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FRANCISCO DAS CHAGAS COSTA

REMODELAÇÃO DO CÓRTEX OVARIANO DURANTE O CULTIVO *IN VITRO* E EFEITOS DE BIOANDAIMES DESCELULARIZADOS E NANOPARTÍCULAS POLIMÉRICAS CARREGADAS COM RESVERATROL NO CRESCIMENTO E VIABILIDADE DE FOLÍCULOS SECUNDÁRIOS BOVINOS

FORTALEZA
2025

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Tese apresentada ao Programa de Pós-Graduação em Biotecnologia – Rede Nordeste de Biotecnologia (RENORBIO), da Universidade Federal do Ceará (UFC), como parte dos requisitos para obtenção do título de Doutor em Biotecnologia. Área de Concentração: Biotecnologia em Agropecuária.

Orientador: Professor Doutor José Roberto Viana Silva.

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"*Eu sou apenas um rapaz latino-americano, sem dinheiro no banco, sem parentes importantes, e vindo do interior''*. (BELCHIOR)

RESUMO

O cultivo *in vitro* de folículos ovarianos pré-antrais bovinos é uma estratégia promissora para obtenção de oócitos, mas ainda é limitado pela incapacidade dos atuais sistemas de cultivo de reproduzir com precisão os estímulos bioquímicos e biomecânicos derivados do microambiente folicular e controlar o ambiente redox. Portanto, caracterizar as alterações da matriz extracelular (MEC) durante o cultivo de tecido ovariano e desenvolver bioandaimes tridimensionais para o cultivo de folículos secundários isolados juntamente com a utilização de antioxidantes biodisponíveis é essencial para desenvolver plataformas que sustentem a foliculogênese *in vitro.* Os objetivos deste estudo foram: Fase 1: avaliar os efeitos do cultivo e da suplementação com antioxidantes sobre a estrutura do córtex ovariano bovino, viabilidade folicular e estado redox *in vitro*; e Fase 2: desenvolver bioandaimes ovarianos bovinos e testar, em combinação com resveratrol nanoencapsulado, seus efeitos sobre folículos secundários isolados cultivados *in vitro*. Na Fase 1, fragmentos de ovário bovino foram cultivados por 6 dias para avaliar a remodelação da MEC, ativação folicular, viabilidade celular e marcadores redox, com ou sem suplementação de ácido ascórbico e resveratrol. Na Fase 2, bioandaimes ovarianos foram obtidos por descelularização e testados como plataformas tridimensionais (3D) para o cultivo *in vitro* de folículos secundários bovinos durante 12 dias em meio suplementado com resveratrol encapsulado em nanopartículas poliméricas (0,02, 0,2 ou 2 μM), avaliando viabilidade, ultraestrutura e expressão de genes antioxidante. Em geral, para a análise estatística, dados quantitativos foram analisados por teste t não pareado ou ANOVA e teste de Tukey ($P < 0,05$). Na Fase 1, o cultivo *in vitro* induziu ativação folicular, mas causou danos à integridade estrutural e celular do tecido ovariano, com maior degeneração, desorganização da MEC e redução de marcadores antioxidantes. A suplementação com ácido ascórbico e/ou resveratrol melhorou a viabilidade celular, preservou a MEC e aumentou a atividade antioxidante. Na Fase 2, a descelularização foi eficaz, mantendo a integridade da MEC. O cultivo em sistema 3D com resveratrol nanoencapsulado aumentou a viabilidade e preservou a morfologia folicular, especialmente com 0,02 μM, que também reduziu a expressão de genes antioxidantes. Em conclusão, o cultivo *in vitro* do tecido ovariano estimula a ativação folicular, mas compromete a integridade tecidual e antioxidante. Porém, a suplementação com ácido ascórbico e/ou resveratrol contribui para a preservação da matriz extracelular e aumento da sobrevivência folicular. Bioandaimes ovarianos descelularizados representam uma plataforma biologicamente ativa capaz de imitar o microambiente ovariano nativo. O sistema de cultivo *in*

vitro 3D utilizando bioandaimes descelularizados e resveratrol nanoencapsulado melhora a viabilidade e morfologia dos folículos secundários isolados, além de contribuir para um microambiente menos oxidativo.

Palavras-chave: Matriz extracelular; descelularização; ovário artificial; folículos ovarianos; estresse oxidativo.

ABSTRACT

The *in vitro* culture of bovine preantral ovarian follicles is a promising strategy for obtaining oocytes, but it is still limited by the inability of current culture systems to accurately reproduce the biochemical and biomechanical stimuli derived from the follicular microenvironment and to control the redox balance. Therefore, characterizing extracellular matrix (ECM) alterations during ovarian tissue culture and developing three-dimensional bioscaffolds for the culture of isolated secondary follicles, together with the use of bioavailable antioxidants, is essential to establish platforms that support *in vitro* folliculogenesis. The aims of this study were: Phase 1: to evaluate the effects of culture and antioxidant supplementation on the structure of the bovine ovarian cortex, follicular viability, and *in vitro* redox status; and Phase 2: to develop bovine ovarian bioscaffolds and test, in combination with nanoencapsulated resveratrol, their effects on isolated secondary follicles cultured *in vitro*. In Phase 1, bovine ovarian fragments were cultured for 6 days to assess ECM remodeling, follicular activation, cell viability, and redox markers, with or without supplementation with ascorbic acid and resveratrol. In Phase 2, ovarian bioscaffolds were obtained by decellularization and tested as three-dimensional (3D) platforms for the *in vitro* culture of bovine secondary follicles for 12 days in medium supplemented with polymeric nanoparticle-encapsulated resveratrol (0.02, 0.2, or 2 µM), evaluating viability, ultrastructure, and antioxidant gene expression. In general, for statistical analysis, quantitative data were analyzed by unpaired t-test or ANOVA followed by Tukey's test ($P < 0.05$). In Phase 1, *in vitro* culture induced follicular activation but caused structural and cellular damage to the ovarian tissue, with increased degeneration, ECM disorganization, and reduced antioxidant markers. Supplementation with ascorbic acid and/or resveratrol improved cell viability, preserved ECM integrity, and enhanced antioxidant activity. In Phase 2, decellularization was effective, maintaining ECM integrity. Culture in a 3D system with nanoencapsulated resveratrol increased viability and preserved follicular morphology, especially with 0.02 µM, which also reduced the expression of antioxidant genes. In conclusion, *in vitro* culture of ovarian tissue stimulates follicular activation but compromises tissue and antioxidant integrity. However, supplementation with ascorbic acid and/or resveratrol contributes to ECM preservation and improved follicular survival. Decellularized ovarian bioscaffolds represent a biologically active platform capable of mimicking the native ovarian microenvironment. The 3D *in vitro* culture system using decellularized bioscaffolds and

nanoencapsulated resveratrol improves the viability and morphology of isolated secondary follicles, while also contributing to a less oxidative microenvironment.

Keywords: Extracellular matrix; decellularization; artificial ovary; ovarian follicles; oxidative stress.

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LISTA DE ABREVIATURAS E SIGLAS

AKAP13	Proteína âncora 13 da A-quinase
AKT	Proteína quinase B
AMH	Hormônio antimülleriano
AMPc	Adenosina 3`, 5`-monofosfato cíclico
AMP	Adenosina monofosfato
ATP	Adenosina trifosfato
ANOVA	Análise de variância
Bim	Inibidor associado à proteína Bcl-2
BMP-2	Proteína morfogenéticas óssea-2
BMP-4	Proteína morfogenéticas óssea-4
BMP-15	Proteína morfogenéticas óssea-15
BIRC	Inibidores baculovirais da repetição da apoptose
BLIMP1	Proteína-1 de maturação induzida por linfócitos B
CAT	Catalase
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CCN	Rede de comunicação celular
CCNB1	Ciclina B1
CCO	Complexo cumulus-oócito
CE	Ceará
CGPs	Células germinativas primordiais
CG	Células da granulosa
CO_2	Dióxido de carbono
c-Kit	Receptor do kit ligante
DMEC	Matriz extracelular descelularizada
DNA	Ácido desoxirribonucleico
E2	Estradiol
EGF	Fator de crescimento epidermal
EGFR	Receptor do fator de crescimento epidermal
FAO	Fundação das Nações Unidas para Alimentação e Agricultura

ERO	Espécie reativa de oxigênio
FasL	Ligante *Fas*
FIOCRUZ	Fundação Oswaldo Cruz
FOXO3a	Fator de transcrição *forkhead box* 3a
FSH	Hormônio folículo estimulante
GAG	Glicosaminoglicano
GDF-9	Fator de crescimento e diferenciação-9
GnRH	Hormônio liberador de gonadotrofina
GPER	Receptor de estrogênio acoplado à proteína G
GPX1	Glutationa peroxidase-1
GR	Glutationa redutase
GSH	Glutationa reduzida
GSSG	Glutationa oxidada
GV	Vesícula germinativa
H2O2	Peróxido de hidrogênio
IGF-1	Fator de crescimento semelhante à insulina-1
KL	*Kit* ligante
LH	Hormônio luteinizante
MI	Metáfase I
MII	Metáfase II
MDA	Malondialdeído
MEC	Matriz extracelular
MEM	Meio essencial mínimo
MMPs	Metaloproteinases de matriz
mTOR	Alvo mamífero da rapamicina
NADPH	Nicotinamida adenina dinucleotídio fosfato
NPP	Nanopartícula polimérica
NRF2	Fator nuclear derivado de eritróide 2
OCT4	Fator de transcrição de ligação ao octâmero 4
$O_2^{-\cdot}$	Radical superóxido
PCL	Policaprolactona

$OH^{\cdot}$	Radical hidroxila
PI3K	Fosfatidilinositol-3-quinase
PDZ	Domínio PDZ
PIP2	Fosfatidilinositol-4,5-bifosfato
PIP3	Fosfatidilinositol-3,4,5-trifosfato
PRDX6	Peroxiredoxina-6
PTEN	Fosfatase homóloga à tensina
P4	Progesterona
P38-MAPK	Proteína quinase ativada por mitógeno
RE	Retículo endoplasmático
RENORBIO	Rede Nordeste de Biotecnologia
RNA	Ácido ribonucleico
RSV	Resveratrol
SOD	Superóxido dismutase
STAR	Proteína reguladora aguda esteroidogênica
TAZ	Coativador com afinidade à PDZ
TCM-199	Meio de cultivo de tecido-199
TEAD	Fator de transcrição de domínio TEA
TNF-α	Fator de necrose tumoral-alfa
TRAIL	Ligante indutor de apoptose relacionado ao TNF
UFC	Universidade Federal do Ceará
UPR	Resposta de proteína mal dobrada
VEGF	Fator de crescimento endotelial vascular
YAP	Proteína associado a *Yes*
ZP	Zona pelúcida

LISTA DE SÍMBOLOS

>	Maior
<	Menor
%	Porcentagem
kg	Quilograma
g	Grama
mg	Miligrama
µg	Micrograma
M	Molar
mM	Milimolar
µM	Micromolar
nM	Nanomolar
mm	Milímetro
µm	Micrômetro
μm^2	Micrometro quadrado
l	Litro
ml	Mililitro
µl	Microlitro
pH	Potencial hidrogeniônico
°C	Graus Celsius
h	horas

SUMÁRIO

1 INTRODUÇÃO

Nos últimos 30 anos, a criação animal tem enfrentado desafios críticos para suprir a crescente demanda por alimentos para sustentar uma população mundial em crescimento constante (Pasquariello *et al*., 2024). Nesse cenário, o refinamento das biotécnicas reprodutivas existentes e o desenvolvimento de novas são imperativos para melhorar a eficiência dos sistemas de produção de animais estratégicos para a pecuária, como os bovinos (Botigelli *et al.,* 2023; Hansen *et al.,* 2023). Nos últimos anos, o desenvolvimento folicular *in vitro* surgiu como uma ferramenta potencialmente capaz de promover o aproveitamento da população de folículos ovarianos pré-antrais para a obtenção de oócitos meioticamente competentes (Malo *et al.,* 2024; Chavoshinezhad *et al.,* 2024). No entanto, alcançar a foliculogênese completa *in vitro* requer a replicação dos eventos fisiológicos chave incluindo a ativação inicial, crescimento e progressão folicular através dos diferentes estágios de desenvolvimento (Vitale *et al.,* 2024). Assim, embora encorajadora, essa abordagem continua sendo extremamente desafiadora, especialmente em mamíferos maiores como os bovinos, uma vez que nem todos os mecanismos envolvidos no desenvolvimento folicular são ainda totalmente compreendidos (Morton *et al.,* 2023; Telfer *et al.,* 2021). Por isso, aprimorar modelos fisiológicos *in vitro* é crucial para delinear os mecanismos subjacentes e definir necessidades foliculares críticas guiando os esforços atuais rumo ao desenvolvimento de sistemas robustos capazes de sustentar a foliculogênese *ex vivo*.

A ativação dos folículos primordiais dormentes para que progridam para os estágios primário e secundário inicial é o primeiro passo para recapitular a foliculogênese *in vitro* (Barbato *et al*., 2023). Após tentativas malsucedidas de cultivo de folículos primordiais isolados (Oktay *et al.*, 1997; Hovatta *et al.,* 1997), consolidou-se o consenso de que a transição dos folículos primordiais para o estágio secundário depende de interações com o estroma ovariano. Avanços recentes em mecanobiologia revelam que a matriz extracelular ovariana (MEC) não é meramente um andaime estrutural, mas um regulador ativo da fisiologia ovariana (Wang *et al.,* 2025). A MEC transmite sinais mecânicos que governam processos-chave, como dormência folicular, ativação e crescimento, moldando em última analise o destino folicular. A remodelação dinâmica e coordenada da MEC fornece as pistas bioquímicas e biomecânicas necessárias para a morfogênese, diferenciação e homeostase folicular (Grosbois *et al.,* 2023). Além disso, foi demonstrado que o

estresse oxidativo impacta a sinalização celular normal, DNA, lipídios e proteínas prejudicando a síntese dos componentes da MEC (Feng *et al.,* 2021). Assim, considerando a intrincada interação entre a estrutura da MEC e a mecanotransdução celular como elo crítico para a foliculogênese, caracterizar a dinâmica da remodelação da MEC durante o cultivo *in vitro* de tecido ovariano e o efeito de antioxidantes nesse processo pode fornecer evidências valiosas para intervenções impulsionadoras dos atuais sistemas de cultivo *in vitro* para folículos primordiais e primários.

Embora a partir do estágio secundário, os folículos possam ser removidos do tecido ovariano e cultivados diretamente sobre uma placa de cultivo bidimensional (2D), em bovinos, a obtenção de oócitos meioticamente competentes a partir de folículos secundários continua sem sucesso (Azevedo *et al*., 2022; Paulino *et al.,* 2018). Apesar de necessidades foliculares críticas terem sido elucidadas com esse sistema de cultivo, tem sido relatado que, uma vez isolados do ovário, os folículos perdem a estrutura de suporte natural promovida pelo tecido ovariano adjacente, interrompendo os mecanismos de mecanossinalização cruciais para o desenvolvimento folicular (Fiorentino *et al.,* 2023). Além disso, o estresse oxidativo *in vitro* continua sendo um fator altamente correlacionado com a baixa qualidade dos folículos secundários após o cultivo (Silva *et al.,* 2023). Para superar esses desafios, sistemas de cultivo tridimensionais (3D) e a suplementação dos meios de cultivo com antioxidantes têm sido indicados como ferramentas estratégicas (Nair *et al.,* 2024; Silva *et al.,* 2023). Nesse campo, a pesquisa ovariana recente vem apontando o uso promissor de andaimes derivados de matriz extracelular ovariana descelularizada como uma estrutura 3D nativa que imita de perto a matriz extracelular ovariana com capacidade de sustentar o desenvolvimento *in vitro* de folículos (Sistani *et al.,* 2023; Park *et al.,* 2023; Mirzaeian *et al.,* 2023). Paralelamente, o equilíbrio redox *in vitro* tem sido melhorado através da adição de compostos antioxidantes como o resveratrol (Silva *et al.,* 2023). Esse composto fenólico natural foi relatado como um atenuador da depleção folicular através de seus efeitos antioxidantes e antiapoptóticos (Macedo *et al.,* 2017, Bezerra *et al.,* 2018). No entanto, sua baixa solubilidade em água e metabolismo rápido podem limitar seus efeitos. Nesse contexto, o encapsulamento dessa molécula em nanocarreadores biocompatíveis e não tóxicos como as nanopartículas poliméricas pode ser uma estratégia promissora para aumentar sua eficácia *in vitro* (Freitas *et al.,* 2023). Entretanto, a eficácia de andaimes ovarianos descelularizados e do resveratrol encapsulado em nanopartículas poliméricas durante o cultivo de folículos secundários ainda não foram elucidados na espécie bovina.

Para contextualizar a relevância desta tese, a revisão de literatura a seguir abordará temas relacionados á pecuária como área estratégica para a segurança alimentar global; aspectos fisiológicos da reprodução de fêmeas bovinas, incluindo a mecanossinalização como uma nova fronteira; e biotécnicas aplicáveis para melhorar o desempenho reprodutivo incluindo limitações atuais e oportunidades.

2 REVISÃO DA LITERATURA

2.1 Biotecnologias reprodutivas na pecuária e segurança alimentar

A segurança alimentar existe quando todas as pessoas, em todos os momentos têm acesso a alimentos suficientes para uma vida ativa e saudável (Callouway *et al.,* 2023). No entanto, atender às necessidades nutricionais de uma população em rápido crescimento dentro das restrições de recursos finitos ainda representa um desafio significativo (Zhu *et al.,* 2025). Até 2050, o setor agrícola deverá ser capaz de produzir 60% a mais do que o atual suprimento de alimentos para sustentar uma população global prevista de 9,3 bilhões de pessoas preservando, ao mesmo tempo, os processos ecossistêmicos essenciais que apoiam o futuro sustentável (FAO, 2023; Michalk *et al*., 2018). Assim, o modelo atual de uma população cada vez maior que depende de recursos finitos é claramente insustentável e exige esforços em pesquisa para produzir recomendações baseadas em evidências e orientar decisões capazes de incrementar a produção de alimentos por setores estratégicos, como a pecuária.

Globalmente, as principais fontes de alimentos mudaram nos últimos 50 anos de grão para proteína de origem animal, tornando a pecuária uma das indústrias agrícolas mais importantes no campo da produção alimentícia (Ambikapathi *et al.,* 2022). Ao longo dos anos, os sistemas de produção de animais foram intensificados em termos de produtividade por animal, e, atualmente, a pecuária responde por cerca de 40% da produção agrícola mundial, além de subsidiar os meios de subsistência e a segurança alimentar de mais de 1,3 bilhões de pessoas (FAO, 2018). No entanto, embora a disponibilidade de alimentos de origem animal tenha aumentado juntamente com o crescimento da população humana nos últimos anos, atualmente, a insegurança alimentar grave ainda afeta cerca de 300 milhões de pessoas (FAO, 2019). Portanto, o desafio da indústria pecuária enquanto área estratégica para a segurança alimentar, requer o desenvolvimento de ferramentas

capazes de impulsionar a produção de alimento de alto valor nutricional para uma população mundial em constante expansão (Cartthy *et al*., 2018).

A eficiência reprodutiva dos rebanhos tem um grande impacto na sustentabilidade econômica da indústria pecuária. À medida que o desempenho reprodutivo ideal é alcançado, outros ajustes no sistema de produção podem ser aplicados com uma forte capacidade de aumentar a sustentabilidade econômica (Lancaster; Larson 2022). No cenário atual, o desenvolvimento do setor pecuário tem sido alimentado por um fluxo consistente de inovações e avanços biotecnológicos, especialmente no âmbito reprodutivo (Li *et al.,* 2025). Entre as ferramentas biotecnológicas atualmente disponíveis, a inseminação artificial (IA), a transferência de embriões (TE) e a produção *in vitro* de embriões (PIVE) destacam-se por sua ampla aplicação e impacto econômico (Mikkola *et al.,* 2024). Essas biotécnicas surgiram como instrumentos transformadores no campo da indústria pecuária e têm sido fundamentais para melhorar a qualidade genética de espécies como bovinos, aumentar a produção de leite e carne e facilitar a disseminação de características genéticas superiores entre diversas raças aumentando a qualidade do rebanho em consideravelmente menos tempo do que os métodos tradicionais de melhoramento (Hambisa, 2024, Shanku, 2022). Outras biotécnicas, como o cultivo *in vitro* de tecido ovariano e de folículos ovarianos pré-antrais isolados têm sido extensivamente estudadas nos últimos anos (Cheng *et al.,* 2024; Vitale *et al.,* 2024). Estudos sobre o cultivo *in vitro* de folículos pré-antrais em animais domésticos são frequentemente realizados para aperfeiçoar o uso de gametas imaturos como fontes de oócitos fertilizáveis, o que pode ajudar a melhorar o manejo reprodutivo nessas espécies (Silva *et al*., 2023; Paulino *et al.,* 2020). Embora até agora nenhum sistema de cultivo definido tenha sido capaz de replicar completamente a foliculogênese *in vitro* na espécie bovina, evidências acumuladas vêm fornecendo informações cruciais sobre as necessidades foliculares e eventos vitais durante o desenvolvimento em ambientes controlados (Converse *et al*., 2023). Esses *insights* são particularmente uteis ao aumentar nossa compreensão da biologia fundamental do folículo em desenvolvimento suprindo lacunas críticas do conhecimento ainda incipiente acerca da foliculogênese em animais mamíferos, orientando direções de pesquisas atuais nesse campo de investigação.

