCM-199 medium supplemented with 10% fetal bovine serum (FBS), 0.2 mM pyruvate, and 100 µg/mL penicillin. Only COCs presenting homogeneous and compact ooplasm, multilayered cumulus cell layers (Grades I and II) were used (Azevedo et al., 2026).

Cumulus cells (CCs) were dissociated from the oocytes by enzymatic digestion with trypsin–EDTA (Sigma-Aldrich) followed by gentle mechanical dissociation. The cell suspension was centrifuged at 500 × g for 5 min at room temperature. The supernatant was discarded, and the cell pellet was resuspended in α-minimum essential medium (α-MEM) supplemented with 10% FBS and penicillin/streptomycin. Cells were cultured in 25 cm^2 flasks at 38.5 °C in a humidified atmosphere containing 5% CO_2, with medium replacement every 48h. When cultures reached approximately 70–80% confluence, cells were detached using trypsin for 3 min, and the first passage was obtained after about 10 days.

For the dose–response experiment, CCs were seeded in 96-well plates at a density of 5,000 cells per well and exposed to different concentrations of thymol (2.5, 25.0, and 250.0 µg/mL). Cells cultured in thymol-free medium served as the control group. After 24 h of incubation at 38.5 °C under 5% CO_2, cell viability was assessed using a LIVE/DEAD assay with 4 µM calcein-AM and 2 µM ethidium homodimer-1 (Molecular Probes, L3224, Invitrogen). Following a 20 min incubation at 37 °C in a 5% CO_2 atmosphere, viable cells were identified by green fluorescence, whereas non-viable cells were identified by red fluorescence. Fluorescence intensity was quantified using ImageJ software (NIH). All assays were performed in triplicate.

Experiment 2: Vitrification of ovarian tissue

Ovarian Tissue Preparation

In the laboratory, the ovarian cortex was dissected from each ovary and cut into small fragments (3 × 3 × 1 mm) using a scalpel in while immersed in α-MEM dissection medium supplemented with penicillin (100 µg/ml) and streptomycin (100 µg/ml). For the fresh control (unvitrified tissue), four fragments of each ovarian pair were randomly selected. Two fragments were immediately fixed in paraformaldehyde (4%) for histological evaluation of follicular morphology and development, while the remaining two fragments were stored at -80 C for subsequent biochemical analyses. All remaining cortical fragments were allocated to the experimental groups and cryopreserved using the solid surface vitrification method, as previously described by Costa et al. (2021).

Vitrification and warming of ovarian tissue

Vitrification was performed according to Costa et al. (2021), with minor modifications. Ovarian cortical fragments were exposed for 5 min to 2 mL of vitrification solution consisting of α-MEM supplemented with 10% fetal bovine serum (FBS), 0.25 M sucrose, 10% DMSO, and thymol (Sigma, CAS-89-83-8) at concentrations of 0, 2.5, 25, or 250 µg/mL. After equilibration, fragments were vitrified using the solid surface method by direct contact with a metal plate precooled in liquid nitrogen and subsequently transferred to 2 mL cryotubes for storage in liquid nitrogen (-196 °C).

After one month of storage, warming was performed by exposing the cryotubes at room temperature for 1 min, followed by immersion in a 37 °C water bath. Cryoprotectant dilution was achieved by sequential exposure to α-MEM supplemented with 10% FBS and decreasing sucrose concentrations (0.5, 0.25, and 0 M) for 5 min each step. After warming, two fragments per treatment were cultured for 24 h in 24-well plates containing α-MEM supplemented with glutamine (2 mM), hypoxanthine (2 mM), BSA (1.25 mg/ml), penicillin/streptomycin (100 µg/ml), selenium (10 µg/ml), transferrin (5.5 µg/ml) and insulin (10 µg/ml) at 38.5 °C, while the remaining fragments were processed for histological, biochemical and extracellular matrix analyses.

Morphological analyses and assessment of in vitro follicular activation

Histological analyses were performed according to Bizarro-Silva et al. (2018), with adaptations. Fresh control (unvitrified), vitrified, and cultured ovarian tissues were fixed in 4% paraformaldehyde, dehydrated in ascending ethanol concentrations, cleared in xylene, embedded in paraffin, and sectioned at 6 µm. Sections were stained with hematoxylin and eosin and examined under a light microscope (Nikon Eclipse TS100, Japan). Only preantral follicles containing a visible oocyte nucleus were analyzed to prevent repeated counting. Follicles were considered morphologically normal when they displayed a circular oocyte surrounded by well-organized granulosa cell layers, without cytoplasmic retraction or nuclear pyknosis. Degenerated follicles were characterized by nuclear pyknosis, disorganization of granulosa cells, and oocyte retraction (Silva et al., 2004).

Follicular developmental stages were classified according to Hulshof et al. (1994) as primordial (oocyte surrounded by a single layer of flattened granulosa cells), primary (oocyte surrounded by a single layer of cuboidal granulosa cells), or secondary (oocyte surrounded by two or more complete layers of cuboidal granulosa cells). The proportion of follicles at each developmental stage was expressed as the percentage of morphologically normal follicles relative to the total follicle count.

Analysis of extracellular matrix (ECM) and Glycosaminoglycans (GAGs)

To evaluate extracellular matrix components, collagen fibers and glycosaminoglycans (GAGs) were histochemically assessed in ovarian cortical tissue sections. Paraffin-embedded sections (6.0 µm) were deparaffinized, rehydrated, and processed according to standard histological procedures. Collagen fiber organization and content were assessed using Picrosirius Red staining (Abcam kit), following the methodology described by Rittié (2017), with minor modifications. Sections were incubated in 0.1% Sirius Red solution for 1 h at room temperature, washed with 0.5% acetic acid, dehydrated, and cleared. Collagen fibers appeared red under light microscopy, while follicles remained unstained. GAGs were identified using Alcian Blue staining (pH 2.5), according to Quintarelli and Dellovo (1965). After rehydration, sections were stained with Alcian Blue solution for 5 min, followed by dehydration and clearing. Microscopic evaluation was performed using a light microscope (Nikon Eclipse TS100, Japan) at 400× magnification. Images were captured with a DS-Ri1 cooled digital camera (Nikon, Japan) and analyzed using ImageJ software (version 1.51p). For both analyses, the percentage of tissue area positive for collagen fibers or GAGs was quantified in ten randomly selected fields per treatment, applying identical image analysis criteria.