2.2 Estrutura e função ovariana

A reprodução de fêmeas mamíferas, como as vacas, depende diretamente do desenvolvimento do ovário (Figura 1), um órgão altamente dinâmico reconhecido por suas funções endócrinas e reprodutivas, incorporadas por seu papel na biossíntese de esteroides e na produção de oócitos, respectivamente (Grosbois *et al.,* 2023). Suas subunidades funcionais, os folículos contendo oócitos, são formadas por diferentes tipos de células somáticas que interagem por meio de extensas redes mediadas por citocinas parácrinas e fatores de crescimento. Essa comunicação coordena tanto o desenvolvimento dos oócitos quanto a produção cíclica de hormônios esteroides, em resposta a estímulos ambientais e sinais específicos do hospedeiro (Soygur *et al.*, 2021). As diferentes funções do ovário são mantidas pelo papel dinâmico do órgão na fisiologia reprodutiva que, por sua vez, é governada pelos arranjos estruturais e pela função de diferentes compartimentos celulares (Zhao *et al*., 2021).

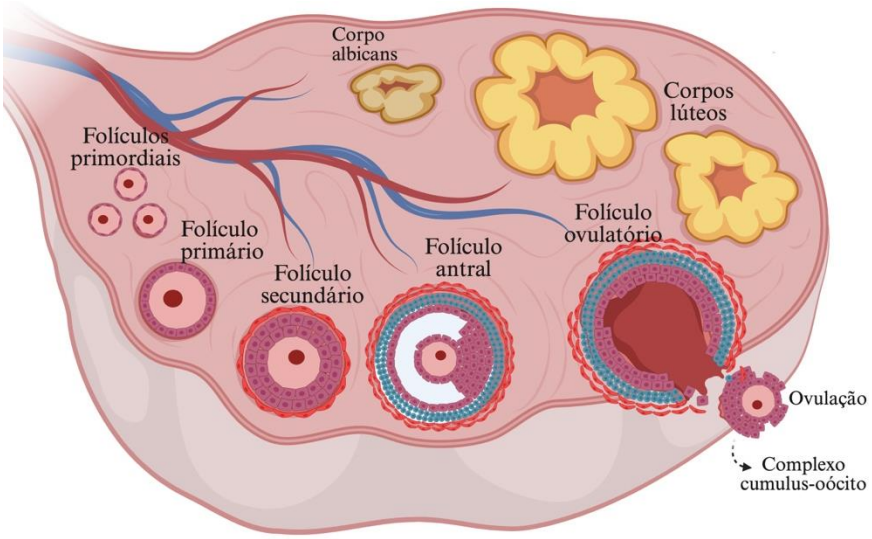

Figura 1: Estrutura ovariana ilustrando a presença de folículos ovarianos em diferentes estágios de desenvolvimento e corpos lúteos. Fonte: Adaptado de Luz *et al.* (2018).

De um ponto de vista estrutural simplificado, o ovário pode ser dividido em três zonas: o epitélio, o córtex e a medula e essa compartimentalização é determinada pela composição, organização e densidade espacial da matriz extracelular (Shah *et al*., 2018). O órgão é envolvido externamente por uma camada esbranquiçada de epitélio de superfície ovariana, derivado do mesoderma e formado por células epiteliais que variam de simples pavimentosas a cúbicas ou de baixa colunar pseudostratificada. Sua integridade é mantida por desmossomos simples, junções

estreitas incompletas, várias integrinas e caderinas (Xu *et al*., 2018). A função principal do epitélio é a regeneração e proliferação repetida de células após a ovulação durante o ciclo reprodutivo (Del Valle *et al*., 2024). Por sua vez, o córtex ovariano é formado por tecido conjuntivo, contendo células do estroma, folículos ovarianos em sua maioria quiescentes e corpos lúteos. Entre as células estromais, as células semelhantes aos fibroblastos produzem moléculas de MEC envolvidas na estruturação e biomecânica do perifolículo; as células fusiformes produzem esteroides, principalmente andrógenos, e participam da modulação folicular (Kinnear *et al.,* 2020). Estudos proteômicos recentes do córtex ovariano identificaram diversas proteínas relacionadas ao matrissoma como colágeno, fibronectina, elastina e laminina conhecidas por regular e remodelar a matriz extracelular ovariana durante o desenvolvimento folicular evidenciando o caráter altamente dinâmico do córtex ovariano durante a foliculogênese (Grosbois *et al*., 2023; Ouni *et al*., 2018). Já a medula é composta por folículos em desenvolvimento, tecido conjuntivo frouxo e ricamente vascularizado, abrigando fibroblastos, fibras elásticas, vasos sanguíneos, vasos linfáticos e fibras nervosas, que desempenham a função de sustentar e nutrir os folículos ovarianos (Shah *et al.,* 2018; Martelli *et al.,* 2017).

2.3 Aspectos básicos da gametogênese em bovinos

A gametogênese em fêmeas mamíferas é o processo pelo qual um oócito, circundado por suas células somáticas de suporte, se desenvolve no ovário até o ponto de ovulação. Por meio desse processo, os oócitos crescem de um estágio imaturo para um oócito maduro e fertilizável (Morton *et al*., 2023). A formação de oócitos funcionais é, sem dúvida, um dos eventos mais importantes no desenvolvimento inicial dos mamíferos, uma vez que fornecem a maior parte dos materiais genéticos e citoplasmáticos para uma reprodução bem sucedida (Biswas *et al.,* 2022). Aspectos da gametogênese em espécies como os bovinos são estudados, sobretudo, para compreender a sucessão de eventos-chave que incluem suporte folicular levando à formação de um oócito competente em termos de desenvolvimento, fertilização, ativação do genoma embrionário e início da diferenciação da linhagem celular (Wang *et al*., 2023). Em vacas, o desenvolvimento oocitário até a aquisição da competência é um processo longo e complexo. Ele resulta de dois processos, foliculogênese e oogênese que, conjuntamente, preparam o cenário para o desenvolvimento embrionário inicial (Pytel *et al.,* 2024). Esses eventos de várias etapas são dois processos paralelos

intrinsecamente ligados caracterizados por estados celulares específicos e estão associados a transições morfológicas e moleculares notáveis rigorosamente controladas que levam o oócito a crescer e amadurecer (Dias *et al.,* 2021). Embora a oogênese e a foliculogênese sejam eventos que ocorrem em paralelo, para estudar a gametogênese de forma sistemática e precisamente definida, uma divisão das duas fases costuma ser introduzida.

2.3.1 Oogênese

A oogênese tem seu início ainda durante o desenvolvimento fetal quando, entre a semana 2 e a semana 8 do desenvolvimento embrionário bovino, um grupo de células que são inicialmente destinadas a um destino mesodérmico somático é induzido pela sinalização de proteínas morfogenéticas ósseas (BMPs) no epiblasto pluripotente tornando-se células germinativas primordias (CGPs) (Kobayashi *et al.,* 2017; Soto e Ross, 2021). Isso leva à regulação positiva dos genes de especificação de CGPs, incluindo *BLIMP1* e *TFAP2C*, acompanhada pela manutenção de OCT4 e reexpressão de muitos fatores de transcrição associados à pluripotência (Ramakrishna *et al*., 2021). Depois disso, as CGPs, orientadas por programas moleculares de motilidade intrínsecos e dicas de orientação externas migram para a crista genital, onde, após a colonização, especificam-se em oogônias e proliferam ativamente por sucessivas divisões mitóticas para formar ninhos interconectados ou cistos de células germinativas. As oogônias, então, entram em meiose, parando na fase de prófase I (estágio de vesícula germinativa – GV), estágio em que passam a ser consideradas oócitos primários. A quebra dos cistos de células germinativas ocorre quando as células somáticas (precursoras das células da granulosa) invadem os cistos e encerram oócitos individuais, formando folículos primordiais e dando início à foliculogênese (Morton *et al.,* 2023). Esses folículos imaturos contendo oócitos primários constituirão a fonte finita de células germinativas femininas conhecidas como reserva ovariana e representam o estágio mais abundante de folículos dentro do ovário em qualquer ponto do desenvolvimento (Kalinderi *et al.,* 2024).

Os oócitos contidos em folículos primordias permanecem em estado de interrupção meiótica até o sinal pré-ovulatório do LH na puberdade. O oócito com prófase interrompida contém um núcleo grande, com um nucléolo proeminente e cromatina parcialmente condensada. A inibição da divisão meiótica neste estágio permite que o oócito passe pela fase de crescimento, durante a qual proteínas e mRNA, necessários para o curso adequado da maturação e os estágios iniciais do

desenvolvimento embrionário, são acumulados no citoplasma (Jentoft *et al.,* 2023; Straczynska *et al.,* 2022; Jaffle *et al.,* 2017). Dentro do folículo a manutenção da parada meiótica do oócito é atribuída principalmente às altas concentrações citoplasmáticas de monofosfato de adenosina cíclico (AMPc). Evidências mostram que a redução na concentração de AMPc desencadeia a retomada da meiose (Pei *et al.,* 2023). Sob a circunstância de que os oócitos são separados dos folículos *in vitro* os níveis de AMPc intra-oócitos diminuem e a meiose é retomada (Medina-Chávez *et al.,* 2021).

Já na fase adulta, quando as fêmeas estão sexualmente maduras, uma pequena porção de folículos primordiais começará a crescer gradualmente. Durante esse período, mudanças morfológicas dinâmicas, ocorrem nas células foliculares com modificações em número, morfologia, atividade metabólica, diferenciação celular e capacidade de resposta a hormônios impulsionando a intercomunicação entre o oócito e seus componentes foliculares o que estimula o crescimento oocitário (Latorraca *et al.,* 2024; Warzych, Lipinska, 2020). Após ser ativado para crescer, o oócito passa por uma fase de transformação molecular e morfológica intensa, envolvendo um aumento de cinco vezes no volume, síntese e reorganização de organelas nucleares e citoplasmáticas, e o estabelecimento do epigenoma e do transcriptoma do oócito, todos os quais devem sustentar a retomada meiótica do oócito e a conclusão posterior da maturação oocitária, fertilização, divisões de clivagem embrionária inicial e ativação do genoma embrionário (Latorraca *et al.,* 2024; Dalbies-Tran *et al.,* 2020; Gu *et al.,* 2019). Mais tarde, os oócitos encerrados em folículos totalmente crescidos em resposta a estimulação de gonadotrofinas retomam a meiose (He *et al.,* 2021). O pico pré-ovulatório de LH leva a uma redução nos níveis de AMPc provocando a desfosforilação e consequente ativação do complexo Fator Promotor da Meiose, que, por sua vez, está envolvido com a ruptura do envelope nuclear, condensação de cromatina, reorganização do citoesqueleto e bloqueio da atividade transcricional (Gahurova *et al.,*2017) A duração desse estágio, ou seja, o crescimento do oócito desde a estimulação dos folículos primordias para o crescimento até o estágio de folículo pré-ovulatório leva cerca de 120 dias em bovinos. Finalmente o processo é finalizado caso ocorra a fecundação do oócito maduro (Fair, Lonergan, 2023).

2.3.2 Foliculogênese

A foliculogênese é um subprocesso separado que acompanha e suporta a oogênese e corresponde a um conjunto de etapas rigorosamente reguladas que conduzem a maturação do folículo do estágio primordial - não crescente - até grandes folículos ovulatórios abrangendo sua ativação, crescimento e progressão até a plena maturação (Straczynska *et al.,* 2022). Esses eventos são controlados por uma extensa rede de sinalização entre as células foliculares, oócito e o microambiente ovariano (Soygur *et al.,* 2021). O desenvolvimento folicular configura-se como um processo gradual, marcado por profundas transformações morfológicas e moleculares dos folículos e oócitos, além de modificações dinâmicas do estroma circundante para acomodar o desenvolvimento e a expansão folicular (Clarck *et al.,* 2022).

O desenvolvimento folicular, a partir do folículo primordial, configura-se como um processo gradual e irreversível com etapas rigidamente reguladas (Zhao *et al.,* 2021). A característica mais específica dos folículos primordiais é sua dormência de longo prazo garantindo a longevidade reprodutiva feminina. Assim, o seu recrutamento inicial em pequenos grupos é uma etapa indispensável da gametogênese, pois determina a vida útil reprodutiva (Vo *et al.,* 2021). A ativação de folículos primordiais é modulada estritamente para garantir que apenas um pequeno número de folículos possa ser ativado em cada ciclo e é desencadeada pela supressão de fatores inibitórios e mantenedores da quiescência folicular que, uma vez silenciados, permitem que um *pool* de folículos primordiais inicie o crescimento (Dovenuto *et al.,* 2020). Embora os mecanismos pelos quais folículos primordiais específicos são escolhidos para ativação ainda estejam sendo investigados (Pauline *et al.,* 2021), alguns eventos marcantes da ativação folicular já foram descritos. Morfologicamente, os folículos primordiais ativados podem ser identificados pela mudança de forma de suas células da granulosa de escamosa para cuboidal e o pelo aumento do volume do oócito. Além disso, nota-se uma maior quantidade de mitocôndrias e o início da formação da zona pelúcida, sendo essas alterações morfológicas reguladas por eventos moleculares específicos rigidamente controlados (Simon *et al.,* 2020).

Como os receptores de gonadotrofina ainda não estão presentes nos folículos primordiais, o recrutamento inicial parece ser independente da hipófise e controlado por vias biomecânicas, autócrinas e parácrinas (Fiorentino *et al.,* 2023). Evidências recentes têm esclarecido o papel de diversas vias de sinalização na regulação da ativação folicular. A recente identificação da via de sinalização fosfatidilinositol 3 quinase (PI3K) / fosfatase e homólogo de tensina deletado no cromossomo 10 (PTEN) como um controlador-chave para ativação folicular reacendeu o debate da

ativação do folículo primordial como um tópico de pesquisa atual no campo da reprodução (Zhao *et al.,* 2021). Essa via é ativada em resposta a sinais extracelulares (Giaccari *et al.,* 2024; Huang *et al.,* 2024; Monte *et al.,* 2021). Esse processo inicia-se quando um fator de crescimento, como o Kit Ligand (KL), se liga ao receptor transmembranar tirosina quinase (c-Kit). Essa interação desencadeia uma cascata de segundos mensageiros intracelulares, que fosforilam a proteína quinase B (AKT) ativando-a. Uma vez ativada, a AKT inibe o fator de transcrição FOXO3a (Forkhead Box O3) no núcleo, promovendo sua translocação para o citoplasma e, consequentemente, iniciando a ativação folicular. Outro regulador-chave dessa via é a fosfatase homóloga à tensina deletada no cromossomo 10 (PTEN), que atua como uma fosfatase de fosfatidilinositol-trifosfato (PIP3). Como um regulador negativo da função PI3K, PTEN reverte PIP3 de volta para PIP2 suprimindo a sinalização PI3K (Salmena *et al.,* 2008) (Figura 2). A inibição de PTEN em fragmentos de tecido ovariano bovino cultivado *in vitro* foi correlacionada com uma proporção significativamente maior de folículos em crescimento em comparação ao controle (Maidarti *et al.,* 2019).

A via Hippo, altamente conservada em mamíferos, também já foi correlacionada com o crescimento e a ativação folicular. A análise de microarray já identificou a expressão de todos os componentes de sinalização Hippo no ovário bovino (Clarck *et al.,* 2022). A via consiste em uma cascata de cinase que regula a atividade dos ativadores transcricionais YAP (*Yes-associated protein*) com motivo de ligação PDZ (TAZ) (Dos Santos *et al*., 2023). Quando a via de sinalização Hippo é interrompida, ocorre a desfosforilação de YAP e um aumento dos níveis nucleares de YAP. A proteína YAP atua em conjunto com fatores de transcrição TEAD que aumentam os níveis de expressão de fatores de crescimento da família CCN, bem como de fatores inibidores de apoptose – BIRC (*baculoviral inhibitors of apoptosis repeat containing*), estimulando o crescimento celular, sobrevivência e proliferação (Clark *et al.,* 2022; Kawamura *et al.,* 2013).

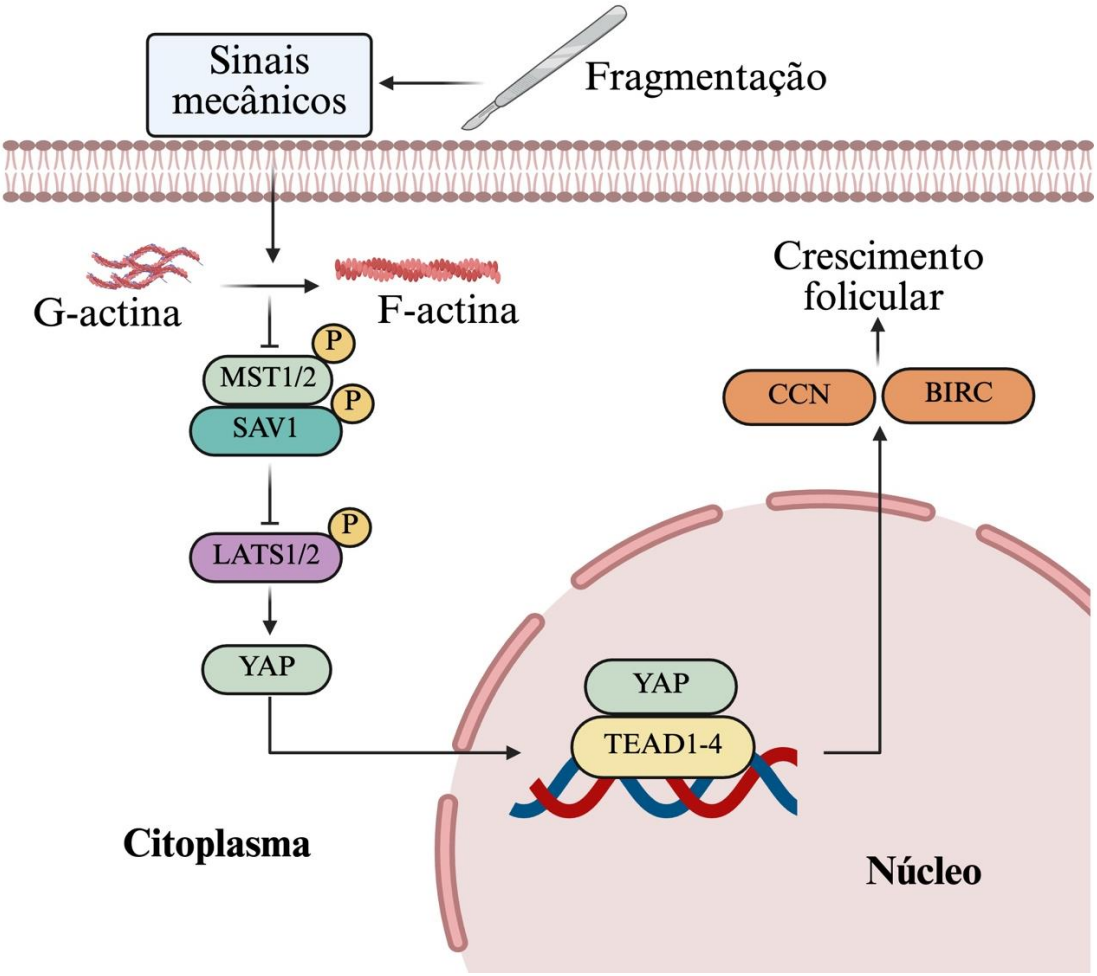

Figura 2. A via Hippo é interrompida por sinalização mecânica. Sinais mecânicos como fragmentação e incisão ovariana, perfuração podem promover a polimerização da actina, interrompendo assim a via Hippo. YAP, proteína associada a Yes; TEAD, domínio associado transcricional aprimorado; CTGF, fator de crescimento do tecido conjuntivo; BIRC, inibidores de apoptose baculoviral contendo repetições; G-actina, actina global; F-actina, actina filamentosa. Fonte: Adaptado de Zhu *et al.* (2023).