Biochemical assays to evaluate redox status and total protein content

Biochemical analyses to evaluate redox status and total protein content were performed according to previously established methods, with minor adaptations (Ellman, 1959; Bradford, 1976). Vitrified ovarian tissue fragments and fresh control samples (100 mg) were homogenized in potassium phosphate buffer (pH 7.5) containing protease inhibitors, followed by centrifugation at 1,500 × g for 10 min at 4 °C. The resulting supernatants were collected and used for spectrophotometric analyses (Genesis 10s UV– Vis; Thermo Scientific) using quartz cuvettes. Total protein concentration was determined by the Bradford method, with absorbance measured at 595 nm and protein content calculated from a bovine serum albumin standard curve. Protein values were used to normalize pro-oxidant and antioxidant parameters, including reduced thiol content and the activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX).

Assessment of redox status through thiol and nitrite levels

Thiol and nitrite levels were evaluated according to established colorimetric methods (Ellman, 1959; Green et al., 1982). Reduced thiol content was determined using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), based on the formation of 2-nitro-5-thiobenzoate (TNB^{2-}), with absorbance measured at 412 nm. Nitrite levels were quantified using the Griess reaction, in which samples were incubated with sulfanilamide and N-(1-naphthyl)ethylenediamine reagents, and absorbance was recorded at 540 nm. Results were expressed relative to protein content or as micromolar concentrations, as appropriate.

Determination of CAT, SOD, and GPX activity

Antioxidant enzyme activities were determined using previously described spectrophotometric assays, following the methodology reported by Costa et al. (2025). Catalase (CAT) activity was assessed by monitoring the decomposition of hydrogen peroxide (H_2O_2) at 240 nm. Superoxide dismutase (SOD) activity was evaluated based on its ability to inhibit adrenaline auto-oxidation in an alkaline medium, with absorbance measured at 480 nm every 10 s for 180 s. Glutathione peroxidase (GPX) activity was determined by measuring the rate of nicotinamide adenine dinucleotide phosphate (NADPH) oxidation at 340 nm in a coupled reaction system containing glutathione reductase and reduced glutathione. Enzyme activities were calculated from changes in absorbance over time and expressed relative to total protein content

Statistical analysis

Statistical analyses were performed using GraphPad Prism software (version 5.0). Data were initially tested for normality and were assessed using the Shapiro–Wilk test. When necessary, data were subjected to logarithmic transformation to achieve normal distribution. Parametric data were analyzed using one-way analysis of variance (ANOVA), followed by Tukey’s post hoc test. Data that did not meet normality assumptions were analyzed using the Kruskal–Wallis test, followed by Dunn’s multiple comparisons test. Categorical data, including follicular morphology and activation, were analyzed using the chi-square (χ^2) test. Differences were considered statistically significant when $P < 0.05$.

RESULTS

Evaluation of thymol dose-dependent in bovine cumulus cells (BCCs)

The dose-response experiment shows that calcein-AM fluorescence intensity in cumulus cells (CCs) cultured with 25 and 250 µg/ml of thymol were significantly increased compared to the control group ($P>0.05$) (figure 1A). Furthermore, ethidium fluorescence intensity was significantly reduced in CCs treated in medium supplemented with 2.5 and 25 µg/ml of thymol, when compared to cultured control ($P<0.05$) (figure 1B). Figure 1C indicates fluorescence images of bovine cumulus cells.

Effects of vitrification on SOD, CAT and GPX enzymes activity

The SOD activity was significantly higher in ovarian tissue vitrified in the presence of thymol at 25 µg/mL compared with unvitrified tissue and vitrified control ($P < 0.05$; Fig. 2B). Tissues supplemented with thymol at 2.5 and 250 µg/mL exhibited similar SOD activity, with no significant differences from the vitrified control. GPX activity was significantly lower in the vitrified control group and in tissues supplemented with thymol at 2.5 and 25 µg/mL compared with unvitrified tissue and 250 µg/mL group ($P < 0.05$; Fig. 2C). In contrast, ovarian tissue vitrified in the presence of thymol at 250 µg/mL showed GPX activity similar to that observed in unvitrified tissue ($P > 0.05$). CAT activity did not differ significantly among experimental groups ($P > 0.05$; Fig. 2A).

Evaluation of pro-oxidant status (thiol) and nitrite levels

Nitrite levels were significantly lower in all vitrified groups compared with unvitrified tissue ($P < 0.05$; Fig. 3A), regardless of thymol supplementation. Thiol content was significantly lower in the vitrified control group and in tissues supplemented with thymol at µg/mL compared with unvitrified tissue and 250 µg/mL thymol group ($P < 0.05$; Fig. 3B). Tissue vitrified in the presence of thymol at 25 µg/mL showed thiol levels with no significant differences from the vitrified control. Thiol content in tissues supplemented with thymol at 250 µg/mL was similar to that observed in unvitrified tissue ($P > 0.05$).

Effects of thymol on follicle morphology 24 hours after vitrification

The percentage of morphologically normal follicles was significantly lower in vitrified ovarian tissue compared with unvitrified tissue ($P < 0.05$; Fig. 4). Ovarian tissue vitrified in the presence of thymol at 2.5, 25, and 250 µg/mL showed significantly higher proportions of morphologically normal follicles compared with vitrified tissue without thymol supplementation ($P < 0.05$). Figure 5 shows representative images of morphologically normal and degenerate follicles from different categories.

Effects of thymol on follicle activation after 24 hours of vitrification

Figure 6 shows the percentage of primordial and developing follicles after 24 hours incubation post vitrification. All vitrified groups showed a significant increase of developing follicles when compared to unvitrified groups, with no differences observed among vitrified groups ($P > 0.05$).

Influence of thymol on ovarian stromal cells after vitrification and 24-hour incubation

Ovarian tissues vitrified in all experimental groups showed a significant decrease in stromal cell density compared to unvitrified tissue ($P < 0.05$). However, tissues vitrified in the presence of 250 µg/ml thymol exhibited a significantly higher stromal cell density compared with vitrified control and those treated with 2.5 µg/ml thymol ($P < 0.05$) (Fig. 7).

Influence of thymol on ECM preservation after ovarian tissue vitrification

Collagen content

No significant differences in collagen content were observed among ovarian tissues vitrified in solution alone or supplemented with thymol at 2.5, 25, or 250 µg/ml when compared to unvitrified tissue ($P > 0.05$) (Fig. 8).

Glycosaminoglycan (GAG) content

Glycosaminoglycan (GAG) levels were significantly higher in ovarian tissue vitrified with thymol at 2.5, 25, and 250 µg/mL compared with unvitrified tissue and vitrified control group ($p < 0.05$; Fig. 9), with no differences among thymol concentrations.

DISCUSSION

The present study provides, for the first time, evidence that supplementing the vitrification solution with thymol modulates the response of bovine ovarian tissue to vitrification by influencing redox balance, extracellular matrix integrity, and follicular dynamics. Although vitrification disrupted antioxidant defenses and altered structural and functional parameters of the ovarian microenvironment, thymol partially mitigated these effects. These findings highlight the relevance of antioxidant modulation as a strategy to improve ovarian tissue cryopreservation.

The improved viability of bovine cumulus cells observed after thymol supplementation suggests that this compound contributes to the preservation of cellular integrity under in vitro conditions, particularly at specific concentrations. This effect is consistent with the modulation of redox-related parameters observed in vitrified ovarian tissue, indicating that thymol may influence different compartments of the ovarian microenvironment. Considering the critical role of cumulus cells in supporting oocyte metabolism and signaling (Richani et al., 2024), the enhanced viability of these cells may represent an important mechanism through which thymol mitigates cryopreservation-induced cellular stress. Similar protective effects of thymol have been reported in different cellular models and are commonly associated with its antioxidant and membrane-stabilizing properties (Nourmohammadi et al., 2022; Amara et al., 2022; Kim et al., 2014) reinforcing the hypothesis that redox modulation plays an important role in the response of ovarian tissue to vitrification.

The reduction in nitrite levels observed after vitrification indicates that cryopreservation disrupts nitric–related metabolism in bovine ovarian tissue. Vitrification and subsequent warming are known to induce profound metabolic disturbances, particularly affecting mitochondrial function and cellular redox balance, which may influence nitric oxide production (Rodrigues et al., 2021). Because nitric oxide synthesis depends on metabolic integrity and the availability of cofactors such as NADPH and tetrahydrobiopterin (Andrew and

Mayer, 1999; Stuehr et al., 2004), alterations in these pathways during cryopreservation may contribute to the decreased nitrite levels detected in this study. Notably, thymol supplementation did not restore nitrite levels, suggesting that its modulatory effects on redox homeostasis may be selective rather than global. Together, these findings support the notion that vitrification impacts multiple metabolic pathways, of which nitric oxide signaling represents a particularly sensitive component.

The present findings indicate that vitrification disrupts the redox balance of bovine ovarian tissue, compromising endogenous antioxidant defenses. Thymol supplementation partially modulated this redox disturbance. The reduction in thiol levels after vitrification suggests an increased oxidative stress, whereas supplementation with 250 µg/mL thymol was associated with thiol levels comparable to those observed in unvitrified tissue. Moreover, thymol exerted differential effects on antioxidant enzymes, with 25 µg/mL being associated with increased SOD activity and 250 µg/mL preserving GPX activity. The elevation of SOD activity at 25 µg/mL may reflect an adaptive response to superoxide generation during the vitrification and warming process, indicating the presence of an oxidative stress. These results highlight that thymol does not uniformly modulate the antioxidant system but rather influences distinct components of the redox network depending on its concentration. Considering the coordinated action of SOD and GPX in the detoxification of reactive oxygen species (Birben et al., 2012; Flohé et al., 2013), the selective modulation of these enzymes by thymol may reflect an adaptive antioxidant response that supports redox homeostasis during vitrification. Similar antioxidant properties of thymol have been reported in different experimental models, reinforcing the notion that this compound can modulate redox pathways under conditions of oxidative stress (Kruk et al., 2000; Saber et al., 2021; Filho et al., 2023; Badr et al., 2025; Khedr et al., 2025). Together, these findings suggest that thymol supplementation contributes to the regulation of oxidative stress in vitrified bovine ovarian tissue, although its effects appear to be parameter- and dose-specific.

Vitrification significantly reduced the proportion of morphologically normal follicles in bovine ovarian tissue, confirming that cryopreservation negatively affects follicular integrity (Morais et al., 2019; Silva et al., 2024; Martins et al., 2026). Thymol supplementation partially attenuated this effect, resulting in a higher proportion of morphologically normal follicles compared with vitrified tissue without antioxidant supplementation. These findings are consistent with the modulation of redox-related parameters observed in this study, suggesting that the preservation of follicular morphology may be associated with reduced oxidative stress during vitrification. Interestingly, vitrification was also associated with an increased proportion of developing follicles compared with unvitrified tissue, indicating that cryopreservation may trigger premature follicular activation (Morais et al., 2019).

In the present study, vitrification followed by short-term incubation resulted in a reduction of stromal cell density, indicating that stromal cells are particularly vulnerable to cryopreservation-associated stress (Morais et al., 2019). Given the role of stromal cells in providing structural support, paracrine signaling, and biomechanical cues to developing follicles (Grosbois et al., 2023; Grubliauskaite et al., 2024), alterations in stromal density likely reflect a broader disruption of the ovarian microenvironment rather than isolated cellular damage. Supplementation of the vitrification solution with thymol at 250 µg/mL partially mitigated this effect, as evidenced by higher stromal cell density after warming. This observation is consistent with other findings of the present study, including improved calcein-AM staining and reduced oxidative stress markers, suggesting that antioxidant-related mechanisms may contribute to stromal cell preservation. In parallel, thymol supplementation preserved collagen levels after warming and increased glycosaminoglycan (GAG) content after 24 h of incubation. While collagen maintenance indicates preservation of the extracellular matrix of the tissue, elevated GAG levels may reflect improved matrix hydration and signaling capacity (Oliveira et al., 2015; Chiti et al., 2022). Together with the preservation of stromal cells, these coordinated effects suggest that thymol contributes to maintaining the functional organization of the ovarian extracellular matrix.

CONCLUSION

Vitrification disrupted redox homeostasis and structural integrity in bovine ovarian tissue, leading to oxidative stress and alterations in follicular morphology, stromal organization, and antioxidant defenses after warming. Thymol supplementation modulated these effects in specific concentrations, which 250 µg/mL thymol contributed to the preservation of thiol levels, GPX activity, stromal cell density, and extracellular matrix components. Therefore, 250 µg/mL thymol emerges as the most promising concentration for future studies aiming to improve ovarian tissue vitrification protocols, due to its broader protective effects on the ovarian cortex microenvironment.