Após a ativação, os folículos progridem para o crescimento e uma camada completa de células da granulosa de morfologia cuboide circunda o oócito formando os folículos primários (Sugiura *et al.,* 2023). Os fatores responsáveis pelo desenvolvimento do folículo primário ainda não são totalmente conhecidos. No entanto, sabe-se que o hormônio antimulleriano derivado das células da granulosa e as ativinas são reguladores importantes desse processo (Andrade *et al.,*

2019). Os folículos primários evoluem para folículos secundários por meio do crescimento contínuo do oócito e da proliferação das células da granulosa, formando uma segunda camada e camadas subsequentes. Nesse estágio, outro tipo de célula folicular somática, as células da teca, é recrutado do estroma ovariano para o folículo. As células da teca se organizam em uma camada externa à lâmina basal, envolvendo as células da granulosa e iniciando o processo de vascularização (Figueiredo *et al.,* 2020). Em estágios mais avançados da foliculogênese, essas células desempenham um papel fundamental na produção de hormônios esteroides dentro do folículo. Além disso, a deposição da zona pelúcida (ZP) se intensifica nesse período, e as camadas mais internas das células da granulosa desenvolvem extensões, chamadas projeções transzonais, que atravessam a ZP para otimizar a comunicação direta com o oócito (Andrade *et al.,* 2019).

À medida que o folículo secundário se desenvolve, novas camadas de células da granulosa são adicionadas, e uma cavidade antral preenchida com fluido folicular começa a se formar entre elas. Com o início da fase antral do crescimento folicular, o folículo passa a ser classificado como terciário. No estágio do folículo antral, o oócito fica cercado por células somáticas especializadas, e os folículos são caracterizados por uma cavidade cheia de fluido rico em moléculas reguladoras. Como tal, o fluido folicular representa um ambiente crucial para o crescimento do oócito (Andrade *et al*., 2019; Da Broi *et al.,* 2018). O fluido folicular contém uma série de biomoléculas (hormônios, fatores de crescimento e metabólitos) que dão suporte à maturação do oócito ao participar da comunicação bidirecional oócito-célula somática durante o desenvolvimento do folículo (Pan *et al.,* 2024). O surgimento do antro promove a diferenciação de subpopulações dentro das células da granulosa. Enquanto algumas células permanecem na parede externa do folículo, outras se especializam, formando as células do cúmulo, que envolvem diretamente o oócito e desempenham um papel fundamental em seu desenvolvimento posterior (Andrade *et al.,* 2019). Em bovinos, o desenvolvimento folicular antral segue um padrão de onda, geralmente duas ou três ondas foliculares (Garcia *et al.*, 2014). O surgimento de uma onda folicular ovariana envolve o crescimento sincronizado de múltiplos pequenos folículos antrais (Aerts *et al.,* 2010). Normalmente, quando o maior folículo atinge um diâmetro de aproximadamente 8,5 mm, ocorre um desvio, caracterizado por uma redução na taxa de crescimento de todos os folículos, exceto o dominante (Ginther *et al.,* 2016). A partir deste momento, este folículo é definido como o folículo dominante que progride para a ovulação, enquanto todos os outros folículos são definidos como

subordinados e destinados ao processo natural de atresia folicular (Garcia *et al.*, 2014; Ginther *et al.,* 2016).

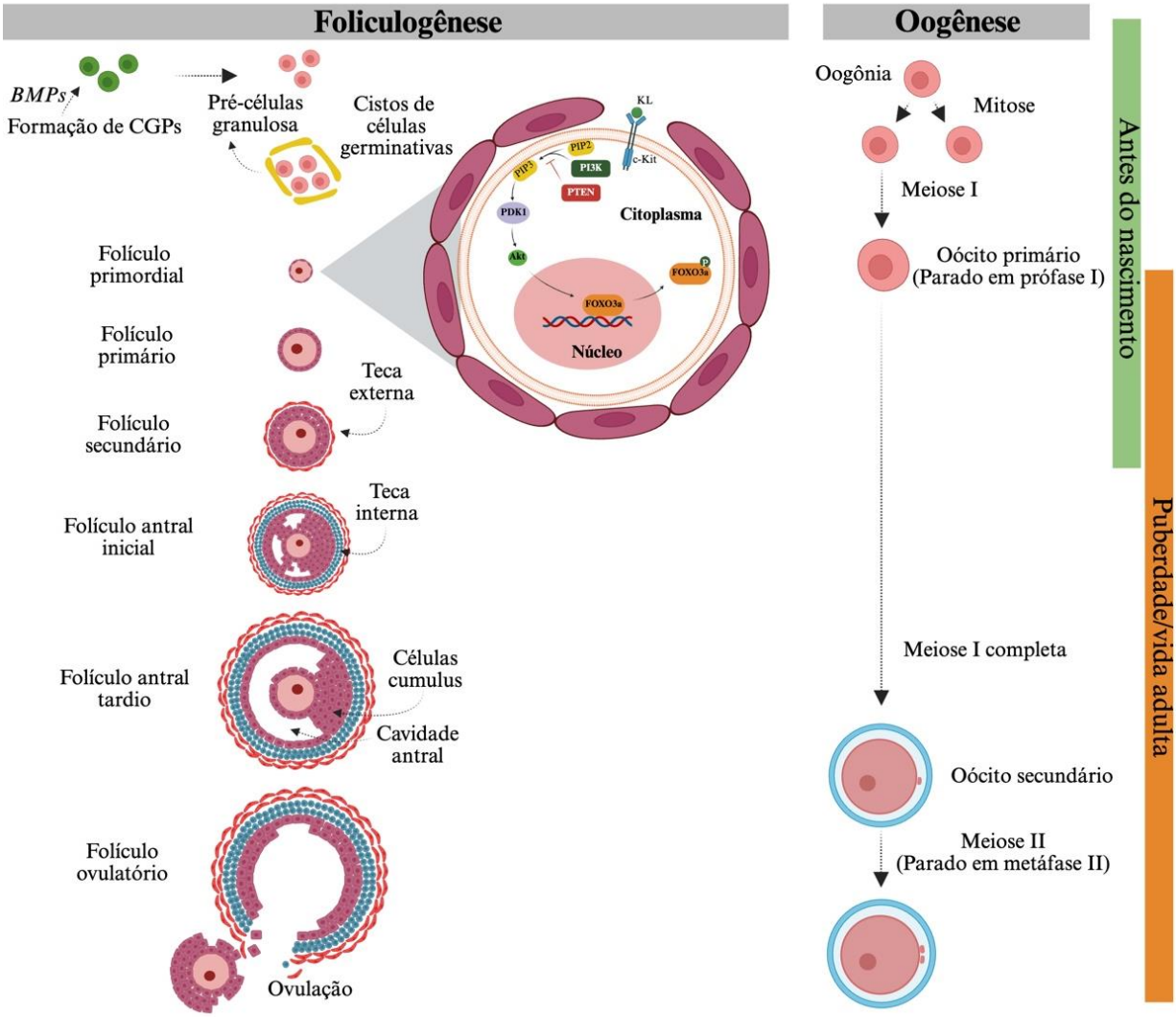

Figura 3. Representação ilustrativa da foliculogênese (à esquerda) e oogênese (á direita) mostrando as características morfológicas dos folículos. No centro, representação esquemática da via de sinalização PI3K/AKT/FOXO3. A ligação do fator KL desencadeia uma cascata de eventos moleculares responsáveis pela regulação da ativação e crescimento dos folículos ovarianos primordiais. Fonte: Elaborada pelo autor.

2.3.3 Mecanossinalização no desenvolvimento folicular

Embora a sinalização hormonal seja conhecida por impactar o desenvolvimento do folículo ovariano, estudos recentes vêm demonstrando que forças mecânicas atuam durante a foliculogênese e sua atividade concertada molda células individuais, tecidos e todo o ovário, representando um conjunto de mecanismos que atua em harmonia com a sinalização endócrina e parácrina, modulando meticulosamente o desenvolvimento folicular (Chan *et al.,* 2021; Shah *et al.,* 2018). Apesar da prevalência, o papel instrutivo potencial da mecânica celular na determinação e padronização do destino das células durante a foliculogênese permaneceu pouco esclarecido durante muito tempo, em parte devido às limitações em traduzir o ambiente mecânico das células em termos moleculares (Wang *et al.,* 2025). A descrição recente dos princípios moduladores da mecânica celular e de como eles impactam na expressão gênica, está, no entanto, esclarecendo o papel desses eventos na biologia ovariana (Grosbois *et al.,* 2023; Piccolo *et al.,* 2022). Em bovinos e outros mamíferos, os processos repetitivos de ativação, crescimento folicular, atresia e ovulação requerem extensa remodelação estrutural da matriz extracelular ovariana (MEC) durante cada ciclo reprodutivo. Esse dinamismo é regulado por mecanismos de síntese e regressão simultâneas dos componentes da MEC sem prosseguir para a degradação completa e fornece os gradientes biomecânicos distintos necessários para a progressão do desenvolvimento folicular (Vasse *et al*., 2025; Parkes *et al.,* 2021; Henning *et al.,* 2021; León-Félix *et al.,* 2025). Nesse processo, as células estromais também parecem desempenhar papeis relevantes na detecção biomecânica. Abir *et al.* (1999) relataram que folículos primordiais humanos isolados não são ativados *in vitro*, mas ativação espontânea foi observada quando os folículos são mantidos dentro do córtex ovariano contendo células estromais. Além disso, Grosbois *et al.* (2023) relataram que a ativação do folículo primordial ocorre concomitantemente com um afrouxamento do córtex ovariano durante a cultura, caracterizado por uma diminuição precoce na densidade de células estromais e acompanhada da remodelação dinâmica da MEC.

Durante a foliculogênese, as células foliculares percebem o seu microambiente por meio de sinais solúveis, mas também por meio de sinais físicos e mecânicos, como o aumento ou diminuição na rigidez da MEC (Grosbois *et al.,* 2023). Córtex e medula correspondem a duas regiões distintas do ovário que diferem em composição da matriz extracelular e consequentemente em propriedades biomecânicas. A MEC do córtex é rica em colágeno e ácido hialurônico, o que o

torna mais rígido, enquanto a medula é menos rica nesses componentes e apresenta MEC mais flexível. Folículos primordiais estão geralmente presentes no córtex rígido. Ao serem ativados e iniciarem o crescimento, os folículos se movem em direção à medula mais macia (Pascoletti *et al.,* 2020; Stewart *et al.,* 2023) (Figura 4). Evidências recentes demonstraram que essa distribuição espacial dos folículos no ovário é crucial para que os folículos permaneçam dormentes ou iniciem o crescimento. A compressão de folículos primordiais na região cortical mantém a localização nuclear de FOXO3a, provavelmente por mecanismos de inibição da via PI3K/Akt. Nagamatsu *et al.* (2019) demonstraram que a degradação da MEC por uma metaloprotease induz a exportação de FOXO3a para o citoplasma induzindo o crescimento folicular. *In vivo,* o processo de neoangiogênese no córtex ovariano durante a ativação folicular aumenta a perfusão vascular o que pode fornecer as metaloproteases que digerem a MEC, favorecendo sua remodelação e o início do crescimento folicular em resposta ao afrouxamento mecânico da matriz no seu entorno (Nagamatsu *et al*., 2019).

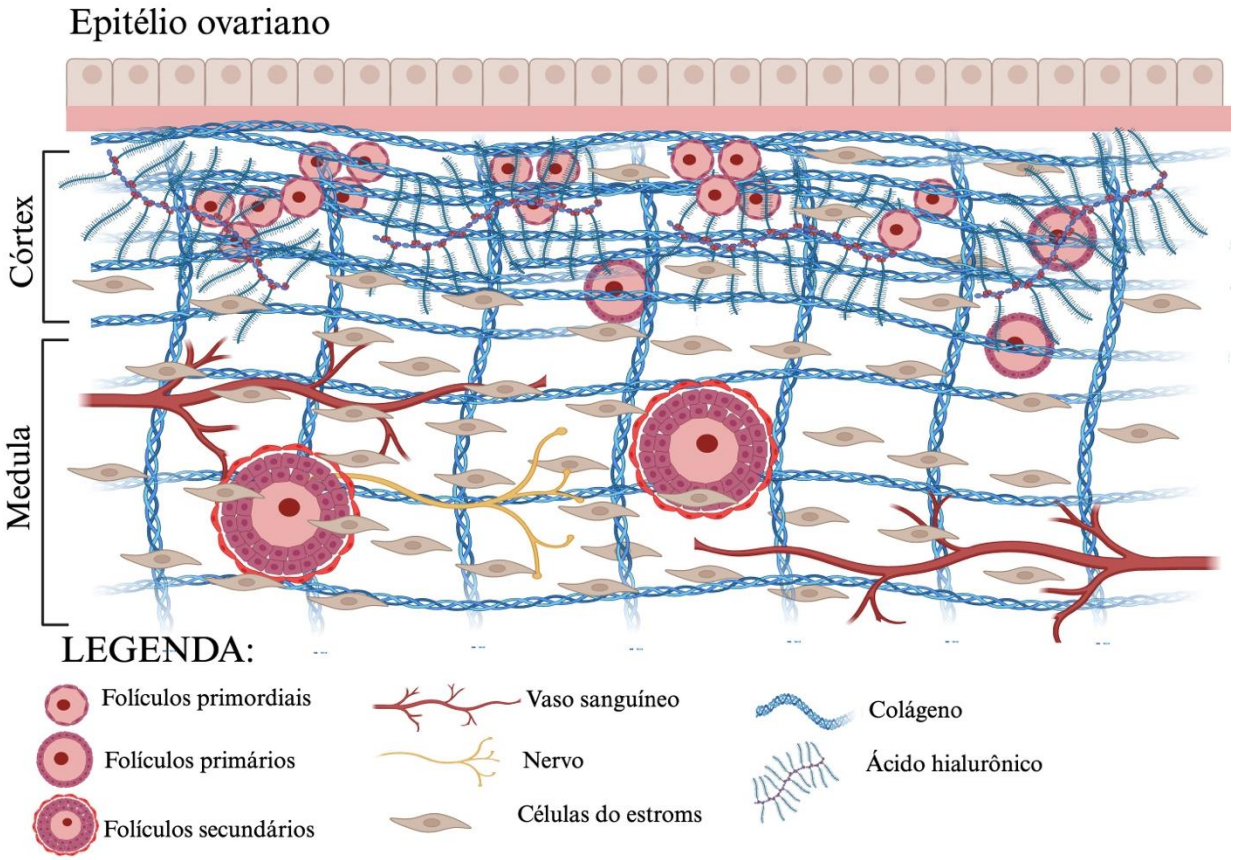

Figura 4. Ilustração da compartimentalização ovariana (córtex e medula) mostrando os principais componentes da matriz extracelular. Os elementos no desenho não estão em escala.
Fonte: Elaborada pelo Autor.

A mecanossensibilização das alterações da rigidez da MEC também pode ser uma entrada causal capaz de modular a via de sinalização Hippo fazendo dessa via um mediador crucial de mecanotransdução dentro do ovário (Zhu *et al*., 2023). Mecanorreceptores de integrina na superfície das células granulosa localizados em ambientes rígidos detectam essa rigidez da MEC e mantém a via Hippo ativada, e, portanto, a quiescência folicular (Xia *et al.,* 2023). Por outro lado, uma diminuição na rigidez ativa uma cascata de mecanotransdução que leva á a polimerização da actina e á inativação da via Hippo com a desfosforilação de YAP e seu consequente aumento no núcleo celular induzindo, em conjunto com fatores de transcrição TEAD, aumento nos níveis de expressão de fatores de crescimento CCN resultando na ativação do folículo primordial (Lunding *et al.,* 2020). Essas constatações são fortemente apoiadas por estudo que mostraram que a fragmentação e ruptura ovariana induzem a polimerização de actina e, consequentemente, à interrupção da via Hippo resultando no recrutamento de folículos primordiais (Kawashima, Kawamura, 2018). YAP e TAZ (coativador transcricional com motivo de ligação PDZ) são, portanto, relés nucleares de sinais mecânicos exercidos pela rigidez da MEC (Vasse et al., 2025). Mudanças na elasticidade da MEC também afetam o citoesqueleto, regulando a expressão de YAP/TAZ. Aragona *et al*. (2013) observaram que, em culturas densas, YAP/TAZ ficam no citoplasma e o crescimento celular cessa, mas o alongamento promove remodelação citoesquelética, ativação nuclear de YAP/TAZ e crescimento celular.

Após a ativação, o crescimento e a sobrevivência dos folículos nos estágios iniciais do desenvolvimento ainda dependem de uma matriz rígida, conforme demonstrado em estudos *in vitro* (Hornick *et al*., 2012). No entanto, com o avanço do desenvolvimento folicular e a aquisição de receptores de gonadotrofina, ele é gradualmente deslocado para a medula mais permissiva (Grosbois *et al.,* 2023; Ouni *et al.,* 2020). A variação na rigidez do ovário parece modular a resposta folicular aos hormônios, desencadeando uma série de processos celulares. Estudos *in vitro* mostram que folículos cultivados em ambientes mais permissivos apresentam maior secreção de estrogênio (Kinnear *et al.,* 2020; West-Farrell *et al.,* 2009; West *et al.,* 2007). A resposta diferencial aos esteroides sexuais pode ser regulada por meio de sinalização mecanicamente responsiva pela

proteína âncora 13 da A-quinase (AKAP13), uma proteína que modula a atividade dos receptores hormonais (Shah *et al.,* 2028; Kino *et al.,* 2010. Esses achados destacam a relação entre a rigidez do ambiente ovariano e a secreção hormonal mediada por sinalização mecânica.

Nos estágios finais da maturação folicular, a MEC desempenha novamente um papel importante na ovulação. À medida que atingem o estágio pré-ovulatório, o pico de LH estimula a produção de plasminogênio pelas células da granulosa que, após sua ativação em plasmina, induz a produção de metaloproteinases de matriz (MMPs) pelas células da teca e a liberação na MEC circundante promovendo a degradação dos componentes da MEC. Esse evento desencadeia a liberação do ligante do fator de necrose tumoral alfa (TNFα) que promove a apoptose das células epiteliais superficiais apicais, facilitando a ruptura folicular e a liberação do oócito (Fiorentino *et al.,* 2023).

2.3.4 Atresia folicular

A foliculogênese, processo contínuo e rigorosamente regulado ao longo da vida reprodutiva dos mamíferos, é marcada por uma redundância significativa, onde o número de folículos recrutados para o crescimento é consideravelmente maior do que o número de folículos que finalmente ovulam (Wu *et al*., 2024; Zang *et al.,* 2024). Notavelmente, mais de 99% desses folículos ativados sofrerá atresia, que é um processo natural complexo que atua como um regulador intrínseco da reserva folicular, influenciando assim o desenvolvimento folicular e abrangendo diferentes mecanismos de morte celular regulada (Xi *et al.,* 2025; Scudieri *et al.,* 2024; Chesnokov *et al.,* 2024). Como a maioria dos folículos é eliminada antes da ovulação, a porcentagem de aproveitamento dos folículos é extremamente baixa em ovários mamíferos (Zaniker *et al.,* 2024). Embora a reserva ovariana possa variar amplamente entre animais individuais, ao nascimento, vacas têm aproximadamente 235.000 folículos em seus ovários. No entanto, ao longo da vida reprodutiva do animal, menos de 1% deles serão selecionados e se desenvolverão em folículos maduros até a ovulação, enquanto os restantes sofrerão atresia (Costa *et al.,* 2023).

Embora os mecanismos que regulam a atresia folicular ainda não sejam completamente compreendidos, muitos processos e reguladores críticos já foram descritos (Song *et al*., 2025). Mudanças no ambiente folicular desempenham um papel crucial na regulação do processo de atresia folicular. Alterações nos níveis hormonais, como a redução nos níveis do hormônio folículo-

estimulante (FSH) e a diminuição da amplitude dos pulsos do hormônio luteinizante (LH), podem comprometer o desenvolvimento e a sobrevivência dos folículos (Wu *et al*., 2022; Chu *et al.,* 2018). Além disso, a redução na disponibilidade de nutrientes e no suprimento sanguíneo pode enfraquecer o microambiente folicular, prejudicando sua capacidade de sustentar processos celulares essenciais (Li *et al.,* 2021; Li *et al.,* 2022). Essas alterações ambientais aceleram coletivamente a atresia folicular, promovendo a degeneração dos folículos não dominantes e contribuindo para o declínio da reserva ovariana (Wu *et al.,* 2024).