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Author contribution statement

Andreza de A. Silva contributed to experimental procedures involving ovarian tissue handling, fragmentation, vitrification and critically revised the manuscript. **Francisco das C. Costa** contributed to experimental procedures involving ovarian tissue handling, fragmentation, vitrification and critically revised the manuscript. **Bianca R. Silva** assisted with experimental execution related to ovarian tissue processing. **Erica C. Marcelino** contributed to experimental procedures and sample handling. **Vitória S. Bezerra** assisted with experimental activities related to ovarian tissue processing and vitrification. **Francisco F. Caetano Filho** contributed to experimental support during ovarian tissue procedures. **Leopoldo R.C.V. da Silva** and **Sueline C. Chaves** assisted with experimental execution and laboratory support. **Venância A. N. Azevedo** contributed to dose–response experiments using bovine cumulus cells treated with thymol and assisted in data analysis and interpretation. **Solano D. Martins and Valdevane R.**

Araújo performed the biochemical analyses, including SOD, CAT, GPX, thiol, and nitrite assays. **José Roberto V. Silva** conceived and supervised the study, contributed to data interpretation, and critically revised the manuscript. All authors reviewed and approved the final version of the manuscript.

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Declaration of Competing Interest

The authors report no declarations of interest

ILLUSTRATIVE IMAGES

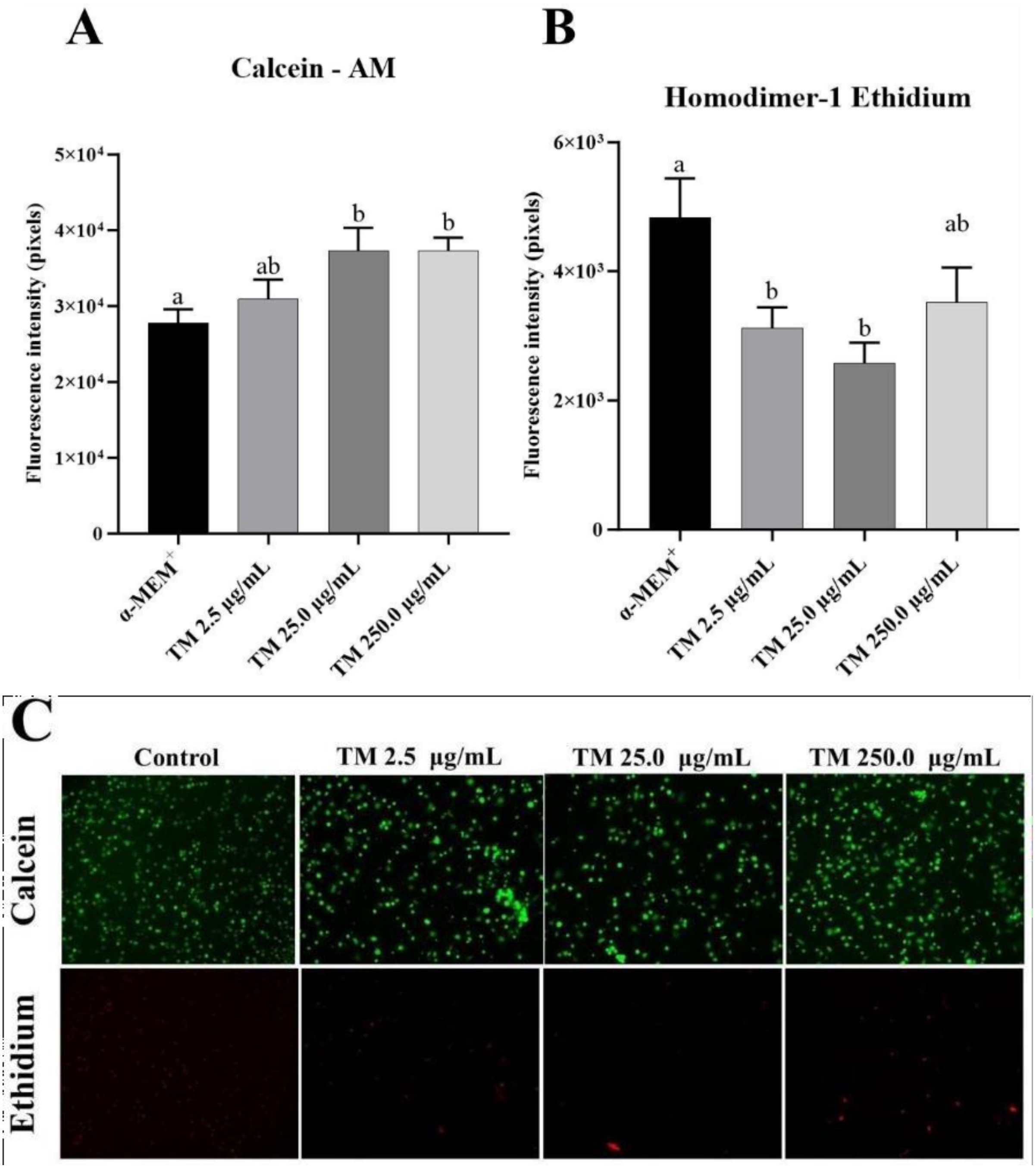

Figure 1. Fluorescence intensity of the calcein-AM (A) and ethidium homodimer (B) in bovine cumulus cells of in vitro culture in α-MEM alone or supplemented with 2.5, 25 and 250 µg/ml thymol (TM). Different letters indicate a statistically significant difference between treatment groups: $P < 0.05$. Letter C indicates fluorescence images of bovine cumulus cells cultured in vitro in the control group and treated with thymol (TM; 2.5, 25.0, and 250.0 µg/mL). Viable cells are stained with calcein-AM (green), and non-viable cells with ethidium homodimer (red).

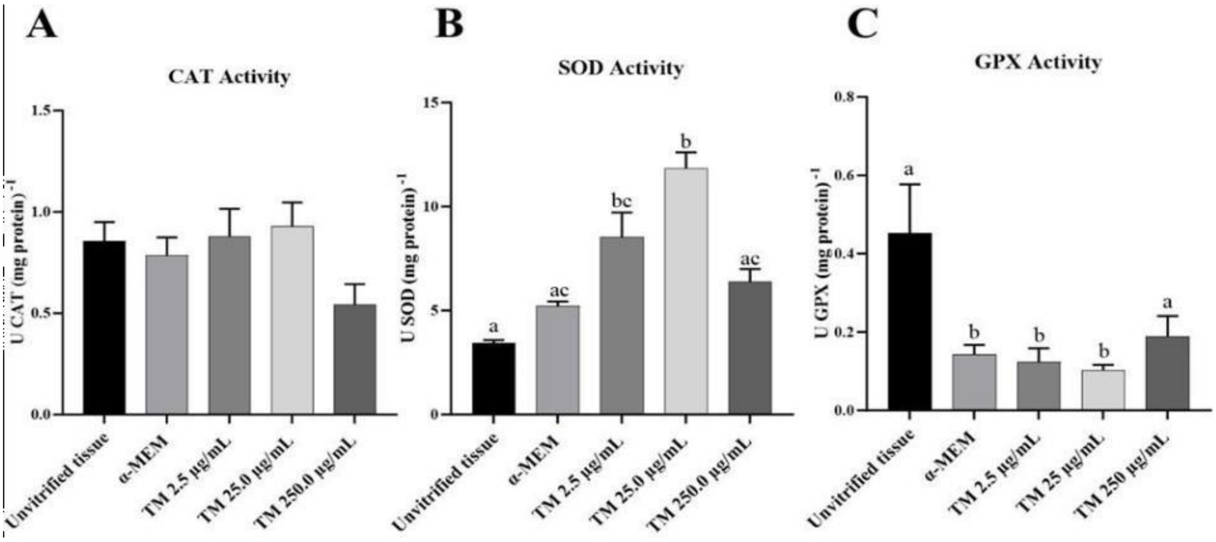

Figure 2. Redox status measures activity of antioxidant enzymes in unvitrified and vitrified tissues. CAT (A), SOD (B) and GPX (C) activity. Different letters indicate a statistically significant difference between treatment groups.

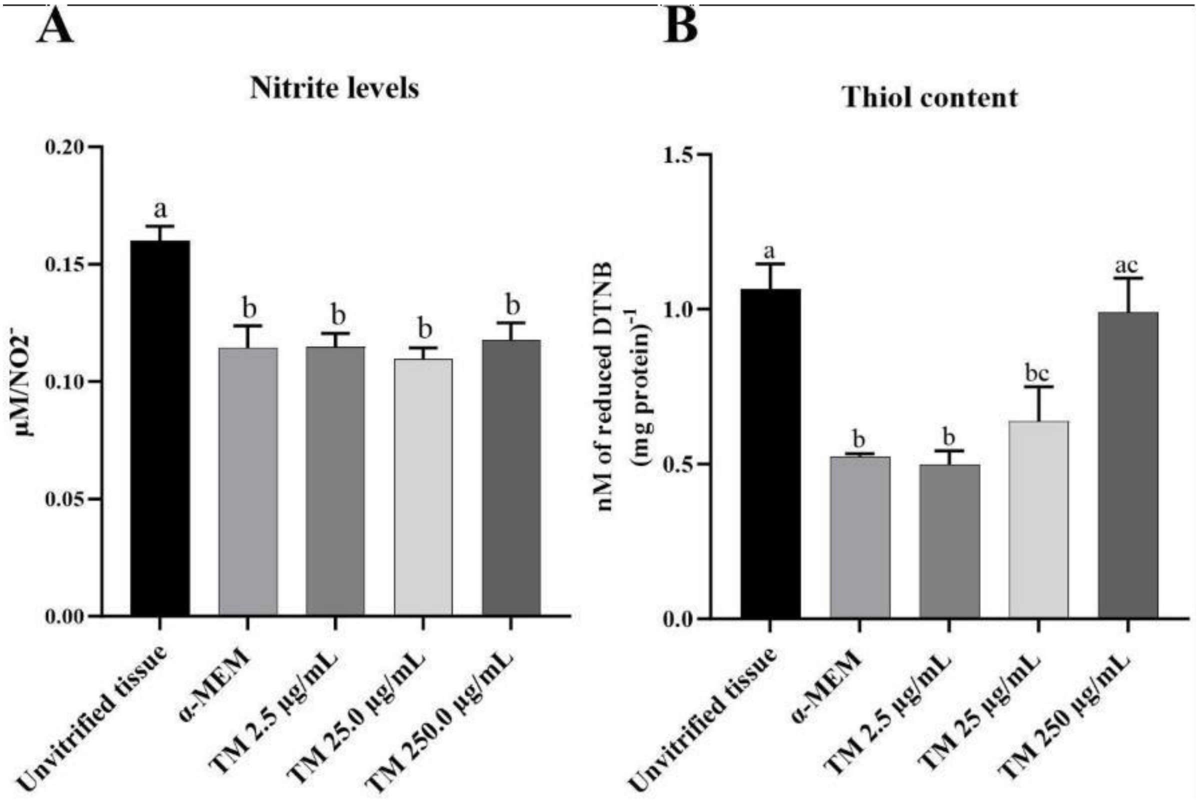

Figure 3. Redox status is measured by nitrite (A) and thiol (B) levels in unvitrified tissue and vitrified tissue with α-MEM alone or supplemented with different thymol (TM) concentrations 2,5, 25 and 250 µg/ml. Different letters indicate a statistically significant difference between treatments groups: *P < 0.05.