A atresia folicular está intimamente relacionada à apoptose de oócitos e células da granulosa podendo ocorrer em qualquer fase do desenvolvimento folicular (Ying *et al.,* 2024). Ao contrário dos folículos primordias, nos quais o ponto focal da atresia é o oócito, o ponto focal da atresia nos folículos em crescimento é a morte das células da granulosa que é regulada por um equilíbrio delicado entre as atividades das vias pró e antiapoptóticas (Liu *et al.,* 2016; Regan *et al.,* 2018). Diversas vias de sinalização, como a proteína quinase A (PKA), proteína quinase B (PKB/AKT), quinase 1 e 2 regulada por sinal extracelular (ERK1/2), proteína quinase ativada por mitógeno (p38-MAPK), EGF e BMPs, atuam na inibição da apoptose das células da granulosa, regulando mais de 100 genes-alvo (Shen *et al.,* 2014) Em contrapartida, a supressão da via PKB/AKT promove a translocação nuclear do fator de transcrição *forkhead box* (FoxO), ativando genes pró-apoptóticos como Bim, TRAIL e FasL, o que desencadeia a apoptose das células da granulosa e a atresia folicular (Yang *et al.*, 2024). Além disso, o aumento do estresse oxidativo dentro do folículo frequentemente resulta em apoptose das células da granulosa e do oócito (Liu *et al.,* 2023; Shen *et al.,* 2016). Além da apoptose, recentemente o papel da autofagia, um mecanismo diferente de morte celular programada, foi demonstrado na atresia folicular (Scudieri *et al.,* 2024). Embora a autofagia normal possa desempenhar um papel autoprotetor durante o desenvolvimento folicular, mantendo a homeostase mitocondrial (Choi *et al.,* 2024), quando excessiva pode induzir atresia folicular (Zhou *et al.,* 2019; Zhou *et al.,* 2017).

2.4 Cultivo *in vitro* de folículos ovarianos pré-antrais

O equilíbrio entre a oogênese e a atresia ainda durante a vida fetal determina o número de gametas contidos nos ovários de mamíferos ao nascimento e sua disponibilidade subsequente. Após o nascimento, a atresia folicular persiste como um fenômeno fisiológico constante e irreversível

que limita consideravelmente a reserva de gametas ao longo da vida reprodutiva da fêmea. Curiosamente, a amplitude das taxas de atresia varia consideravelmente dependendo do estágio folicular. Em geral, a incidência de atresia folicular em bovinos aumenta drasticamente além do estágio do folículo secundário em até 60% nos folículos antrais médios e aumenta ainda mais à medida que o folículo progride no crescimento (Dey *et al*., 2024). Como, em geral, apenas um folículo se desenvolve até o estágio ovulatório por ciclo, o destino da maioria (99%) dos folículos antrais em mamíferos é a atresia (Costa *et al*., 2023). Assim, a reserva ovariana dos folículos pré-antrais representa uma fonte de gametas abundante, em comparação aos folículos antrais, disponíveis para serem explorados visando aumentar a eficiência reprodutiva em espécies com impacto econômico relevante, como os bovinos. Portanto, o aprimoramento de biotécnicas reprodutivas capazes de explorar a reserva folicular pré-antral ainda não extensivamente afetada pela atresia é um campo de pesquisa atual em pleno desenvolvimento com potencial capaz de oportunizar o uso ideal da reserva folicular em mamíferos, revolucionando os procedimentos atuais de criação animal (Barbato *et al.,* 2023).

Dentre as estratégias biotecnológicas que estão sendo exploradas atualmente, o cultivo *in vitro* de folículos ovariano pré-antrais vem concentrando esforços de pesquisa consideráveis. Esta técnica consiste no resgate dos folículos pré-antrais do ovário e o posterior cultivo *in vitro* desses folículos para que progridam no crescimento, funcionando, portanto, como um ovário artificial potencialmente capaz de recapitular a foliculogênese fora do corpo (Figueiredo *et al*., 2020). Considerando a necessidade de mimetizar *in vitro* o microambiente único do folículo ovariano, diferentes sistemas de cultivo estão sob investigação (Simon *et al.,* 2020). Recapitular a foliculogênese *in vitro* requer, inicialmente, que os folículos primordiais dormentes sejam ativados para que progridam através do estágio primário e secundário inicial (Barbato *et al*., 2023). Após tentativas frustradas de cultivar folículos primordiais isolados (Oktay *et al.,* 1997; Hovatta *et al.,* 1997) estabeleceu-se o consenso de que a progressão dos folículos primordiais até o estágio secundário requer interações com o estroma ovariano e, portanto, desenvolvê-los *in vitro* só é possível mediante o cultivo do córtex ovariano intacto. A partir do estágio secundário, no entanto, os folículos podem ser removidos do tecido ovariano e cultivados isoladamente.

2.4.1 Cultivo in vitro de folículos pré-antrais inclusos no tecido ovariano

O cultivo do córtex ovariano vem sendo desenvolvido em várias espécies de animais de produção (caprinos: Sousa *et al.,* 2021; ovinos: Monte *et al.,* 2021; bovinos: Silva *et al*., 2024, Caetano Filho *et al.,* 2024, Bezerra *et al.,* 2024) e é uma promessa significativa para vários procedimentos reprodutivos (Chavoshinezhad *et al.,* 2024). Embora o cultivo de fragmentos corticais remova os folículos da influência endócrina e parácrina *in vivo*, esse sistema ainda é considerado promissor por sua maior proximidade fisiológica com o ambiente de crescimento *in vivo*, proporcionando melhor capacidade de simulação das interações foliculares entre si e com o seu microambiente circundante representado pelas células estromais e elementos da matriz extracelular ovariana como colágeno e glicosaminoglicanos o que parece acelerar o início do desenvolvimento folicular. Além disso, é um método relativamente mais fácil de executar e minimiza a exposição prolongada das células ao ambiente externo (Chen *et al.,* 2024; Ghezelayagh *et al.,* 2022).

O modelo mais amplamente usado para a cultura de fragmentos de tecido cortical ovariano ainda é a placa de cultivo convencional. Nessas placas, os fragmentos de tecido são cultivados sob uma camada estática de meio de cultivo em uma atmosfera gasosa contendo oxigênio (O_2) e dióxido de carbono (CO_2) (Catapano *et al.,* 2019). Essencialmente em grandes mamíferos, o cultivo do córtex ovariano usando sistemas estáticos vem fornecendo importantes *insights* sobre os fatores e mecanismos que regulam a ativação dos folículos primordiais quiescentes abrindo novas perspectivas para o uso conjunto de folículos primordiais em tecnologias de reprodução assistida (Silva *et al*., 2024). Diversos estudos vêm esclarecendo o efeito de diferentes fatores sobre o crescimento de folículos primordiais. Na espécie bovina, alguns estudos *in vitro* demonstraram que o timol (Caetano filho *et al.* 2024; punicalagina (Bezerra *et al.,* 2024), melatonina (Silva *et al.,* 2024; Cavalcante *et al*., 2019), FSH (Ribeiro *et al.,* 2015) e ativina A (McLaughlin *et al*., 2020) podem influenciar as taxas de ativação folicular no tecido ovariano cultivado *in vitro*. Além disso, os inibidores de fosfatase e homólogo de tensina (PTEN) e ativadores de PI3K/Akt são conhecidos por favorecer a ativação folicular *in vitro* (Grosbois *et al*., 2018; Kawamura *et al.,* 2013), embora possam comprometer o desenvolvimento folicular em bovinos (Maidarti *et al.,* 2019).

Além da influência de diferentes fatores, a compreensão atual de que estímulos biomecânicos influenciam a foliculogênese por interações bidirecionais coordenadas (Matsuzaki,

2021) vem destacando o possível papel dos estímulos biomecânicos na ativação e crescimento de folículos primordiais dormentes durante o cultivo *in vitro* de tecido ovariano. Observações experimentais recentes introduziram novos conceitos na biologia do folículo que vêm desafiando a premissa de que fatores endócrinos e parácrinos são os únicos controladores do desenvolvimento do folículo. Grosbois *et al*. (2023) investigaram os efeitos do alongamento do córtex ovariano *in vitro*, observando afrouxamento e remodelação da MEC, com redução do colágeno na área cortical externa. Nos primeiros dias de cultivo, a foliculogênese foi ativada, reduzindo folículos primordiais e aumentando os em desenvolvimento. Os autores sugerem que o alongamento da MEC pode mecanorregular o desenvolvimento folicular, aumentando a difusão de fatores de crescimento e oxigênio. No entanto, a remodelação pode estar associada à interrupção das vias PI3K-Akt e Hippo (Vasse *et al.,* 2025). Em bovinos, Pascoletti *et al.* (2020) sugerem que, devido à alta compactação do córtex ovariano e à presença de folículos primordiais que expressam fatores inibitórios do crescimento folicular, como a AMH, o alongamento do tecido pode reduzir a densidade das fibras colágenas estromais, enfraquecendo suas interações. Esse processo pode diminuir o efeito inibitório entre folículos próximos, aumentando a distância entre eles e potencialmente influenciando sua ativação.

Embora o cultivo de fragmentos corticais em placas de cultivo estático seja o método mais amplamente utilizado para bovinos, biorreatores dinâmicos também já foram utilizados com esse propósito. No estudo de Fragomeni *et al.* (2024), tecido ovariano bovino foi cultivado em um biorreator de polipropileno citocompatível que preservou a viabilidade do folículo e promoveu melhor desenvolvimento folicular quando comparado à cultura estática. Biorreatores também já foram utilizados para cultivar o ovário bovino inteiro, embora estudos com esse propósito ainda sejam escassos. Para isso, um biorreator dinâmico foi desenvolvido para fornecer meio de cultivo para o ovário durante 48 horas através do isolamento de um ramo da artéria ovariana principal. Embora folículos e oócitos não tenham sido avaliados diretamente, esse método de cultivo demonstrou manter a viabilidade celular com menores taxas de apoptose no tecido ovariano (Zanotelli *et al*., 2016). De modo geral, biorreatores vêm sendo desenvolvidos para superar as atuais limitações associadas ao cultivo estático como, por exemplo, a ausência de suprimento contínuo de oxigênio e nutrientes e a não remoção contínua de metabólitos residuais (Barbato *et al.,* 2023). No entanto, resultados inconsistentes demandam por mais estudos sobre a eficácia desses sistemas (Catapano *et al.,* 2019).

2.4.2 Cultivo in vitro de folículos pré-antrais isolados

Considerando que os folículos pré-antrais compreendem uma ampla gama de estágios foliculares e categorias biológicas com diferentes requisitos de crescimento, é essencial que o sistema de cultivo possa ser ajustado de forma específica para garantir sua sobrevivência em cada etapa, além de fornecer uma oportunidade para investigar os processos biológicos que governam a foliculogênese individual de cada categoria folicular, bem como permitir investigar a resposta individual do folículo frente a medicamentos e produtos químicos diversos (Dey *et al.,* 2024; Babayev *et al*., 2022; Taghizabet *et al.,* 2022). Para isso, o cultivo de folículos pré-antrais isolados, especialmente folículos secundários, vem sendo amplamente investigado na espécie bovina (Jachter *et al.,* 2022; Paulino *et al.,* 2022; Azevedo *et al.,* 2022). Protocolos dessa natureza incluem uma sequencia coordenada de etapas e a configuração de um protocolo otimizado requer controle rigoroso de todas as variáveis.

O primeiro passo para cultivar folículos isoladamente é a separação do folículo do tecido ovariano e, idealmente, a técnica utilizada deve fornecer um equilíbrio perfeito em termos de quantidade e qualidade (Chiti *et al.,* 2017). Os métodos de isolamento mais amplamente aplicados incluem o isolamento enzimático, o mecânico, ou uma combinação dos dois (Babayev *et al.,* 2022). O método enzimático envolve a digestão proteolítica do estroma ovariano usando proteases como colagenase e liberasse com os protocolos, variando em relação às enzimas específicas utilizadas, sua concentração e a duração do tratamento (Simon *et al.,* 2020). Por esse método, o número de folículos obtidos é normalmente maior em comparação com métodos mecânicos e requerem menos tempo. No entanto, os folículos são mais propensos a serem danificados pela atividade das enzimas utilizadas (Taghizabet *et al.,* 2022). No método de separação mecânica, utilizam-se agulhas especiais para isolar os folículos do estroma ovariano (Paulino *et al.,* 2022; Azevedo *et al.,* 2022), além de moedores de tecido, homogeneizadores e filtros de células. Esse método causa menos danos aos folículos em comparação com a separação enzimática, preservando melhor a camada da teca e a morfologia folicular. No entanto, sua principal desvantagem é o tempo prolongado necessário para o processo. Uma combinação dos métodos mecânicos e enzimáticos também pode ser aplicada. Para isso, uma curta etapa de digestão enzimática é utilizada seguida de separação mecânica objetivando manter a estrutura folicular e maximizar o número de folículos obtidos (Babayev *et al.,* 2022). Em todos os casos, o rendimento dos protocolos é variável, sendo

influenciado pela idade do animal, volume de tecido disponível e densidade folicular individual (Dey *et al.*, 2024).

A escolha do meio base, por exemplo, α-MEM, TCM199, McCoy's, volume do meio e seus componentes é o próximo passo e deve ser uma decisão precisamente calculada. A composição e o volume do meio demonstraram afetar diretamente a morfologia e a morfometria de folículos pré-antrais cultivados no modelo bovino (Jimenez *et al.,* 2016). Em humanos foi demonstrado que o baixo volume do meio apoiou a progressão folicular (Bjarkadottir *et al.,* 2021). Além disso, a suplementação do meio base é crucial e o efeitos de hormônios, fatores de crescimento e produtos naturais já foi extensivamente investigada na espécie bovina. Hormônio folículo estimulante (FSH) (Passos *et al.,* 2013; Vasconcelos *et al.,* 2013), fator de necrose tumoral alfa (TNF- α) (Paulino *et al.,* 2018), fator de crescimento epidérmico (EGF) (Paulino *et al.,* 2020); proteínas morfogenéticas ósseas 2, 4 e 15 (Cunha *et al* 2028; Passos *et al.,* 2013), fator de crescimento endotelial vascular (VEGF) (Araújo *et al.,* 2014), fator de diferenciação do crescimento-9 (GDF9) (Vasconcelos *et al.,* 2013), ativina (McLaughlin *et al.,* 2010), extrato de *aloe vera* (Azevedo *et al.,* 2022); eugenol (Vasconcelos *et al.,* 2021) foram relatados como promotores do crescimento, formação de antro, maiores taxas de viabilidade, menores taxas de extrusão folicular e melhora do estresse oxidativo em folículos secundários bovinos cultivados *in vitro*.

Atualmente, a maioria das investigações no campo do cultivo de folículos isolados é baseada em sistemas bidimensionais estáticos (2D), com troca manual de meio a cada 24 a 72 horas (Malo *et al*., 2024). Sistemas 2D convencionais incluem o método de gotícula onde os folículos são cultivados inseridos em gotas de meio de cultivo cobertas por óleo mineral (Paulino *et al.,* 2018, Paulino *et al.,* 2020; Nascimento *et al.,* 2022*.,* Azevedo *et al.,* 2022). Embora resultados promissores com esses sistemas tenham sido obtidos, tem sido relatado que o cultivo 2D convencional falha em manter a estrutura esférica do folículo, interrompendo as interações celulares entre o oócito e as células da granulosa, o que compromete o desenvolvimento folicular *in vitro* (Malo *et al.,* 2024). Além disso, uma vez isolados do ovário, os folículos perdem a estrutura de suporte natural promovido pelo tecido ovariano adjacente, interrompendo os mecanismos de mecanossinalização cruciais para o desenvolvimento folicular (Wang *et al.,* 2025). Para superar esses desafios, sistemas de cultivo tridimensionais (3D) vêm sendo desenvolvidos visando criar um nicho artificial para o cultivo de folículos. Esses sistemas envolvem a incorporação de folículos

individuais ou em grupos em biomateriais, permitindo o monitoramento de sua progressão. Entre os biomateriais naturais utilizados para a cultura folicular, destacam-se proteínas como fibrina e colágeno, além de moléculas de polissacarídeos, como hidrogel e alginato e resultados promissores têm sido relatados (Dey *et al.,* 2024; Malo *et al.,* 2024; Zheng *et al.,* 2023; Joo *et al.,* 2016). Como nenhum desses materiais demonstrou a habilidade de replicar todas as funções do microambiente nativo, o uso de matriz extracelular ovariana descelularizada (DMEC) tem sido apresentada como material de escolha para essa função (León-Félix *et al*., 2024; Amjadi *et al.,* 2022; Nikniaz *et al.,* 2021; Franko *et al.,* 2024). No entanto, se andaimes 3D derivados de DMEC fornecem um microambiente funcional para o cultivo *in vitro* de células ovariana ainda é controverso demandando investigações adicionais.

2.5 Capítulo 1

Advances in the application of decellularized ovarian scaffolds as biomimetic platforms for follicular development: a systematic review

[Avanços na aplicação de andaimes ovarianos descelularizados como plataformas biomiméticas para o desenvolvimento folicular: uma revisão sistemática]

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Advances in the application of decellularized ovarian scaffolds as biomimetic platforms for follicular development: a systematic review

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Abstract

Decellularized ovarian tissue is currently being investigated as a promising platform potentially capable of providing a biomimetic microenvironment that supports follicular development and advances artificial ovary technologies. This systematic review aims to critically evaluate the effectiveness of decellularized ovarian scaffolds in supporting the survival and development of ovarian follicles, ovarian cells, and stem cells. Following PRISMA guidelines, we conducted a comprehensive literature search focusing on *in vivo* and *in vitro* studies that employed decellularized ovarian tissue as a bioscaffold to support the development of ovarian cells, stem cells, or ovarian follicles. This study highlights that the use of decellularized ovarian tissue as a biomimetic scaffold supports key biological processes such as cellular adhesion, migration, differentiation, and survival. The formation of follicle-like structures and the expression of granulosa, theca, and oocyte-specific markers were also demonstrated. Moreover, *in vivo* transplantation of recellularized scaffolds restored hormonal levels and promoted organized follicular architecture. In conclusion, decellularized ovarian scaffolds represent a biologically active platform capable of mimicking the native ovarian microenvironment and guiding follicular organization and function.

Keywords: Decellularized ovarian tissue; Artificial ovary; 3D follicle culture; *Ex vivo* folliculogenesis

1 Introduction

The development of *ex vivo* models that support follicular growth under controlled conditions plays a key role to improve the strategies to utilize the ovarian follicle reserve in animals and humans. In animals, the models are primarily aimed at financial gains or biomedical research (Di Berardino *et al.,* 2024). In humans, growing attention has been directed toward artificial reconstructed ovaries as emerging strategies for fertility preservation in young cancer patients or individuals with ovarian failure (Sistani *et al.,* 2024). By recreating a biomimetic microenvironment, these systems can support *in vitro* follicular growth and oocyte maturation enabling the subsequent transplantation of the engineered constructs for fertility restoration while minimizing the risk of malignant cell reintroduction associated with ovarian tissue transplantation. Additionally, they serve as valuable platforms for studying folliculogenesis (Chavoshinezhad *et al.,* 2024; Malo *et al.,* 2024; Wu *et al.,* 2023; Figueiredo *et al.,* 2018).

Despite advancements in platforms designed to replicate follicle development across various animal models, effectively managing folliculogenesis *in vitro* remains a major challenge (Hatekar *et al.,* 2025; Vitale *et al.,* 2024). The limited outcomes achieved thus far underscore the inherent complexity of replicating the *in vivo* follicular environment under *in vitro* conditions, encouraging multidisciplinary approaches to drive further advances in the field. Recent studies have demonstrated that the regulation of follicle development involves mechanisms extending beyond the follicular unit itself (Vasse *et al.,* 2025; Grosbois *et al.,* 2023; Parkes *et al.,* 2021). Thus, although the most commonly used two-dimensional culture models have provided valuable insights into aspects of reproductive biology, their static and oversimplified nature limits their ability to reproduce the dynamic and complex ovarian features (Martinez *et al.,* 2025; Francés-Herrero *et al.,* 2022; Dehghani-Ghobadi *et al.,* 2022). These limitations have been stimulating research strategies focused on the development of more dynamic and physiologically relevant platforms for *in vitro* follicular growth (Kim *et al.,* 2025; Chavoshinezhad *et al.,* 2024)

In an attempt to mimic the complex *in vivo* microenvironment, three-dimensional (3D) artificial ovaries have been proposed to encapsulate and preserve follicular spherical architecture, an essential feature for maintaining cell development (Nair *et al.,* 2024; Healy *et al.,* 2021; Wu *et al.,* 2022). To achieve this, searching for the ideal scaffold to support follicle development, and different kinds of 3D matrixes is gained attention (Nikniaz *et al.,* 2021). Different biomaterials can serve as structural supports for follicular development, such as fibrin and/or alginate-based matrices, three-dimensional (3D) printed cross-linked gelatin fibers, and polyethylene glycol (Xiang *et al.,* 2021; Vanacker and Amorim, 2017; Laronda *et al.,* 2015). However, despite substantial technological advancements and progress in polymer science, the majority of artificial scaffolds still fail to meet the requirements for a bioactive support capable of providing both cell-instructive and cell-responsive functions. As such, they represent a limited alternative for application in larger mammals, which require a highly dynamic microenvironment for proper follicular development (Alshaikh *et al.,* 2020). In response to this limitation, ovarian tissue bioengineering is unlocking new frontiers in the study of female reproduction *in vitro*, driving the development of sophisticated 3D models that may more accurately mimic the ovary complex architecture and functionality, thereby advancing scientific discovery in the field.