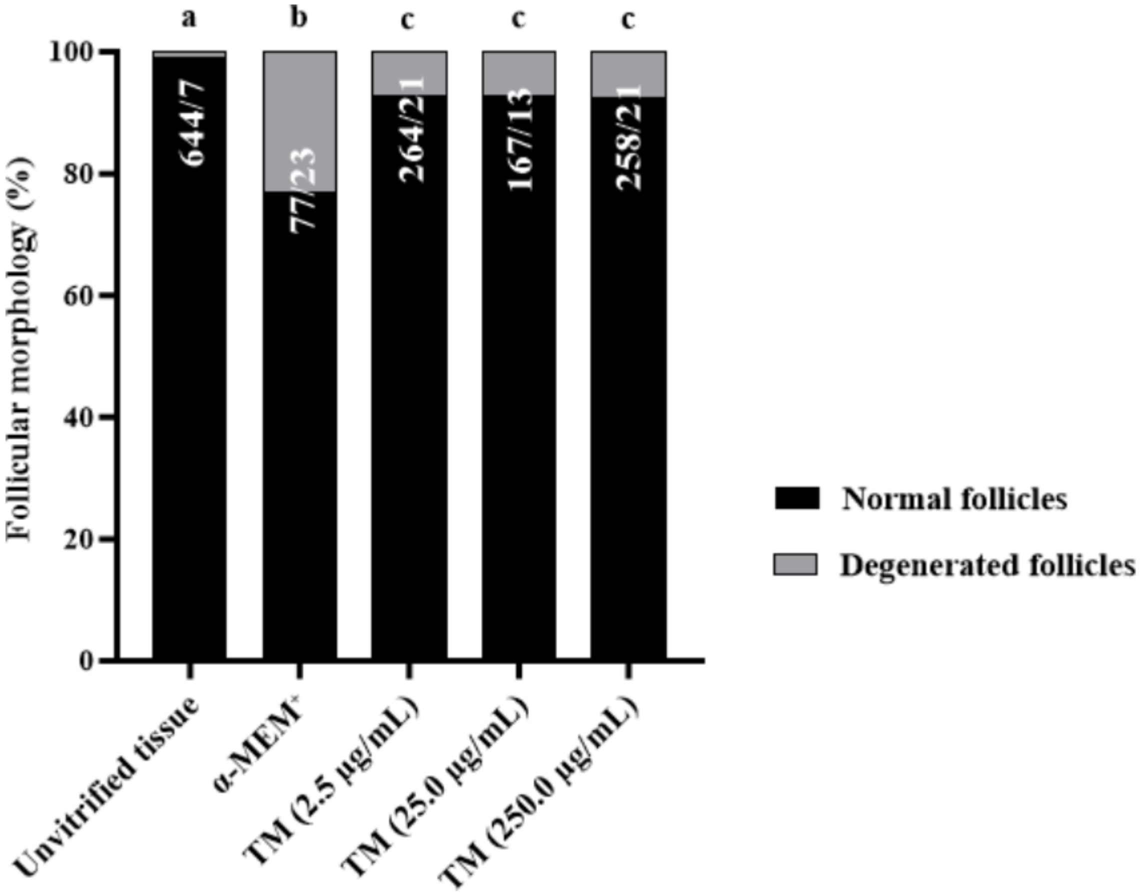

Figure 4. Percentage of morphologically normal follicles in bovine ovarian tissue vitrified in α-MEM+ alone or supplemented with 2.5, 25 and 250 µg/ml thymol (TM). Different letters indicate a statistically significant difference between treatment groups.

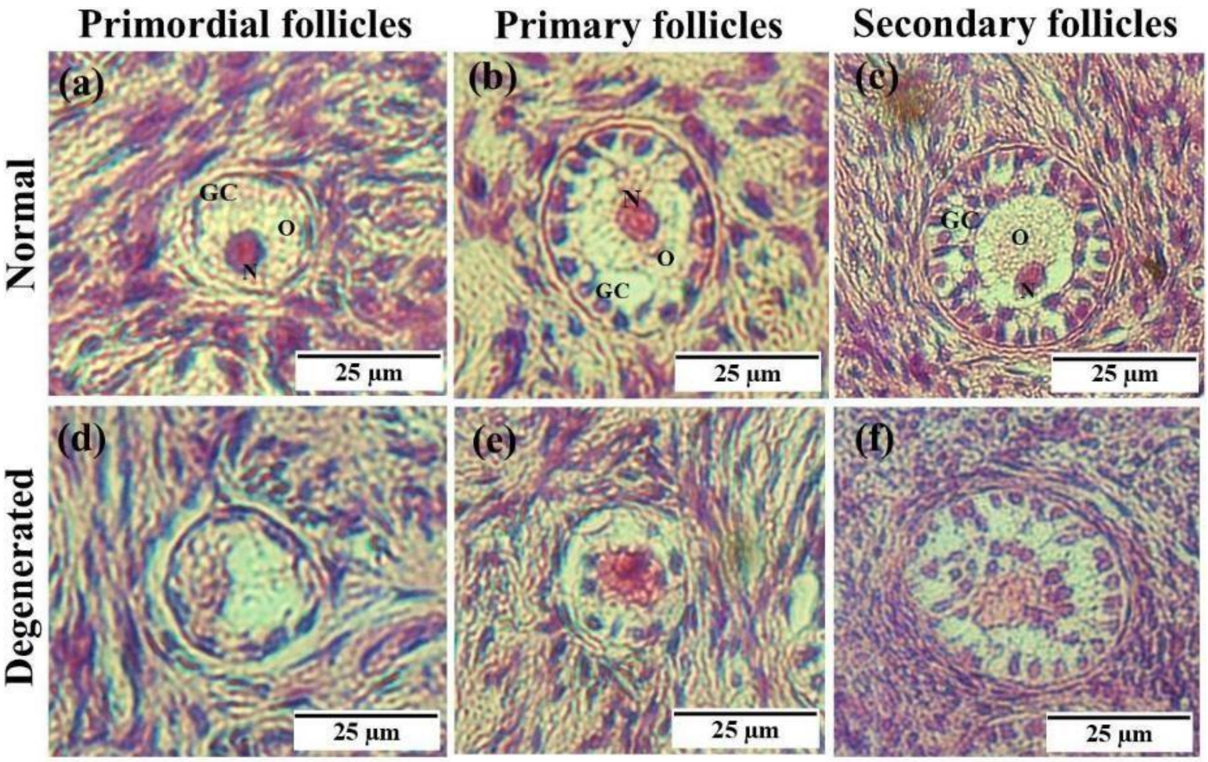

Figure 5. Representative images of sections of bovine ovarian tissue showing morphologically normal and degenerate follicles from different categories stained with hematoxylin and eosin. Normal (a) and degenerated (d) primordial follicles, normal (b) and degenerated (e) primary follicles; normal (c) and degenerated (f) secondary follicles. Granulosa cells (GC); oocyte (O); oocyte nucleus (N). Scale bar: 25 µm.

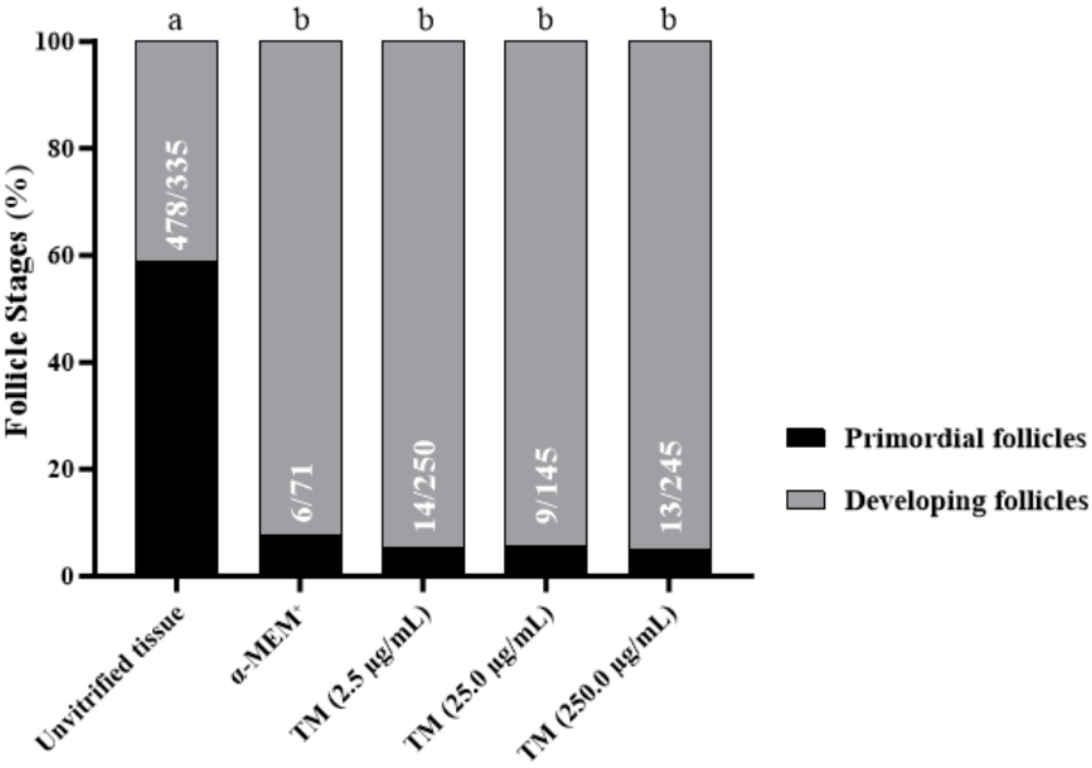

Figure 6. Percentage of primordial follicles and developing follicles in unvitrified tissues and vitrified tissues after 24 hours of incubation, in vitrification solution alone or supplemented with thymol at concentrations of 2.5, 25 and 250 µg/ml. Different letters indicate a statistically significant difference between treatment groups: ($p < 0.05$).

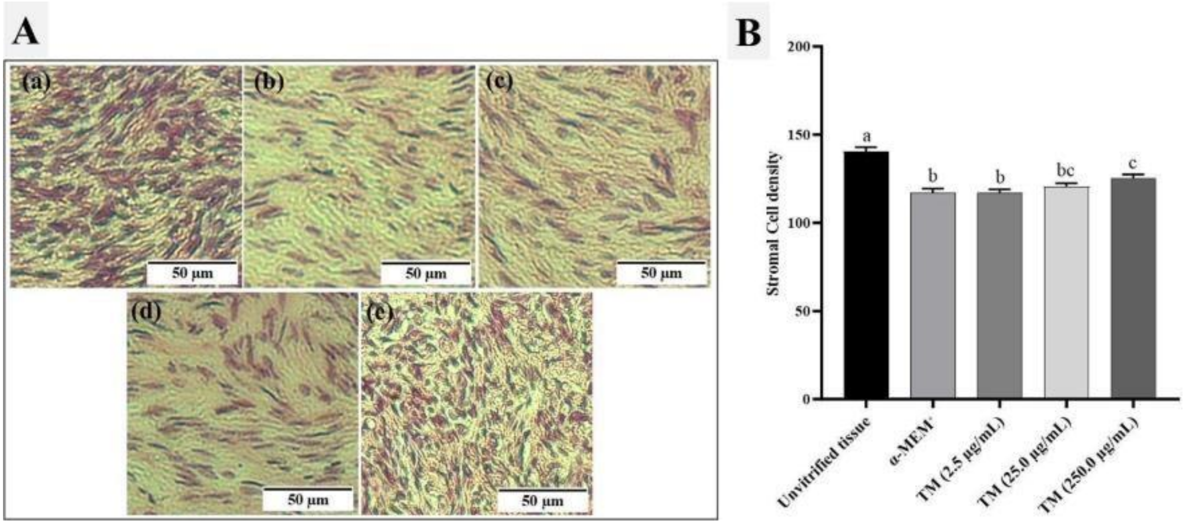

Figure 7. Effects of thymol supplementation on stromal cell density in vitrified bovine ovarian tissue. (A) Representative histological images of ovarian stromal cells stained with hematoxylin

and eosin. Unvitrified tissue (a); vitrified control tissue without thymol (b); vitrified tissue supplemented with thymol at 2.5 µg/mL (c); 25.0 µg/mL (d); and 250.0 µg/mL (e). Scale bar = 50 µm. (B) Quantitative measurement of stromal cell density before and 24 hours after vitrification. Data are expressed as mean ± SEM. *Different letters indicate statistically significant differences between groups. *P<0.05.