In recent years, extracellular matrix (ECM)-based platforms derived from decellularized tissues have garnered considerable attention in ovarian research (Swinehart *et al.,* 2016). Once stripped of cellular components, decellularized ECM (dECM) primarily consists of water-insoluble structural proteins, such as collagens, laminins, and elastins, but also retains appreciable levels of endogenous bioactive molecules, including growth factors (Theocharis *et al.,* 2016; Jakus *et al.,* 2017). Furthermore, the decellularization process eliminates immunogenic components, enabling the *in vivo* assessment of ovarian follicles and cells seeded *in vitro* through xenotransplantation into animal models, such as mice. Additionally, dECM scaffolds have been reported to direct stem cell differentiation toward ovarian lineages (Sistani *et al.,* 2021; Alshaikh *et al.,* 2020). Thus, dECM scaffolds form a structurally supportive network embedded with biomolecular cues that finely regulate cellular behavior (Kolliopoulos *et al.,* 2025; Hoshiba, 2021). However, although this strategy has been increasingly discussed in female reproductive bioengineering, fueling growing interest in the development of a functional artificial ovary capable of supporting *ex vivo* follicular development, the application of decellularized tissues as scaffolds for follicle and ovarian cell development is preliminary. We hypothesize that decellularized ovarian scaffolds provide a

biomimetic environment that guides the organization and functional development of follicles, ovarian cells, and stem cells seeded onto the scaffold.

This systematic review aims to compile recent findings on the effectiveness of decellularized extracellular matrix-derived ovarian scaffolds in supporting the survival of seeded follicles, ovarian cells, or stem cells, offering valuable insights to guide future research toward the development of physiologically relevant artificial ovarian models.

2 Methodology

2.1 Sources of information and search strategy

This systematic review followed the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines to ensure methodological rigor, clarity, and reproducibility (Page *et al.,* 2021). The research question to guide this review was: "Can scaffolds derived from decellularized ovarian tissue provide a biomimetic microenvironment that supports the survival and development of ovarian cells or stem cells, thereby contributing to the design of a functional artificial ovary?" All data analyzed in this review were obtained from previously published studies available in international peer-reviewed journals. A systematic search was conducted in the MEDLINE (PubMed), EMBASE, and Web of Science electronic medical databases on March 22, 2025, using a search strategy based on the following concepts combined with Boolean operators: 'decellularized ovary', decellularized ovarian tissue', 'ovarian decellularization', 'ovarian recellularization', 'ovarian tissue decellularization', 'decellularized ovarian scaffold', 'ovarian scaffold', 'ovarian decellularized bioscaffold', 'ovarian tissue scaffold', ovarian bioscaffold'. Medical Subject Heading – MeSH on PubMed, Emtree terms on EMBASE and synonymous were used to outspread the search. The database-specific search strategies are detailed in Supplementary Table S1. All studies considered relevant and published up to March 2025, with full-text availability and indexing, were included in this systematic review. The search strategy had no chronological restrictions. Additionally, a manual screening of the reference lists from the selected articles was conducted to identify further studies not captured by the electronic database search.

2.2 Eligibility criteria

The eligibility criteria for article selection in this study were defined based on the PICO framework (Table 1). The following studies were deemed not relevant and therefore excluded: (i) non-original articles (e.g., literature reviews, editorials, letters, notes, and conference abstracts); (ii) studies without accessible full text; (iii) research not involving ovarian tissue as the source of the decellularized scaffold.; and (iv) studies that evaluated only the decellularization method without seeding ovarian follicles, ovarian cells or stem cells. The exclusion criteria were applied to both electronic database searches and manual screening

Table 1. Eligibility criteria for article selection based on the PICO framework (Population, Intervention, Comparator, and Outcome).

PICO structure	
Population (P)	Ovarian follicles, ovarian cells, or stem cells from humans or animals.
Intervention (I)	Reseeding of ovarian follicles, ovarian cells (including stromal, granulosa, and theca cells), or stem cells into scaffolds derived from decellularized extracellular matrix, aiming to develop an artificial ovary.
Comparator (C)	Development of ovarian follicles, ovarian cells, or stem cells in models that do not involve recellularized scaffolds, such as two-dimensional cultures or, in the case of *in vivo* assessments, xenotransplantation of dECM not repopulated with cells or simulated surgeries.
Outcome (O)	Effects of dECM scaffolds on cell and follicle survival, growth, oocyte maturation, steroid hormone production, and the expression of genes related to folliculogenesis, endocrine function, and apoptosis.

2.3 Study selection and data extraction

A comprehensive two-stage approach was employed to identify the relevant studies. Initially, titles and abstracts were independently screened by Costa, F.D.C, and Silva, B.R. based

on predefined inclusion and exclusion criteria. Using Rayyan© software (https://rayyan.qcri.org), studies were categorized as "included", "excluded", or "maybe" after duplicate records were removed. The exclusion criteria at this stage included: ‘off-topic’: referring to studies addressing unrelated subjects; and ‘wrong publication’: applied when the recovered works were reviews, abstracts, editorials, letters, notes, or conference abstracts. Articles that met the initial screening criteria proceeded to a full-text review. In this second stage, articles were categorized as ‘included in review’ if they met all inclusion criteria (Table 1) or excluded based on: ‘wrong population’, when cells other than ovarian follicles, ovarian cells, or stem cells were used; ‘wrong study design’ when seeding of follicles, ovarian cells or stem cells has not been performed; and ‘wrong result’, when studies did not report outcomes related to follicle or cells survival, growth, oocyte maturation, steroid hormone production, or expression of genes related to folliculogenesis, endocrine function, and apoptosis. Any discrepancies during either stage were resolved through discussion and consultation with Silva, J.R.V. A standardized data extraction form was employed to ensure consistency and minimize errors. Information was retrieved from the Results and Methods sections, as well as from tables, graphs, and figures. The extracted data included study characteristics (first author, year of publication), study design, and intervention-related details such as animal species, type of intervention, exposure duration, control group, and primary outcomes.

2.4 Quality assessment

For the studies involving *in vivo* models, two independent reviewers (Costa, F.D.C. and Silva, B.R.) assessed study quality using the SYRCLE risk of bias tool, which is adapted from the Cochrane Collaboration’s Risk of Bias guidelines and tailored for animal research (Hooijmans *et al.,* 2014). SYRCLES`s risk of bias tool assesses ten domains: (1) sequence generation; (2) baseline characteristics; (3) allocation concealment; (4) random housing; (5) binding of investigator; (6) random outcome assessment; (7) blinding of outcome assessor; (8) incomplete outcome data; (9) selective outcome data; and (10) other sources of bias. ‘Yes’, ‘no’, ‘unclear’ represent low, high, and uncertain risk of bias, respectively (Supplementary Table S2). To the best of our knowledge, no specific checklist for assessing the risk of bias in *in vitro* studies has been validated (Almeida *et al.,* 2021). For the *in vitro* studies included in this review, we adapted quality assessment criteria based on the SciRAP (http://www.scirap.org/) and Parsifal (https://parsif.al) platforms

(Vasconcelos *et al.,* 2024; Waspe *et al.,* 2021). SciRAP provides structured criteria for evaluating methodological quality, while Parsifal assists in designing and conducting systematic reviews, allowing the creation of tailored questions in line with the study objectives. The questions used to evaluate each article were adapted from SciRAP with minor modifications and are presented in Supplementary Table S3. Each study received an overall score based on the evaluation of individual criteria, following the SciRAP rating system: "fulfilled" (score 1), "partially fulfilled" (score 0.5), and "not fulfilled" (score 0). The maximum achievable score for each article was 17 calculated based on the number of questions and on the answer of greater weight. Scores were then converted into percentages. In accordance with the classification proposed by Almeida *et al.* (2023), studies were categorized into Klimisch reliability levels as follows: "reliable without restrictions" (score above 80%), "reliable with restrictions" (score above 60%), and "unreliable" (score below 60%).

3 Results

3.1 Study identification and selection

A total of 1,763 studies were retrieved from three databases: Embase (n = 467), PubMed (n = 507), and Web of Science (n = 789). After removing 780 duplicates, the remaining studies underwent eligibility screening. Based on title and abstract evaluation, 700 records were excluded for being unrelated to the research topic (Offtopic), and 248 were excluded for being reviews, abstracts, editorials, letters, notes, or congress summaries (Wrong publication). Consequently, 35 studies underwent to full-text review, of which 17 were classified as “wrong study design”, and 5 presented “wrong population” being then excluded. Therefore, 13 studies were included in the final analysis. The detailed PRISMA flow diagram is presented in Figure 1.

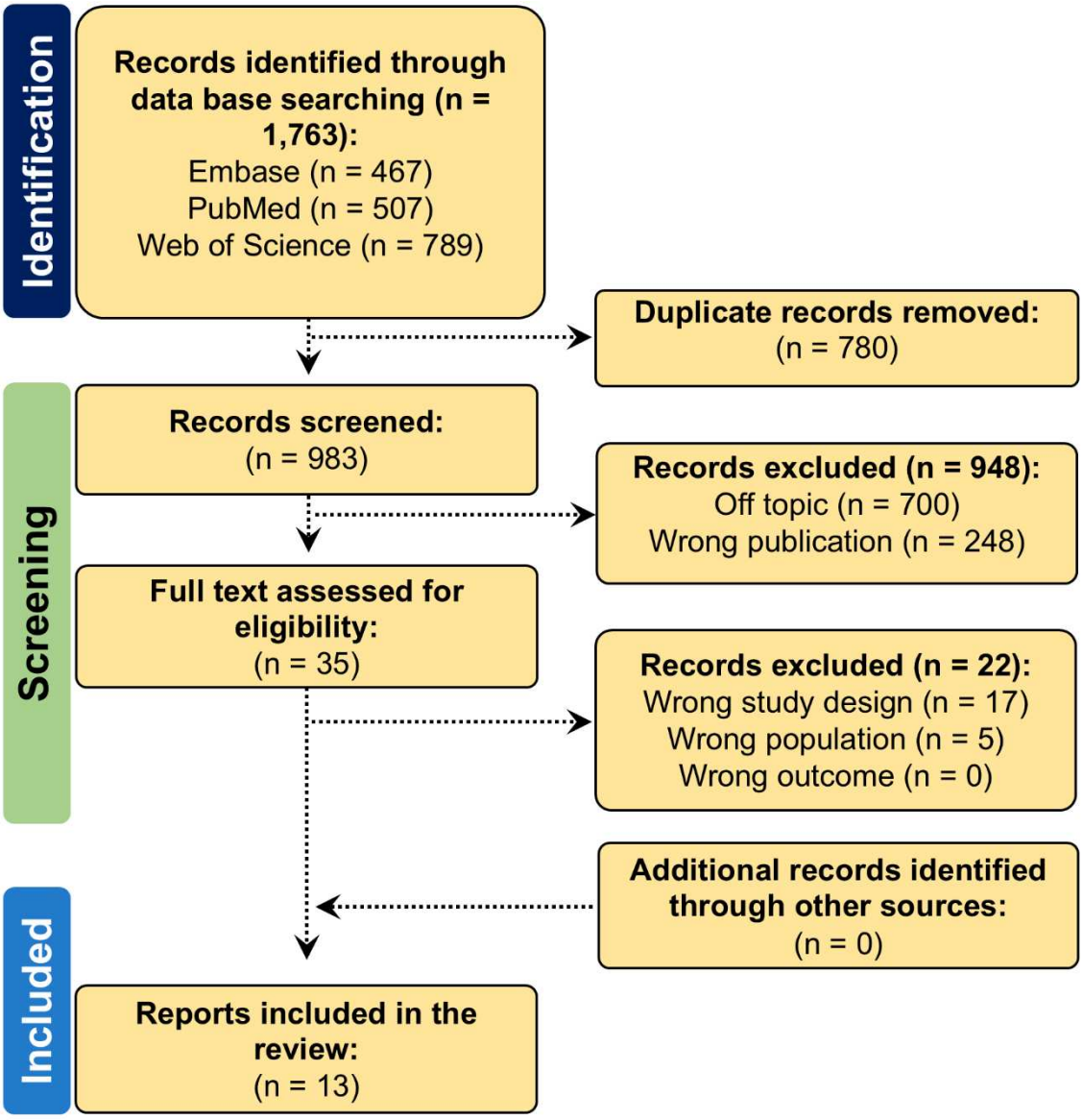

Figure 5. PRISMA diagram showing the identification, screening, eligibility, and inclusion of articles in the systematic review.

3.2 Basic characteristics and quality of the included studies

Out of the 13 eligible studies, 54% (n = 7) conducted *in vitro* approaches (Alshaikh *et al.,* 2020; Alaee *et al.,* 2021; Sistani *et al.,* 2021; Pennarossa *et al.,* 2021; Francés-Herrero *et al.,* 2023; Park *et al.,* 2023; Sistani *et al.,* 2023), whereas 46% (n = 6) evaluated the efficacy of dECM

following transplantation into rats (Laronda *et al.,* 2015; Liu *et al.,* 2017; Hassanpour *et al.,* 2018; Pors *et al.,* 2019; Wu *et al.,* 2022; Mirzaeian *et al.,* 2023). The decellularized scaffold was derived from human ovarian tissue in 5 studies (Hassanpour *et al.,* 2018; Pors *et al.,* 2019; Sistani *et al.,* 2021; Mirzaeian *et al.,* 2023; Sistani *et al.,* 2023), from porcine tissue in 4 studies (Liu *et al.,* 2017; Pennarossa *et al.,* 2021; Wu *et al.,* 2022; Park *et al.,* 2023), from bovine tissue in 2 studies (Laronda *et al.,* 2015; Francés-Herrero *et al.,* 2023), or from rat tissue in 2 studies (Alshaikh *et al.,* 2020; Alaee *et al.,* 2021) (Figure 2A). For the recellularization of DOT, various cell types were employed: primary ovarian cells (POCs) or granulosa cell (GCs) from mice or swine (Laronda *et al.,* 2015; Hassanpour *et al.,* 2018; Liu *et al.,* 2017; Wu *et al.,* 2022; Pennarossa *et al.,* 2021), GCs, ovarian stromal cells (SCs), and preantral (PF) follicles from human or mice, secondary follicles from mice (Pors *et al.,* 2019; Alaee *et al.,* 2021; Francés-Herrero *et al.,* 2023; Park *et al.,* 2023), mesenchymal stem cell (MSCs) from mice or human (Alshaikh *et al.,* 2020; Sistani *et al.,* 2021), ovarian cells (OCs), peritoneal stem cells (PMCs), and bone marrow stem cell (BMCs) from mice (Mirzaeian *et al.,* 2023), and fetal ovarian cells from mice (Sistani *et al.,* 2023) (Figure 2B). Baseline characteristics of the included analyses are summarized in Table 2. Regarding the quality assessment of the included, all evaluated *in vitro* studies reached scores above 80%, considered reliable without restriction following the Klimisch criteria (Supplementary table 3). Risk of bias of animal studies was assessed by SYRCLE`s tool. None of the studies was rated as having a high risk of bias in any of the evaluated domains. All studies were assessed as having a low risk of bias regarding selective outcome reporting and conflict of interest. The risk of bias related to allocation concealment and blinding of interventions was unclear in all studies. For the remaining domains, most studies were classified as having either an unclear risk of bias (Supplementary table 2).

3.3 ECM-based scaffold microarchitecture supports cell adhesion and promotes cellular bioactivity

Five studies assessed the biological interaction between decellularized ovarian tissues (DOTs) and various seeded cell populations. *In vitro*, swine primary ovarian cells (POCs) were able to adhere to and gradually migrate into porcine DOTs. Gene expression analysis revealed active transcription of classic granulosa cell markers—steroidogenic acute regulatory protein (STAR), cytochrome P450 family 11 subfamily A member 1 (CYP11A1), cytochrome P450 family

19 subfamily A member 1 (CYP19A1), anti-Müllerian hormone (AMH), FSH receptor (FSHR), and LH receptor (LHR) —at levels comparable to those observed in native tissue (Pennarossa *et al.,* 2021). Porcine DOT also supported the migration of GCs from murine ovarian tissue, with infiltration initiating by day 6 and intensifying by day 8; by day 12, GCs formed aggregates. Additionally, DOT-implanted ovarian tissue secreted higher levels of estradiol (E2) compared to isolated tissue (Liu *et al.,* 2017). Isolated murine GCs also proliferated actively and formed aggregates on DOT after 15 days of culture, exhibiting low apoptosis and positive CYP19A1 immunostaining, indicative of hormonal synthesis. However, these constructs lost functionality upon xenotransplantation into mice, likely due to an exacerbated immune response (Wu *et al.,* 2022). Furthermore, murine bone marrow-derived mesenchymal stem cells (BMSCs) displayed robust proliferation and recolonized large areas of murine DOT, including deeper layers, while showing low cleaved caspase-3 expression (Alshaikh *et al.,* 2020). Similarly, human MSCs colonized both the surface and inner regions of human-derived DOT scaffolds (Sistani *et al.,* 2021).

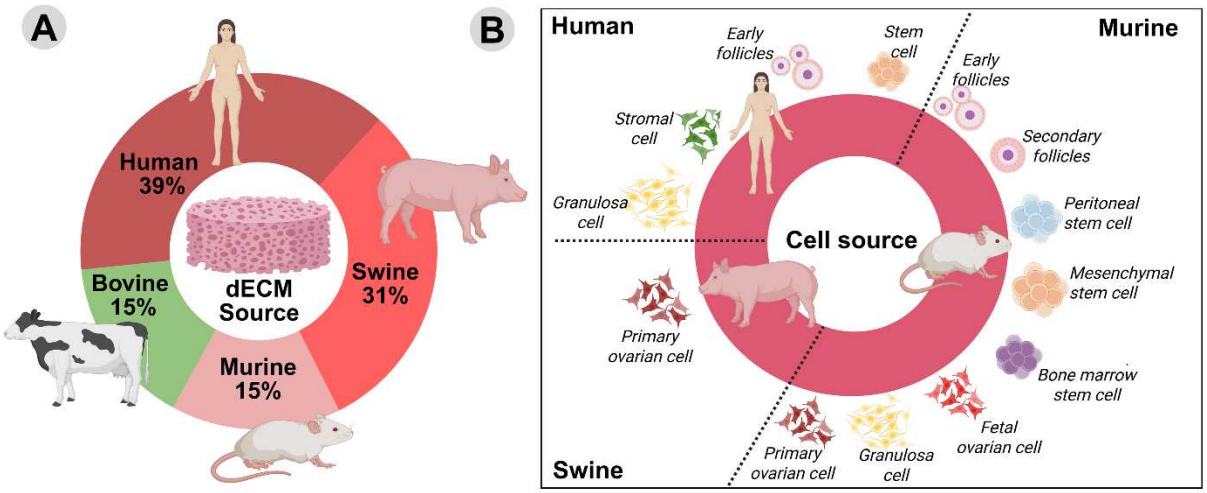

Figure 6. (A) Schematic overview of the tissue origins used for ovarian decellularization, including human (n = 5), swine (n = 4), bovine (n = 2), and murine (n = 2) sources. **(B)** Summary of cell types and their origins employed for scaffold recellularization in the included studies.

Table 2. Basic characteristics of the studies included in the systematic review

Study ID	DOT	Seeded cells/specie	Intervention	Exposure	Control	Primary outcomes
Laronda *et al.* (2015)	Bovine	POCs (mice)	POCs seeded in DOT and transplanted into mice	*In vitro* culture for 2 days, and transplantation for 2-4 weeks	DOT transplant without PCO	Follicle-like structures *in vitro,* and E2 secretion *in vivo*
Liu *et al.* (2017)	Swine	GCs and ovarian tissue (mice)	CGs co-cultured with DOT, and OT inserted into DOT	5-12 days	CGs and OT grown without DOT	Penetration of CGs in DOT and increased production of E2
Hassanpour *et al.* (2018)	Human	POCs (mice)	*In vivo* grafting of DOT containing POCs into mice	4 weeks	Grafting of DOT without POCs	Follicle-like structures. Expression of steroid hormone markers. Increased expression of E2 and P4
Pors *et al.* (2019)	Human	GCs, SCs and PF (Humans); PF (mice)	*In vitro* culture, and *in vivo* grafting of DOT containing OCs into mice	*In vitro* culture for 10-30 days, and grafting for 2-3 weeks	Non-co-cultured OCs. DOT grafts without OCs	Survival and growth of PFs *in vivo* and *in vitro*
Alshaikh *et al.* (2020)	Mouse	MSCs (mouse)	MSCs seeded into DOT and *in vitro* cultured	14 days	DOT without MSCs	MSCs were extensively distributed throughout the constructs

Alaee *et al.* (2021)	Mouse	PF (mouse)	PF seeded into DOT and *in vitro* cultured	12 days	PF cultured in 2D system	DOT supports PF growth, steroidogenesis, and oocyte maturation
Sistani *et al.* (2021)	Human	MSCs (human)	MSCs seeded onto DOT and *in vitro* cultured	7 days	MSCs not exposed to DOT	MSCs successfully interacted with DOT
Pennarossa *et al.* (2021)	Swine	POCs (swine)	POCs seeded onto DOT	7 days	POCs not exposed to DOT, and native tissue.	Expression of CG markers STAR, CYP11A1, CYP19A1, AMH, FSHR, and LHR
Wu *et al.* (2022)	Swine	CGs (mice)	CGs and OF were seeded onto DOT and cultured *in vitro* or grafted *in vivo* into mice	*In vitro* culture for 15 days, and grafting for 4 weeks	GCs and OF not exposed to DOT, and grafting of DOT without cell	Proliferation of GCs. Severe immune response in grafted constructs
Francés-Herrero *et al.* (2023)	Bovine	PFs (mice)	PFs cultured *in vitro* into DOT hydrogel	8 days	PF cultured in 2D system and *in vivo* maturation	DOT hydrogel coating allows 3D follicular growth and oocyte maturation.

Park *et al.* (2023)	Swine	Secondary follicles (mice)	Secondary follicles cultured into DOT hydrogel	9 days	Secondary follicles on day 0 of culture and follicles cultured in collagen or Matrigel	High rates of antral follicle and MII oocyte
Mirzaeian *et al.* (2023)	Human	OSCs, PMSCs e BMSCs (mice)	Stem cell seeded into DOT and grafted *in vivo* into mice	8 weeks	Non-transplanted ovariectomized mice	Follicle-like structures in all groups. Differentiation ability to oocyte and granulosa cells and angiogenesis capability
Sistani *et al.* (2023)	Human	Fetal OCs (mice)	Fetal OCs seeded onto DOT and *in vitro* cultured	7 days	Fetal OCs non-seeded onto DOT	Follicular-like structure; genes and proteins related to oocyte and follicular cells

Abbreviations: OF: ovarian follicles; OSCs: oogonial stem cell; PMSCs: peritoneal stem cell; BMSCs: bone marrow stem cell; MSCs: mesenchymal stem cell; OCs: ovarian cells; POCs: Primary ovarian cells; GCs: granulosa cells; PF: preantral follicles; DOT: decellularized ovarian tissue; SCs: stromal cells; OT: ovarian tissue.

3.4 ECM-based scaffold guide primary ovarian cells or stem cells toward follicular fate

Four independent studies reported the capacity of decellularized ovarian tissue (DOT) to guide primary ovarian cells and stem cells toward a follicular fate. Primary ovarian cells from CD1 mice seeded onto bovine DOT and cultured for 48 h expressed granulosa (FOXL2, α-inhibin) and theca (CYP17) cell markers, and some cells organized into follicle-like structures. Upon xenografting into ovariectomized mice, the constructs restored normal or elevated E2 levels within 2–4 weeks (Laronda *et al.,* 2015). Fetal mouse ovarian cells seeded onto decellularized scaffolds derived from human ovarian tissue and cultured *in vitro* for 7 days colonized the scaffold, organized in primordial follicle-like structures, and expressed oocyte- and follicle-related genes such as DEAD-box helicase 4 (DDX4), and growth differentiation factor 9 (GDF9) (Sistani *et al.,* 2024). Human DOT seeded with primary rat ovarian cells and transplanted into ovariectomized mice also induced a spatial organization of cells that mimicked follicular structures, along with the expression of E2 and progesterone (P4) receptors and increased levels of these hormones after 4 weeks of grafting (Hassanpour *et al.,* 2018). Human decellularized ovarian tissue also promoted the differentiation of oogonial stem cells, peritoneal stem cells, and bone marrow-derived stem cells. All seeded cell formed follicle-like structures after being xenografted into mice for 8 weeks, and the expression of key oocyte- and follicle-related markers, including DDX4, LHR, ZP3 (zona pellucida glycoprotein 3), and GDF9 was reported (Mirzaeian *et al.,* 2023).

3.5 ECM-based scaffold provides a favorable microenvironment for follicular development

The design of a microenvironment conducive to the development of isolated preantral follicles *in vitro* and *in vivo* was reported in four studies. Human scaffolds recellularized with stromal cells and co-cultured with human preantral follicles supported follicular survival for up to 30 days without significant signs of apoptosis, although low survival rates were observed following xenotransplantation into mice (Pors *et al.,* 2019). Murine preantral follicles cultured *in vitro* for 12 days within DOT survived and grew at similar rates to those cultured in droplets under mineral oil. However, the DOT microenvironment favored antrum formation, oocyte maturation, and E2 and P4 secretion. Notably, the expression levels of GDF9, BMP15, and BMP6 did not differ between the two culture systems (Alaee *et al.,* 2021). Hydrogels derived from ovarian extracellular matrix

have also been reported as favorable microenvironments for follicular development. A hydrogel developed from porcine DOT provided a favorable microenvironment for *in vitro* follicular development. Murine secondary follicles cultured in this hydrogel showed higher rates of antral follicle formation and MII oocyte maturation compared to those cultured in Matrigel and alginate. Moreover, embryos were generated following parthenogenetic activation (Park *et al.,* 2023). Although murine secondary follicles cultured on a hydrogel coating derived from bovine DOT showed a lower capacity for antrum formation by day 8 of culture, their E2 and P4 production was not impaired. Under these conditions, higher rates of MII oocytes and embryonic development were also reported (Francés-Herrero *et al.,* 2023). Figure 3 summarizes the main findings from the systematic review.

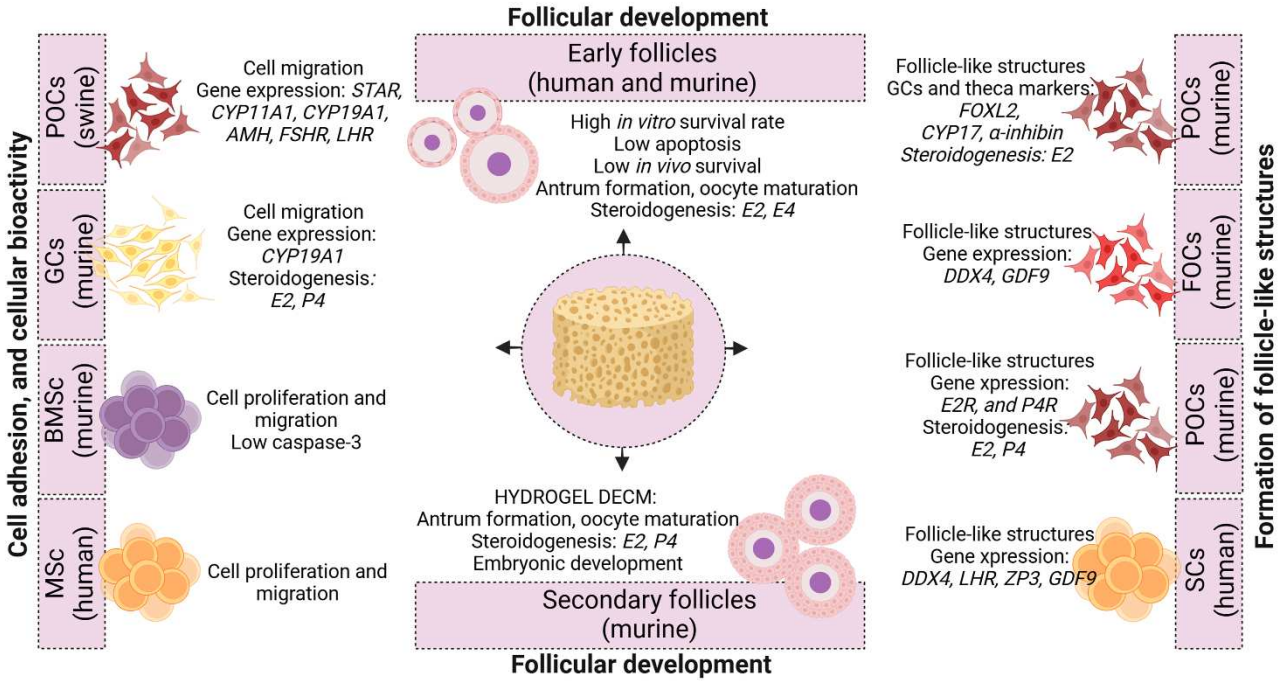

Figure 7. Summary of the main findings from the systematic review. Abbreviations: POCs: Primary ovarian cells; GCs: granulosa cells; BMSCs: bone marrow stem cell; MSCs: mesenchymal stem cell; FOCs: fetal ovarian cell; SCs: stromal cells; DECM: decellularized extracellular matrix; CYP11A1: cytochrome P450 family 11 subfamily A member 1; CYP19A1: cytochrome P450 family 19 subfamily A member 1; AMH: anti-Müllerian hormone; FSHR: FSH receptor; LHR: LH receptor; E2: estradiol; P4: progesterone; FOXL2: forkhead box L2; CYP17: cytochrome P450

family 17; DDX4: DEAD-box helicase 4; GDF9: growth differentiation factor 9; E2R: estrogen receptor; P4R: progesterone receptor; ZP3: zona pellucida glycoprotein 3.

4 Discussion

Tissue-engineered ovarian scaffolds and ovarian culture systems have been proposed as strategies to harness the ovarian reserve of economically important animals and to restore ovarian function in cases of reproductive impairment, thereby reinforcing the concept of artificial ovary development. Although an artificial ovary represents a pivotal advancement in the field of reproductive biology, its success still depends on the accurate recapitulation of ovarian folliculogenesis (Di Berardino *et al*., 2024; Almeida *et al.,* 2023; Gandolfi *et al*., 2020). Artificial ovary is typically constructed by encapsulating healthy ovarian follicles or cells within biomaterial-based scaffolds, forming a functional structure capable of supporting *ex vivo* folliculogenesis (Kim et al., 2025; Aydin *et al.,* 2024). This systematic review compiles strong evidence supporting the potential of decellularized ovarian tissue as a promising biomaterial for the development of three-dimensional artificial ovaries.

The ability for cell roaming is an expected effect of any biomaterial intended to provide a microenvironment capable of mimic folliculogenesis *ex vivo* (Henning *et al.,* 2021). However, the ability of cells to migrate through the scaffold is highly dependent on its porosity, as adequate culture medium perfusion throughout the entire structure is essential (Mirzaeian *et al.,* 2020). Our systematic data show that the microarchitecture of ovarian ECM-based scaffolds provides a supportive framework for cell adhesion, homing into the scaffold, and enhanced cellular bioactivity. Although it has been reported that the applied decellularization method may alter tissue microarchitecture (Tabatabai *et al.,* 2024), optimal organ decellularization preserves the native ECM with ideal porosity, rigidity, and elasticity. It also retains growth factors and a modifiable microenvironment that can be reshaped by cells after reconstruction, along with peptide domains that play a crucial role in integrin-mediated cell adhesion (Guo *et al.,* 2024; Nicolas *et al.,* 2020). The reported results here demonstrate that nutrient diffusion through the scaffold is sufficient to sustain cell viability, even within its deeper regions. Moreover, although integrin-mediated signaling was not reported in the studies analyzed, it is reasonable to consider its role in the cell/dECM adhesion process. Beyond mediating cell adhesion, integrins play a pivotal role in

regulating key cellular processes, including cell viability and differentiation, by modulating multiple signaling pathways (Kanchanawong *et al*., 2023; Bachmann *et al.,* 2019).

Experiments using murine primary ovarian cells and stem cells have shown that dECM scaffolds exert an inductive effect follicular formation as the same as *in vivo* condition. Notably, upon seeding onto the dECM, the cells self-organized into characteristic structures reminiscent of follicular morphology. In addition, the expression of follicular marker genes and steroidogenic hormones was reported. Cells are highly responsive to the chemical, physical, and mechanical properties of their surrounding microenvironment (Nakayama *et al.,* 2015). Both the composition and architecture of the ECM regulate access to mechanotransduction signals that trigger adaptive intracellular responses in the cytoplasm and nucleus, ultimately influencing cellular behavior, function, and fate determination (León-Félix *et al.,* 2025). Accordingly, the preservation of mechanical cues within the dECM may play a key role in regulating cell remodeling and guiding differentiation within the dECM-based artificial ovary toward a follicular phenotype. Stem cells exhibit remarkable plasticity and the capacity for multi-lineage differentiation, making them a valuable model for investigating how interactions with the ECM influence downstream regulation of cell survival, proliferation, differentiation, and function. Thus, it is also reasonable to speculate that the dECM provides essential cues that promote the differentiation of ovarian-resident oogonial stem cells into germ cells, enabling their subsequent entry into the follicular development pathway (Mirzaeian *et al.,* 2023).

Independent reports have also demonstrated the ability of dECM to support the survival of ovarian follicles either in isolation or in co-culture with stromal cells (Pors *et al.,* 2019; Alaee *et al.,* 2021; Park *et al.,* 2023; Francés-Herrero *et al.,* 2023). These findings highlight the sustained follicular performance observed during three-dimensional culture in dECM, emphasizing the intricate regulation of key folliculogenesis-related gene expression and steroid hormone production throughout follicular growth, as well as their critical contribution in supporting the development of competent oocytes and subsequent reproductive events. Although hormonal signaling plays a key role in follicle development, recent studies have shown that mechanical forces also contribute to regulate folliculogenesis (Vasse *et al.,* 2025). The highly dynamic nature of the ovarian ECM plays a pivotal role in regulating various cellular features during folliculogenesis, such as morphology, steroid hormone production, cell survival, and proliferation (Grosbois *et al.,* 2023). In this context, the ovarian decellularization process preserves the native ECM, which retains unique components

that favor follicular development. For instance, glycosaminoglycans stimulate cumulus cells to produce cytokines such as GDF9 (Watson *et al.,* 2012), and also play critical roles in cumulus cell expansion and oocyte maturation during *in vitro* culture (Marei *et al.,* 2012). As a result, follicles embedded within these scaffolds are exposed to a more dynamic and responsive microenvironment that supports the bidirectional interactions necessary for follicular growth.

Despite these promising findings, immune response following *in vivo* xenotransplantation appears to be a critical challenge. Wu *et al.* (2022) reported host inflammatory reactions after xenotransplantation of decellularized ovarian. Immune rejection may result from multiple factors, including tissue heterogeneity, which can affect decellularization efficiency and lead to residual nuclear material within the scaffold. Incomplete removal of organelle debris and lipids, such as mitochondrial and endosomal remnants, may also trigger immune activation (Kabirian *et al.,* 2020). Furthermore, although most ECM proteins are conserved across species, unique ECM components may elicit immunogenic responses. Structural alterations in the ECM, particularly changes in collagen morphology, alignment, and thermal stability, could further contribute to innate immune activation (Pan *et al.,* 2018).

5 Conclusions and perspectives

Bioscaffolds derived from decellularized ovarian tissue provide a suitable niche for the growth of ovarian cells and stem cells, potentially recreating the complex architecture and functionality of the native ovary, representing a biotechnological innovation with potential to address unmet challenges in female reproductive health such as harnessing reproductive potential or restoring ovarian function. However, the ability of dECM-based scaffolds to support the survival and coordinated long-term development of multiple follicles has yet to be fully demonstrated, particularly in reproductively complex species such as farm animals and humans. Achieving this milestone, along with the standardization of decellularization protocols – which remains a critical challenge – is essential to ensure consistency, and reproducibility. This can be addressed by developing consensus-based guidelines, implementing comparative studies of decellularization methods, adopting automated and computationally guided platforms, and establishing standardized quality control criteria for assessing ECM preservation, and biocompatibility. Importantly, translating these experimental advances into clinical practice will require well-designed preclinical

models and carefully controlled human studies to validate the safety, efficacy, and functional outcomes of dECM-based ovarian scaffolds in fertility restoration strategies.

6 Limitations

Although most decellularized scaffolds described in the included studies were derived from human ovaries or large animals such as cattle and pigs, the majority of the cellular models used were of murine origin. This discrepancy limits the extrapolation of findings to other species, as murine folliculogenesis is less complex and may not accurately reflect the dynamics observed in larger mammals. Additionally, the absence of standardized experimental protocols may introduce significant inter-study and inter-experimental variation. Finally, the heterogeneity of the cellular models, along with differences in study objectives and methodological designs, resulted in substantial variability among the included studies, which prevented the performance of a meta-analysis.

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Declaration of competing of interest

The authors declare that they have no conflicts of interest.

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Supplementary Tables:

Table 3. Specific search terms of databases

EMBASE **467 records**	'decellularized ovarian extracellular matrix' OR (decellularized AND ovarian AND extracellular AND ('matrix'/exp OR matrix)) OR 'ovarian recellularization' OR 'decellularized ovary' OR (decellularized AND ('ovary'/exp OR ovary)) OR 'decellularized ovarian tissue' OR (decellularized AND ovarian AND ('tissue'/exp OR tissue)) OR 'ovarian decellularization' OR (ovarian AND ('decellularization'/exp OR decellularization)) OR 'ovarian tissue decellularization' OR (ovarian AND ('tissue'/exp OR tissue) AND ('decellularization'/exp OR decellularization)) OR 'decellularized ovarian scaffold' OR (decellularized AND ovarian AND ('scaffold'/exp OR scaffold)) OR (decellularized AND ovarian AND ('matrix'/exp OR matrix)) OR 'ovarian scaffold':ti,ab,kw OR 'ovarian decellularized bioscaffold' OR (ovarian AND decellularized AND ('bioscaffold'/exp OR bioscaffold)) OR (ovarian AND ('tissue'/exp OR tissue) AND ('scaffold'/exp OR scaffold)) OR 'ovarian bioscaffold' OR (ovarian AND ('bioscaffold'/exp OR bioscaffold)) OR 'ovarian tissue decm' OR (ovarian AND ('tissue'/exp OR tissue) AND decm) OR 'ovary decm' OR (('ovary'/exp OR ovary) AND decm) OR 'bioscaffold extracellular matrix ovarian' OR (('bioscaffold'/exp OR bioscaffold) AND extracellular AND ('matrix'/exp OR matrix) AND ovarian)
PUBMED **507 records**	"decellularized extracellular matrix"[MeSH Terms] OR (("decellular"[All Fields] OR "decellularization"[All Fields] OR "decellularize"[All Fields] OR "decellularized"[All Fields] OR "decellularizes"[All Fields] OR "decellularizing"[All Fields]) AND ("ovarial"[All Fields] OR "ovary"[MeSH Terms] OR "ovary"[All Fields] OR "ovaries"[All Fields] OR "ovary s"[All Fields])) OR

	(("decellular"[All Fields] OR "decellularization"[All Fields] OR "decellularize"[All Fields] OR "decellularized"[All Fields] OR "decellularizes"[All Fields] OR "decellularizing"[All Fields]) AND ("ovarian"[All Fields] OR "ovarians"[All Fields]) AND ("tissue s"[All Fields] OR "tissues"[MeSH Terms] OR "tissues"[All Fields] OR "tissue"[All Fields])) OR (("ovarian"[All Fields] OR "ovarians"[All Fields]) AND ("decellular"[All Fields] OR "decellularization"[All Fields] OR "decellularize"[All Fields] OR "decellularized"[All Fields] OR "decellularizes"[All Fields] OR "decellularizing"[All Fields])) OR (("ovarian"[All Fields] OR "ovarians"[All Fields]) AND ("recellularization"[All Fields] OR "recellularize"[All Fields] OR "recellularized"[All Fields] OR "recellularizing"[All Fields])) OR (("recellularization"[All Fields] OR "recellularize"[All Fields] OR "recellularized"[All Fields] OR "recellularizing"[All Fields]) AND ("ovarial"[All Fields] OR "ovary"[MeSH Terms] OR "ovary"[All Fields] OR "ovaries"[All Fields] OR "ovary s"[All Fields])) OR (("ovarian"[All Fields] OR "ovarians"[All Fields]) AND ("tissue s"[All Fields] OR "tissues"[MeSH Terms] OR "tissues"[All Fields] OR "tissue"[All Fields]) AND ("decellular"[All Fields] OR "decellularization"[All Fields] OR "decellularize"[All Fields] OR "decellularized"[All Fields] OR "decellularizes"[All Fields] OR "decellularizing"[All Fields])) OR (("decellular"[All Fields] OR "decellularization"[All Fields] OR "decellularize"[All Fields] OR "decellularized"[All Fields] OR "decellularizes"[All Fields] OR "decellularizing"[All Fields]) AND ("ovarian"[All Fields] OR "ovarians"[All Fields]) AND ("scaffold"[All Fields] OR "scaffold s"[All Fields] OR "scaffolded"[All Fields] OR "scaffolder"[All Fields] OR "scaffolders"[All Fields] OR "scaffolding"[All Fields] OR "scaffoldings"[All Fields] OR "scaffolds"[All Fields])) OR "ovarian scaffold"[All Fields] OR (("ovarian"[All Fields] OR "ovarians"[All

	Fields]) AND ("decellular"[All Fields] OR "decellularization"[All Fields] OR "decellularize"[All Fields] OR "decellularized"[All Fields] OR "decellularizes"[All Fields] OR "decellularizing"[All Fields]) AND ("bioscaffold"[All Fields] OR "bioscaffolds"[All Fields])) OR (("ovarian"[All Fields] OR "ovarians"[All Fields]) AND ("tissue scaffolds"[MeSH Terms] OR ("tissue"[All Fields] AND "scaffolds"[All Fields]) OR "tissue scaffolds"[All Fields] OR ("tissue"[All Fields] AND "scaffold"[All Fields]) OR "tissue scaffold"[All Fields])) OR (("ovarian"[All Fields] OR "ovarians"[All Fields]) AND ("bioscaffold"[All Fields] OR "bioscaffolds"[All Fields])) OR (("ovarian"[All Fields] OR "ovarians"[All Fields]) AND ("tissue s"[All Fields] OR "tissues"[MeSH Terms] OR "tissues"[All Fields] OR "tissue"[All Fields]) AND "decm"[All Fields]) OR (("ovarial"[All Fields] OR "ovary"[MeSH Terms] OR "ovary"[All Fields] OR "ovaries"[All Fields] OR "ovary s"[All Fields]) AND "decm"[All Fields])
WEB OF SCIENCE 789 records	((((((((((TS=(decellularized ovary)) OR TS=(ovarian recellularization)) OR TS=(ovary recellularization)) OR TS=(decellularized ovarian tissue)) OR TS=(ovarian decellularization)) OR TS=(ovarian tissue decellularization)) OR TS=(decellularized ovarian scaffold)) OR TS=(ovarian scaffold)) OR TS=(ovarian decellularized bioscaffold)) OR TS=(ovarian tissue scaffold)) OR TS=(ovarian bioscaffold)) OR TS=(ovarian tissue DECM)) OR TS=(ovary DECM)

Table 4. Risk of bias of animal studies assessed by SYRCLE`s tool.