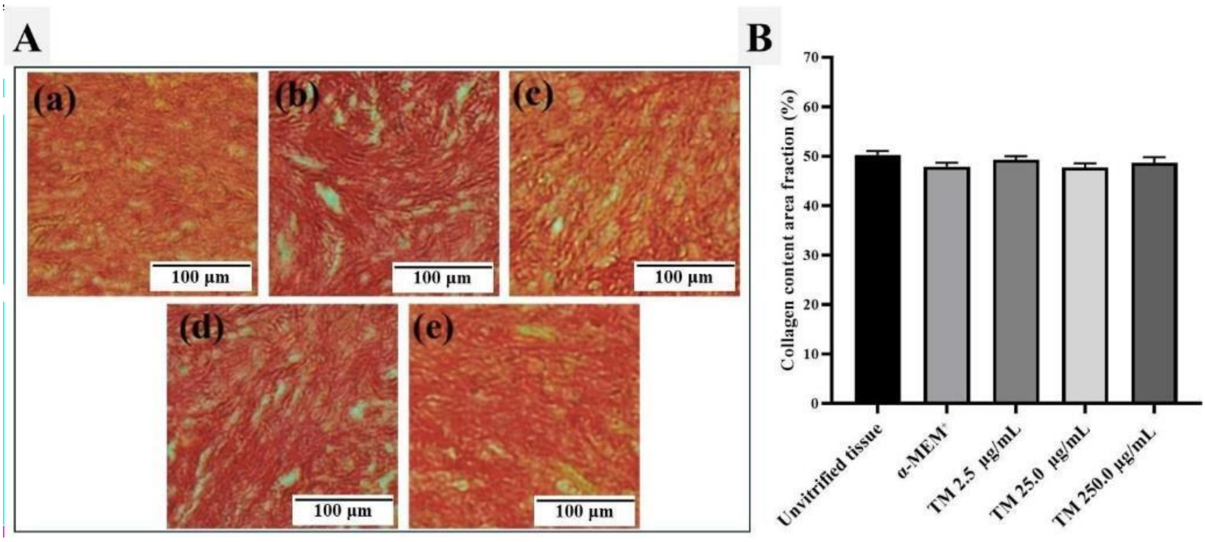

Figure 8. (A) Representative images of collagen fibres labelled by a red picrosirius stain. Unvitrified tissue (a); Vitrified control tissue without thymol (MEM+) (b); vitrified tissue supplemented with thymol at 2.5 µg/mL (c); 25.0 µg/mL (d); and 250.0 µg/mL (e). Scale bar = 100 µm. (B) Percentage of collagen fibers in ovarian tissue in unvitrified tissue and after vitrification: without thymol (MEM+); or with 2.5 µg/ml thymol (TM 2.5) and 25.0 µg/ml (TM 25.0), 250.0 (TM 250.0) µg/ml of thymol.

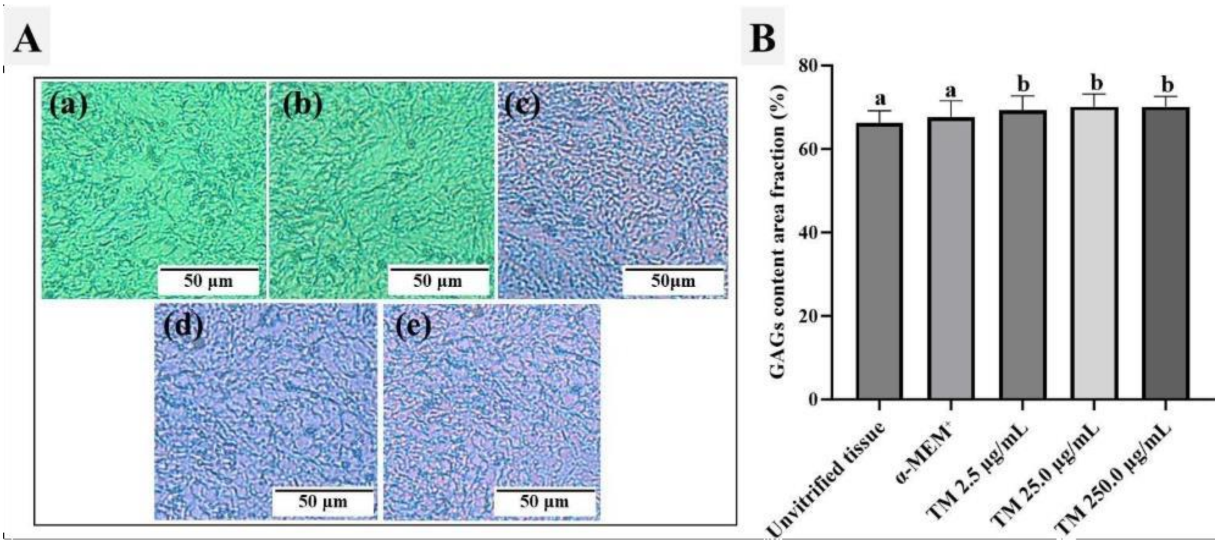

Figure 9. (A) Representative images of glycosaminoglycans (GAGs) stained by alcian blue. Unvitrified tissue (a); vitrified control tissue without thymol (MEM+) (b); vitrified tissue supplemented with thymol at 2.5 μg/mL (c), 25.0 μg/mL (d), and 250.0 μg/mL (e). (B) GAGs content of ovarian tissue in unvitrified tissue and vitrification solution alone (MEM); or supplemented with 2.5, 25.0 and 250.0 µg/ml of thymol. Different letters indicate a statistically significant difference between treatment groups.

7 CONCLUSÕES GERAIS

- A abordagem dose–resposta em células do cúmulus bovinas demonstrou que o timol, nas concentrações de 25 e 250 μg/mL, aumentou a intensidade de fluorescência do calcein-AM, indicando maior viabilidade celular, enquanto as concentrações de 2,5 e 25 μg/mL reduziram significativamente a intensidade de fluorescência do etídio, sugerindo menor dano à membrana plasmática. Esses achados indicam que o timol exerce efeitos dependentes da concentração sobre a viabilidade e a integridade celular, os quais podem ter contribuído para os desfechos redox e estruturais observados posteriormente no tecido ovariano vitrificado.

- A presença de 250 μg/mL de timol no meio de vitrificação foi eficaz na preservação dos níveis de tióis e da atividade da glutationa peroxidase (GPX), mantendo valores semelhantes aos observados no tecido não vitrificado, o que indica melhor manutenção do equilíbrio redox. Esta concentração de timol esteve associada a desfechos biológicos relevantes, incluindo maior proporção de folículos morfologicamente normais, preservação da densidade de células do estroma ovariano, manutenção do conteúdo de colágeno e aumento dos níveis de glicosaminoglicanos (GAGs) após o reaquecimento e a incubação.

8 PERSPECTIVAS

Os resultados obtidos neste estudo fornecem subsídios importantes para o aprimoramento dos protocolos de vitrificação de tecido ovariano, ao demonstrar que a suplementação com timol modula o equilíbrio redox e preserva a integridade estrutural do tecido de forma dependente da concentração. Esses achados indicam que a incorporação de antioxidantes naturais, como o timol, pode representar uma estratégia viável para reduzir os danos associados ao estresse oxidativo durante a criopreservação.

Com base nesses resultados, futuras investigações podem explorar a aplicação do timol em protocolos de vitrificação visando avaliar a funcionalidade de longo prazo do tecido ovariano preservado, incluindo a capacidade de crescimento folicular, a manutenção da atividade esteroidogênica e a competência de desenvolvimento oocitário após períodos prolongados de cultivo ou após transplante. Além disso, a compreensão mais aprofundada dos mecanismos envolvidos na ação do timol poderá contribuir para o refinamento das estratégias antioxidantes, especialmente no que se refere à regulação de vias sensíveis ao estado redox, à função mitocondrial e aos processos de sobrevivência celular.

Adicionalmente, os dados gerados neste estudo abrem perspectivas para a avaliação do uso combinado do timol com outros antioxidantes ou abordagens complementares, com o objetivo de potencializar a preservação do microambiente ovariano e ampliar a eficiência dos protocolos de criopreservação aplicados tanto à reprodução animal quanto à preservação da fertilidade feminina.

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