Study ID	Selection bias			Performance bias		Detection bias		Attrition bias	Reporting bias	Other bias
	Sequence generation	**Baseline characteristics**	**Allocation concealment**	**Random housing**	**Blinding to interventions**	**Random outcome assessment**	**Blinding outcome assessor**	**Incomplete outcome data**	**Selective outcome data**	**Conflict of interest**
Laronda *et al.* (2015)	Y	U	U	U	U	U	Y	U	Y	Y
Liu *et al.* (2017)	Y	U	U	U	U	U	U	U	Y	Y
Hassanpour *et al.* (2018)	Y	Y	U	U	U	Y	Y	Y	Y	Y
Pors *et al,* (2019)	Y	Y	U	Y	U	U	U	U	Y	Y
Wu *et al.* (2022)	Y	Y	U	U	U	Y	Y	Y	Y	Y
Mirzaeian *al.* (2023)	U	U	U	U	U	U	U	U	Y	Y

Abbreviations: Y = yes (low risk of bias); U = unclear (uncertain risk of bias).

Table 5. Questions used to guide the quality assessment of each *in vitro* article evaluated during the systematic review stages using the SciRAP tool.

	Study ID						
Questions	Alshaikh *et al.* (2020)	Alaee *et al.* (2021)	Sistani *et al.* (2021)	Pennarossa *et al.* (2021)	Francés-Herrero *et al.* (2023)	Park *et al.* (2023)	Sistani *et al.* (2023)
Test compound and controls:							
The chemical name or other identification of the culture medium was given?	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Was the concentration of each substance used described?	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Experimental system:							
The test system (e.g., cell line / cells / tissue) was described?	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Was the effect of DOT scaffolds on ovarian cells or stem cell development evaluated?	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Was the duration of treatment indicated?	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Composition of media was described, including use of serum, antibiotics, etc?	1.0	1.0	1.0	1.0	1.0	1.0	0.5
The number of replicates/repetitions were stated?	1.0	1.0	1.0	0.0	0.0	0.0	1.0
Incubation temperature, humidity, and CO2 concentration were described?	0.0	1.0	1.0	1.0	1.0	1.0	1.0
Test and controls:							
Cell density or number of cells used during treatment was described?	1.0	1.0	1.0	0.0	0.0	0.0	1.0

The duration of intervention was stated?	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Data collection and results:							
The tests and/or analytical methods used were sufficiently described to allow for evaluation of reliability of results?	1.0	1.0	1.0	1.0	1.0	1.0	1.0
The statistical methods and software used were described?	0.5	1.0	1.0	0.5	1.0	0.5	1.0
The statistical methods were clearly described and do not seem inappropriate, unusual or unfamiliar?	1.0	0.5	1.0	0.5	1.0	0.5	1.0
All results were clearly presented?	1.0	0.5	1.0	1.0	1.0	1.0	1.0
Funding and competing interests:							
The funding sources for the study were stated?	1.0	1.0	0.0	1.0	1.0	1.0	0.0
Any competing interests were disclosed or it was explicitly stated that the authors did not have any competing interests?	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Regarding improvement in cells survival (review question):							
Was the method of assessing survival clearly described?	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Total score:	16.5	16.0	16.0	14.0	15.0	14.0	15.5
(%) Score:	>80%	>80%	>80%	>80%	>80%	>80%	>80%

Each value in the table represents the score assigned to the study for each SciRAP item evaluated: 1.0: fulfilled; 0.5: partially fulfilled; 0.0: no fulfilled.

2.6 Estresse oxidativo e antioxidantes no cultivo *in vitro* de folículos ovarianos pré-antrais

Espécies reativas de oxigênio (EROs), ou radicais livres, são espécimes químicos oxidantes formados quando o oxigênio é reduzido de forma incompleta e incluem ânions superóxido (O^{2-}), peróxido de hidrogênio (H_2O_2), íons hidróxido (OH^-) e radicais hidroxila (•OH) (Xia *et al.,* 2025). Cada um desses EROs pode ser gerado em diferentes compartimentos celulares, mas as mitocôndrias são a principal fonte, embora o retículo endoplasmático (RE) seja também uma fonte relevante. À medida que o ATP é gerado na mitocôndria, uma taxa baixa, mas contante de escape eletrônico na cadeia transportadora de elétrons fornece a fonte de elétrons que reduzem as moléculas de O_2 para O^{2-} (EROs primários) que são posteriormente convertidos em moléculas como H_2O_2 e •OH (EROs secundárias) por meio de diferentes reações catalíticas (Yang *et al.,* 2019). Nesse processo, os complexos I e III da cadeia transportadora de elétrons produzem principalmente EROs primários (O^{2-}). O O^{2-} é, então, convertido em H_2O_2 pela superóxido dismutase de Mn mitocondrial (SOD2) na matriz mitocondrial ou pela superóxido dismutase de Cu-Zn (SOD1) no espaço intermembranar (Keane *et al.,* 2024; Kim *et al.,* 2021). Além da mitocôndria, o RE, conhecido por seu papel vital no dobramento de proteínas recém sintetizadas, é uma fonte adicional de EROs (Camargo *et al.,* 2024). O acúmulo de proteínas mal dobradas no RE ativa a resposta de proteína mal dobrada (UPR) que desencadeia uma cascata de produção de EROs pela participação da proteína disssulfeto isomerase, ER oxidoredutina 1 e do complexo nicotinamida adenina dinucleotídio fosfato (NADPH) oxidase (NOX) (Zeeshan *et al.,* 2016). O estresse do ER também induz uma liberação maciça de Ca^{2+} na mitocôndria, interrompendo EROs mitocondrial normal e aumentando O^{2-} (Senft *et al.,* 2015).

É bem documentado que as EROs desempenham funções críticas em processos fisiológicos-chave ao atuar como moléculas de sinalização que regulam direta ou indiretamente o crescimento celular, apoptose, necrose e sobrevivência, que fazem parte de funções metabólicas fundamentais (Sies *et al.,* 2022). No ovário, EROs regula várias funções fisiológicas relevantes que se estendem do crescimento do oócito à fertilização, incluindo meiose, ovulação e manutenção e regressão do corpo lúteo (Liang *et al.,* 2023). Durante o crescimento folicular, o aumento da secreção de esteroides ativa o citocromo P450, induzindo síntese de EROs, enquanto a maior secreção de estradiol (E_2) estimula a expressão de catalase (CAT), equilibrando EROs e antioxidantes (Yan *et al.,* 2022). A estimulação de EROs regula o progresso da meiose I em oócitos; em contraste, a progressão da meiose II é fortemente regulada por antioxidantes (Agarwal *et al.,* 2012). Antes da ovulação, o aumento do LH eleva os níveis

de precursores inflamatórios no ovário, intensificando a produção de ROS, que induzem apoptose nas células da granulosa, levando à ruptura folicular e à ovulação (Shkolnik *et al.,* 2010). Da mesma forma, a apoptose de células da granulosa luteinizadas induzida por EROs também contribui para a regressão do corpo lúteo (Liang *et al.,* 2023).

Como destacado, a manutenção fisiológica de eventos essenciais no ovário é dependente de um balanço delicado entre a síntese de EROs e a capacidade das células de mantê-las em níveis equilibrados. Para manter a homeostase e prevenir danos, sistemas eficientes de resposta ao estresse celular detectam desvios nos níveis de oxidantes em diferentes compartimentos celulares e ativam contramedidas adequadas. *In vivo,* esse papel é desempenhado por sistemas antioxidantes enzimáticos e não enzimáticos. As defesas antioxidantes enzimáticas consistem predominantemente em enzimas antioxidantes poderosas como a catalase (CAT), a superóxido dismutase (SOD) e a glutationa peroxidase (GPX) que atuam eliminando radicais livres e equilibrando o sistema redox celular (Silva *et al*., 2024) Em menor grau, compostos de baixa massa molecular, incluindo vitaminas, micronutrientes e cofatores também atuam nesse processo como componentes não enzimáticos. Esses sistemas juntos moldam a paisagem redox, tendo papéis benéficos ao eliminar o excesso de oxidantes prevenindo o sofrimento oxidativo e as consequências dele decorrentes (Sies *et al.,* 2022).

Embora EROs influenciem positivamente as funções ovarianas normais, está bem estabelecido que concentrações suprafisiológicas de EROs, como aquelas observadas durante o cultivo *in vitro* de folículos ovarianos pré-antrais, perturbam o equilíbrio pró-oxidante-antioxidante induzindo um estado de estresse oxidativo (EO) que induz reações não específicas de EROs com biomacromoléculas celulares como proteínas, lipídios, ácidos nucleicos e carboidratos, além de gerar outras espécies reativas com consequências potencialmente tóxicas (Silva et al., 2023). Não surpreendentemente, o insulto celular induzido por EO é fortemente associado a baixa qualidade de folículos cultivado *in vitro* em diferentes espécies, incluindo bovinos (da Silva *et al.,* 2024; Bergamo *et al.,* 2021). De fato, durante o cultivo *in vitro* de folículos pré-antrais inclusos no tecido ovariano ou isoladamente, a exposição folicular às condições isquêmicas após a coleta dos ovários, exposição excessiva à luz e excesso de manipulação durante os processos de fragmentação do tecido e isolamento folicular, variações de temperatura e pH dos meios de cultivo e trocas de meio podem comprometer o equilíbrio redox *in vitro*, induzindo danos significativos no tecido, folículos e oócitos. Em conjunto, esses fatores podem induzir superprodução de EROs concomitantemente a uma redução da atividade de sistemas antioxidantes endógenos controladores do ambiente redox (Silva *et al.,* 2024; Silva

et al., 2023; Soto-Heras *et al.,* 2020; Cacciottola *et al.,* 2018; Lins *et al.,* 2017; Wang *et al.,* 2017).

O tecido ovariano contém diversos tipos de células com exigências nutricionais e hormonais específicas. Esses requisitos geralmente não são todos atendidos durante o cultivo *in vitro*, elevando a produção de EROs (Silva *et al.,* 2025). Tem sido relatado que o cultivo *in vitro* de folículos ovariano pré-antrais inclusos no tecido ovariano pode resultar em perdas folicular consideráveis fortemente associadas à biossíntese exacerbada de EROs (Silva *et al.,* 2022; Sá *et al.,* 2018). Além disso, a MEC pode ser severamente afetada quando exposta a níveis elevados de EROs. Embora o impacto deletério do EO na remodelação da MEC ovariana ainda esteja sendo caracterizado, estudos já mostraram que a síntese e/ou estabilidade de vários colágenos, incluindo colágeno I, a proteína fibrosa mais abundante da MEC, são afetados pelo estresse oxidativo (Martins *et al*., 2021). Além disso, a sobrecarga de EROs pode induzir desregulação da síntese de metaloproteinases de matriz (MMPs) que contribuem para a degradação do colágeno (Fu *et al.,* 2019; Ali *et al.,* 2019). Também já foi demonstrado que o EO induzido por produtos finais de glicação avançada pode causar reticulação anormal de colágeno e elastina na matriz extracelular, comprometendo a proliferação e sobrevivência das células da granulosa (Mouanness *et al.,* 2022). Por sua vez, em folículos isolados cultivados *in vitro*, insultos de EO têm sido associados à baixa viabilidade folicular, fragmentação de DNA, dano mitocondrial, erros cromossômicos em oócitos e peroxidação lipídica demonstrada por níveis aumentados de malondialdeído (MDA) (Silva *et al.,* 2023).

Como visto, no ambiente *in vitro,* os gametas são expostos a um nível elevado de EROs, excedendo a capacidade antioxidante das células para combate-las. Assim, dentre as estratégias atualmente sendo estudadas para aprimorar o cultivo *in vitro* de folículos ovarianos pré-antrais, a suplementação dos meios de cultivo com antioxidantes, especialmente aqueles de origem natural, vêm sendo fortemente incentivada para reduzir ou prevenir os dados decorrentes das injurias do EO. Antioxidantes não enzimáticos, como vitaminas E, A e C, flavonoides, carotenoides, glutationa e melatonina, são suplementos naturais que interrompem as reações em cadeia dos radicais livres. Presentes em alimentos como frutas, vegetais, cereais, cogumelos, bebidas, flores, especiarias e ervas medicinais, esses antioxidantes vêm sendo estudados há décadas, com evidências de benefícios no cultivo *in vitro* de tecido ovariano e folículos isolados (Caetano Filho *et al.,* 2024, Bezerra *et al*., 2024; Silva *et al.,* 2024; Sá *et al.,* 2018). Embora a combinação de antioxidantes ideais, concentrações e tempos de exposição ainda demandem investigações adicionais, importantes efeitos durante o cultivo *in vitro* de folículos pré-antrais já foram relatados. A tabela 1 resume avanços documentados na espécie bovina.

Tabela 6. Principais efeitos da suplementação antioxidante no cultivo *in vitro* de folículos pré-antrais bovino

Antioxidante	**Concentração**	**Sistema de cultivo**	**Duração do cultivo**	**Resultados**	**Referência**
Resveratrol	20 µM	Tecido ovariano	6 dias	Melhor integridade da MEC e sobrevivência folicular, maior expressão da atividade da enzima CAT e maiores níveis de tiol	Costa *et al.* (2025)
Timol	400 µg/ml	Tecido ovariano	6 dias	Maior ativação folicular, preserva células estromais e fibras de colágeno no tecido	Filho *et al.,* (2024)
Punicalagina	10 µM	Tecido ovariano	6 dias	Maior ativação folicular e maiores níveis de tiol e atividade das enzimas SOD, CAT e GPX.	Bezerra *et al.* (2024)
Melatonina	1000 pM	Tecido ovariano	6 dias	Maior ativação folicular, preservou	Silva *et al.* (2024)

				colágeno na MEC, maior expressão de mRNA para CAT.	
Anetol	1 µg/ml	Tecido ovariano	6 dias	Maior sobrevivência folicular, preserva colágeno na MEC e induz maiores níveis de tiol.	da Silva *et al.* (2024)
N-acetil cisteína	1mM	Folículos secundários isolados	18 dias	Maior viabilidade e crescimento foliculares	Nascimento *et al*. (2022)
Ácido ascórbico	50 µg/ml	Tecido ovariano	8 dias	Maior sobrevivência folicular e proliferação celular	Andrade *et al.* (2012)
	50 µg/ml	Folículos secundários isolados	12 dias	Melhor sobrevivência folicular e crescimento	Thomas *et al.* (2001)
Eugenol	5 µM	Folículos secundários isolados	18 dias	Maior viabilidade folicular e formação de antro e maior expressão de	Vasconcelos *et al.* (2021)

				mRNA para SOD e GPX.	
Ácido alfa-lipóico	100 ng/ml	Tecido ovariano	12 dias	Aumento da sobrevivência e ativação foliculares.	Bergamo *et al.* (2021)
Alfa-pineno	10 µg/ml	Tecido ovariano	6 dias	Preserva colágeno na MEC, aumenta a expressão de mRNA para NRF2 e PRDX6	Azevedo *et al.* (2024)

Fonte: Autor.

2.7 Funções biológicas do resveratrol durante o crescimento de folículos ovarianos

O resveratrol (3,5,4'-tri-hidroxi-trans-estilbeno) cujo nome químico (nome IUPAC) é (4-hidroxiestiril) benzeno-1-3-diol é uma fitoalexina aromática, metabólito secundário de plantas gerado como uma reação adaptativa a estressores abióticos e bióticos, como irradiação ultravioleta, danos e infecções fúngicas, sendo comumente encontrado em uvas, amendoins, frutas vermelhas, soja e pistache (Kadry *et al.,* 2024; More *et al*., 2024; Salehi *et al.,* 2018). Apresenta-se como um pó branco (comumente extraído com metanol) com um ponto de fusão de 253 – 255 ºC com um peso molecular baixo de 228,25 g/mol (Francioso *et al.,* 2014; Kulkarni *et al.,* 2015). O RSV pertence à família estilbeno de polifenóis, na qual os grupos fenil são substituídos por grupos hidroxilas nas posições 3, 5 e 4. A estrutura química do RSV (Figura 5) consiste em dois anéis aromáticos ligados por uma ponte de metileno. A molécula apresenta uma ligação que pode ser isomerizada *cis* ou *trans*, sendo a forma *trans* mais prevalente com diferentes atividades biológicas associadas a ele e a mais amplamente investigada por ser a mais abundante e mais biologicamente ativa do que a forma *cis* (Herrero *et al.,* 2021; Zhang *et al*., 2021; Pasquariello *et al.,* 2020). O resveratrol deriva da fenilalanina por meio da ativação da enzima estilbeno sintase e pode passar por múltiplas alterações estruturais após a sua síntese quando as plantas estão sob circunstâncias particulares ou durante o período pós-colheita. Por exemplo, sua forma *trans* pode isomerizar o resveratrol quando exposta à luz UV (Islam *et al.,* 2022).

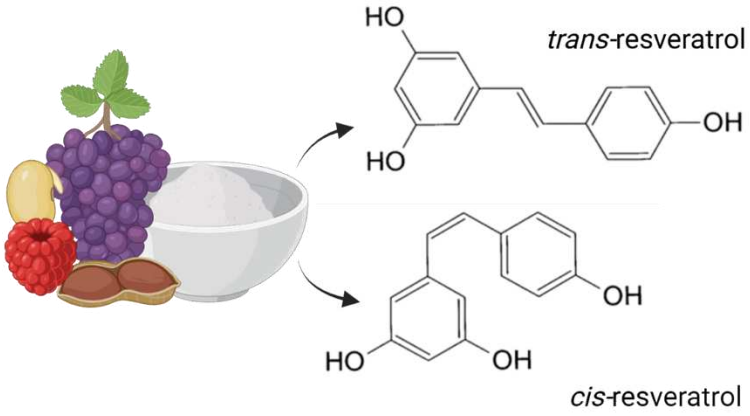

Figura 8. Diagrama esquemático mostrando as principais fontes e a estrutura química das duas formas isoméricas do resveratrol. Fonte: Autor.

Diversos estudos vêm evidenciando o papel do resveratrol como um modulador potente da reprodução de fêmeas *in vivo* e *in vitro* através de múltiplos mecanismos regulatórios que incluem neutralização do declínio da fertilidade relacionado à idade e retenção epigenômica, estímulos proliferativos, antiapoptóticos e de proteção ao DNA, melhora da função mitocondrial, e efeito antioxidante robusto (Podgrajsek *et al.,* 2024) Embora o envelhecimento reprodutivo em mamíferos fêmeas seja um processo irreversível associado ao declínio da quantidade e qualidade dos oócitos (Bertoldo *et al.,* 2020), a administração oral de resveratrol demonstrou resgatar a fertilidade de camundongos fêmeas durante o envelhecimento reprodutivo (Liu *et al.,* 2013). O mecanismo subjacente a esse efeito foi investigado recentemente por Gou *et al.* (2023) que relataram importante efeito do resveratrol no resgate transcriptômico de oócitos de camundongos, ou seja, oócitos de camundongos velhos alimentados com resveratrol apresentaram transcriptoma notavelmente semelhante ao de camundongos de meia-idade.

O resveratrol age também em nível epigenômico. Foi relatado que o resveratrol retém um epigenoma ovariano mais jovem em oócitos de camundongos estendendo a vida útil reprodutiva natural (Gou *et al.,* 2023). O efeito do resveratrol na modulação da acetilação de histonas em oócitos imaturos também já foi descrito. Comizzoli *et al.* (2009), usando um modelo de gato doméstico, verificaram que oócitos em vesícula germinativa vitrificados na presença de 1 mmol/L de resveratrol apresentaram maiores níveis de desacetilação de histonas, aumentando a compactação da cromatina. Curiosamente, esse efeito não prejudicou a competência de desenvolvimento do oócito, sugerindo que essas modificações foram transitórias e exerceram efeito protetor da cromatina do oócito durante o evento estressor da vitrificação. Relatório anterior também já evidenciou que o resveratrol retarda o

envelhecimento dos ovócitos em camundongos ao ativar a mitofagia, melhorando a reserva ovariana (Zhou *et al.,* 2019).

Evidências crescentes vêm demonstrando o papel do resveratrol na função ovariana e na modulação da esteroidogênese mediada por sirtuínas (Ochiai *et al.,* 2019; Ortega *et al.,* 2015). O resveratrol é o ligante natural mais potente do gene sirtuína-1 (*SIRT1*) encontrado em células da granulosa, células do cumulus, oócitos e embriões (Nishigaki *et al.,* 2021). No sistema reprodutivo, *SIRT1* desempenha um papel na apoptose de CGs durante a atresia folicular e tem sido associado à preservação da reserva folicular e à extensão da vida útil ovariana e é um fator essencial na ativação da esteroidogênese associada à luteinização (Morita *et al.,* 2012). Eventos estressantes estimulam a ativação da *SIRT1*, promovendo sua interação com diversos alvos moleculares, como NF-κB, proteína tumoral p53, FOXO, PGC-1α, receptor X do fígado e HIF-2α (Finkel *et al.,* 2009). Ao modular essas vias por meio da SIRT1, o resveratrol exerce um papel essencial na regulação da homeostase energética, na manutenção da estabilidade genômica e na promoção da sobrevivência celular. Estudos recentes relataram o efeito positivo do resveratrol na síntese de ATP e na promoção da biogênese mitocondrial em CGs de humanos e bovinos, além de aumentar a expressão da proteína SIRT1 e o conteúdo de ATP em oócitos bovinos, resultando em melhores índices de fertilização (Ragonese *et al.,* 2021; Sugiyama *et al.,* 2015). Em oócitos humanos, o resveratrol aumenta a taxa de emissão do primeiro corpúsculo polar e reduz a porcentagem de fuso com morfologia anormal (Liu *et al.,* 2018). Essa melhora na normalidade do fuso pode estar correlacionada ao papel do resveratrol ao regular positivamente diversos genes relacionados à actina como *Myo1b, Myh11, Ablim* e *Abl2* (Gou *et al.,* 2023).

O resveratrol apresenta uma estrutura química semelhante à de alguns estrogênios, como o dietilestilbeno, sendo, portanto, considerado um fitoestrogênio natural. Sua configuração molecular, incluindo dois anéis de seis carbonos com substituintes hidroxila, pode imitar o estradiol. Dessa forma, esse fitoquímico pode atuar como agonista dos dois subtipos de receptores estrogênicos: receptor de estrogênio alfa (REα) e beta (REβ) (Meng *et al.,* 2023). Os fundamentos teóricos de suas interações com receptores de estrogênio foram descritos em estudos que investigaram o seu acoplamento *in sílico* a esses receptores (Kobylka *et al.,* 2022). Os grupos hidroxilas presentes na estrutura molecular do resveratrol são igualmente responsáveis por sua interação aos receptores de estrogênio (Shah *et al.,* 2022; Chakraborty *et al.,* 2013). Recentemente, Tinglin *et al.* (2023) forneceram importantes *insights* sobre o efeito modulador do resveratrol na esteroidogênese de CGs humanas. Nesse estudo, foi relatado que o resveratrol atua também por meio do receptor de estrogênio acoplado à proteína G (GPER) e

regula positivamente a expressão da proteína reguladora aguda esteroidogênica (StAR) e a produção de progesterona (P4) nas CGs com envolvimento da cinase 1/2 regulada por sinal extracelular (ERK1/2). Além disso, a expressão de um repressor transcricional, Snail, foi regulada negativamente por RSV, o que contribuiu para a ação indutora do resveratrol na expressão de StAR e na produção de P4 (Song *et al.,* 2023). Outro estudo relatou efeito semelhante do resveratrol induzindo a produção de P4 em células da granulosa de iaque (*Bos grunniens*) e estimulando a expressão de genes relacionados à síntese de P4 (*StAR*, *HSD3B1* e *CYP11A1*) (Jiang *et al.,* 2024). Em bovinos, Wang *et al.* (2012) descobriram que o meio de maturação *in vitro* suplementado com resveratrol aumentou a expressão de P4 e as taxas de maturação oocitária.

O resveratrol também atua no ovário por meio da modulação do *status* redox celular ao agir como um potente antioxidante (Jiang *et al.,* 2024; Saber *et al.,* 2024; Bertoldo *et al.,* 2024; Silva *et al.,* 2023). Essa relevante atividade biológica do resveratrol está fortemente correlacionada às características redox de seus grupos OH fenólicos e com a possibilidade de deslocalização de elétrons no nível de sua estrutura química, o que favorece sua atuação na inibição direta da produção de EROs (Constantinescu *et al.,* 2023; Rudrapal *et al.,* 2022). Além disso, a indução da expressão gênica de enzimas antioxidantes chave como *SOD, GPX* e *CAT*, bem como a regulação de vias e genes de defesa antioxidante também está incluída no rol de mecanismos pelos quais o RSV atua como antioxidante no ovário mamífero (Karimian *et al.,* 2024). Como um agente antioxidante direto, o resveratrol elimina diversas espécies de EROs com mecanismos de transferência de átomo de hidrogênio e transferência sequencial por perda de prótons, protegendo assim as biomoléculas celulares de danos oxidativos (Truong *et al.,* 2018). O efeito do resveratrol na redução dos níveis de EROs vem sendo descrito por vários estudos recentes. Cai *et al.* (2023) relataram que o resveratrol mitiga o estresse oxidativo celular em CGs de ratos reduzindo os níveis de EROs, com consequente redução de MDA e aumento da capacidade antioxidante traduzida pelo aumento da SOD. Esse mesmo efeito foi observado em CGs de *Bos grunniens* associado ao aumento do conteúdo de GSH, expressão reduzida de *Bax* e *Casp3* e aumentada de *Bcl-2*, aliada à maior expressão de *CAT, SOD2* e *GPX1* e maior produção de ATP (Jiang *et al.,* 2024).

Rocha *et al.* (2018) relataram efeito positivo do resveratrol contra a degeneração de folículos pré-antrais após vitrificação de tecido ovariano bovino. Nesse estudo, a adição de 20 μM de RSV ao meio de vitrificação aumentou a proporção de folículos morfologicamente normais no tecido. Em tecido ovariano ovino cultivado *in vitro* a suplementação do meio de cultivo com 2 μM de resveratrol foi eficaz em aumentar a ativação do folículo primordial ao

mesmo tempo em que reduziu as taxas de fragmentação do DNA e estimulou a proliferação das células da granulosa por meio da ativação da via de sinalização PI3K/Akt (Bezerra *et al.,* 2018). No entanto, os potenciais benefícios do resveratrol sobre o ovário parecem ser dependentes da dose. Macedo *et al.* (2017) relataram que folículos ovarianos isolados de ovinos são capazes de crescer até o estágio antral após cultivo *in vitro* em meio contendo 2 µM de resveratrol, mantendo as mesmas taxas de danos ao DNA, níveis de GSH e função mitocondrial que o meio controle. Entretanto, a adição de 30 µM de resveratrol induziu um aumento significativo nos níveis de fragmentação do DNA e estresse oxidativo, além de diminuir a atividade mitocondrial. Relatório de Wang *et al.* (2010) corroboram esses achados. Nesse estudo, após a cultura *in vitro* de células teca-intersticiais de rato, o tratamento com resveratrol (70 ou 100 µM) reduziu a viabilidade celular e promoveu um efeito pró-apoptótico de maneira dose-dependente, evidenciado pelo aumento da ativação das caspases-3/7 e pela intensificação da fragmentação do DNA. O efeito dose-dependente do resveratrol também já foi relatado em oócitos em um modelo de gato doméstico. Oócitos imaturos vitrificados e cultivados na presença de 1,5 mmol/L de resveratrol apresentaram baixa capacidade de atingir metáfase II e de formar blastocistos do que aqueles cultivados em dose mais baixa (1 mmol/L) (Comizzoli *et al.,* 2009). Na modulação da atividade antioxidante resveratrol pode se comportar como um antioxidante ou pró-oxidante dependendo de muitos parâmetros, incluindo a dose e o microambiente (Shaito *et al.,* 2020). Alguns estudos têm demonstrado que o resveratrol tem efeitos bifásicos dependentes da concentração, sendo um antioxidante em baixas doses e pró-oxidante em altas doses (Posadino *et al.,* 2015).

2.8 Limitações físico-químicas do resveratrol e uso de nanopartículas poliméricas

A pluralidade de alvos moleculares do resveratrol no ovário evidencia seus múltiplos benefícios na reprodução de fêmeas. No entanto, apesar das vantagens, existem alguns problemas associados à natureza físico-química do resveratrol que podem limitar sua aplicabilidade plena no campo da preservação da função reprodutiva *in vivo* e *in vitro*. Inicialmente, convém destacar que o resveratrol é altamente lipofílico e apresenta solubilidade em água de apenas 0,03 mg/ml, o que é considerada uma das principais razões para a baixa biodisponibilidade desse composto, o que pode reduzir as atividades do resveratrol em sistemas biológicos (Radeva *et al.,* 2025; Banshoya *et al*., 2024). Outro problema associado é sua baixa estabilidade química em diferentes condições de pH e temperatura. Foi relatado que o resveratrol é quimicamente mais estável em condições ácidas. Particularmente, mais de 70% da

substância permaneceu estável em pH ácido (5-6) e neutro por mais de 200 dias, enquanto pH básico (8-10) reduziu o percentual de estabilidade do composto para cerca de 2% em apenas 2 meses (Robinson *et al.,* 2015). Foi relatado também que o pH determinada o efeito da temperatura sobre a estabilidade do composto. Em meios ácidos nos quais o resveratrol é estável, a temperatura teve impacto mínimo na degradação. Contrariamente, em pH alcalino, temperaturas mais altas (37 °C) aceleram ainda mais a taxa de degradação do resveratrol (Zupancic *et al.,* 2015). Além disso, o resveratrol é fotoinstável e propenso à auto-oxidação e à geração subsequente se semiquinonas complexas, quininas e fenantrenoides tóxicos limitando suas aplicações *in vitro* e *in vivo* (El-Sayed *et al.,* 2025; Santos *et al.,* 2019). Kumar *et al.* (2017) relataram que a exposição do resveratrol à luz induz modificações estruturais no composto. Notavelmente, foi observado que quando o *trans*-resveratrol é irradiado com luz UV ou exposto à luz visível a forma *trans* é convertida para a forma *cis*.

Dadas as importantes limitações inerentes à natureza físico-química do resveratrol, diferentes grupos de pesquisa vêm empreendendo esforços significativos para explorar e melhorar a estabilidade e a biodisponibilidade desse composto *in vivo* e *in vitro* ampliando seu potencial biomédico. Para isso, a utilização de sistemas carreadores de fármacos baseados em nanotecnologia vem sendo relatados como estratégias promissoras para aumentar o potencial farmacológico do resveratrol (Ali *et al.,* 2024; Najafiyan *et al.,* 2024; Zaki *et al.,* 2024). Nesse contexto, a nanotecnologia consiste na manipulação intencional de materiais em escala nanométrica (normalmente variando em escala de tamanho de 1 a 100 nm), permitindo sua reorganização em nanosistemas com propriedades funcionais aprimoradas (Nasrollahzadeh *et al.,* 2019). As nanopartículas são o resultado final da modificação tecnológica da matéria e, dependendo de seus tamanhos, são alguns graus maiores do que um átomo, consequência do processamento molecular da matéria. Como possuem características aprimoradas, como estabilidade autorreativa e autorremontagem, são facilmente adaptáveis e podem ser modificadas para atingir uma característica específica ou propriedades pretendidas, como alta área de superfície quando comparadas a substâncias convencionais (Yusuf *et al.,* 2023; Zaki *et al.,* 2024). A utilização de nanopartículas como agentes nanocarreadores tem como objetivo principal transportar moléculas desejadas para locais alvos maximizando seus efeitos ao mesmo tempo em que melhoram a estabilidade e solubilidade dos compostos, controlando a sua liberação e minimizando sua toxicidade (Hamimed *et al.,* 2022).

O nano-aprisionamento do resveratrol pode aumentar sua estabilidade ao atuar como uma barreira forte contra elementos externos capazes de induzir sua deterioração química, melhorando sua biodisponibilidade e, consequentemente, seus efeitos biológicos associados

(Sarfraz *et al.,* 2023). No entanto, a escolha dos materiais precursores de nanocarreadores para esse fim é um fator crítico para otimizar a aplicabilidade e os resultados desses sistemas, pois esses materiais devem atender a critérios fundamentais, como biocompatibilidade, biodegradabilidade e baixa imunogenicidade (Essa *et al.,* 2020). A família dos nanocarreadores inclui, atualmente, nanocarreadores baseados em lipídios (Jakoby *et al.,* 2015), dendrímeros (Vidal *et al.,* 2015), nanotubos de carbono (Zygouri *et al.,* 2023), nanocarreadores inorgânicos como as nanopartículas de prata e ouro (Asl *et al.,* 2023) e nanopartículas poliméricas (Li *et al.,* 2023). Estas últimas são os materiais mais comumente explorados para a construção de sistemas de entrega de fármacos versáteis baseados em nanopartículas (Scicluna *et al.,* 2020) e diversas investigações recentes vêm avaliando a eficácia dessas plataformas na encapsulação do resveratrol com diversas finalidades (Mohammadi *et al.,* 2025; Cavalcante de Freitas *et al.,* 2023; Zhang *et al.,* 2024).

As NPPs são nanoestruturas sólidas coloidais com tamanho variando de 10 a 1000 nanômetros projetadas a partir de polímeros sintéticos como policaprolactona e poliacrilato (Aguilera-Correa *et al.,* 2021). Polímero naturais como alginato e quitosana ou mesmo proteínas como a albumina também podem ser usados com esse propósito, embora esses compostos naturais ofereçam menor estabilidade, especialmente em temperaturas e/ou pressões elevadas (Singh *et al.,* 2017). Dentre os polímeros sintéticos, a policaprolactona (PCL) vem sendo relatada como uma plataforma biocompatível para o desenvolvimento de nanopartículas carreadoras (Lukasiewicz *et al.,* 2021). Devido a sua natureza hidrofóbica, o encapsulamento de ativos hidrofóbicos, como o resveratrol é bastante fácil. Além disso, comparado com outros materiais poliméricos, a biodegradação do PCL é mais lenta, permitindo o desenvolvimento de sistemas de liberação controlada de ativos (Elmowafy *et al.,* 2023). NPPs têm como vantagens maior estabilidade e versatilidade no design podendo ser projetadas para ter propriedade específicas, como tamanho, forma e carga de superfície (Tariq *et al.,* 2023). Aliado a isso, apresentam maior biocompatibilidade que outros materiais, apresentam baixa ou nenhuma toxicidade e podem ser biodegradáveis, tornando-as candidatas promissoras para compor sistemas de nanoentrega (Freitas *et al.,* 2023). Como sistema de entrega, NPPs baseadas em PCL ou outros polímeros têm permitido a elaboração de plataformas com alto potencial de aprisionamento e estabilidade melhorando a solubilidade e biodisponibilidade de compostos ativos, além de proteção aprimorada do ativo farmacológico contra luminosidade, oxigênio, ataque enzimático e variações de pH e temperatura, além de perimir a liberação prolongada de compostos bioativos (Sristi *et al.,* 2024; Wang *et al.,* 2024; Salla *et al.,* 2024; Elmowafy *et al.,* 2023; Yee *et al.,* 2022). Aliado a isso, a encapsulação parece potencializar o efeito do fármaco

em doses reduzidas, prevenindo a toxicidade inerente de compostos quando utilizadas doses altas (Geszke-Moritz *et al.,* 2024). Dados os efeitos negativos do resveratrol sobre células ovarianas quando administrado em concentrações maiores (Macedo *et al.,* 2017; Wang *et al.,* 2010; Comizzoli *et al.,* 2009), a utilização de NPPs parece ser uma estratégia promissora para reduzir sua toxicidade e aproveitar todo o potencial desse composto no ovário.

Embora os efeitos da encapsulação de resveratrol em NPPs durante o cultivo *in vitro* de folículos ovarianos ainda não tenham sido suficientemente descritos na literatura, os resultados atuais obtidos com essas plataformas apontam direções de pesquisa encorajadoras para o campo da pesquisa ovariana. Por exemplo, Gonçalves *et al.* (2021) deram um importante passo ao relatar que NNPs de (Poli (ácido láctico-co-glicólico) podem ser absorvidas por oócitos bovinos por via transcelular (via células *cumulus* e projeções transzonais) e paracelular (via zona pelúcida) e não comprometeram a viabilidade celular nem o desenvolvimento embrionário, indicando pouca ou nenhuma toxicidade aumentando o entusiasmo em relação ao uso dessa ferramenta para entrega de fármacos ou moléculas visando células ovarianas. Especificamente em relação ao resveratrol, Sarma *et al.* (2022) encapsularam o resveratrol em NPPs, o que resultou em maior biodisponibilidade e melhor atividade de eliminação de radicais livres *in vitro* do que o resveratrol livre. Sanna *et al.* (2012) desenvolveram NPPs carregadas com resveratrol que forneceram proteção significativa contra a degradação induzida pela luz. Da mesma forma, Detoni *et al.* (2012) mostraram que o encapsulamento de resveratrol em NPPs forneceu proteção contra a radiação UV, melhorando sua biodisponibilidade para vários alvos. Abordagens usando cultivo de células *in vitro* também já demonstraram efeitos melhorados do RSV após encapsulação em NPPs. Em linhagem celular de neuroblastoma humano (SH-SY5Y) cultivadas *in vitro*, Katila *et al.,* (2022) observaram efeito potencializado do resveratrol nanoencapsulado em NPPs na redução de EROs e melhora da sobrevivência celular, além de melhorar o potencial de membrana mitocondrial na comparação com o resveratrol livre (não encapsulado). Além disso, NPPs baseadas em poli(N-vinilpirrolidona) -b-poli(ε-caprolactona) carregadas com resveratrol demonstraram maior neuroproteção do que uma dose equivalente de resveratrol livre e um efeito protetor contra ROS em cultura de células corticais de ratos (Lu *et al.,* 2013).

3 JUSTIFICATIVA

A eficiência reprodutiva dos rebanhos é determinante para o desenvolvimento da indústria pecuária, tornando a aplicação de biotécnicas reprodutivas uma estratégia fundamental para o setor. Em bovinos, as biotécnicas atuais dependem fortemente de oócitos provenientes de um conjunto restrito de folículos antrais, deixando uma porcentagem significativa de folículos pré-antrais no ovário inexplorada. O cultivo *in vitro* de tecido ovariano bovino e de folículos secundários isolados surgiram como abordagens promissoras para gerar oócitos a partir de folículos pré-antrais, com potencial para revolucionar os procedimentos atuais de reprodução animal. Também podem fornecer um sistema modelo *in vitro* para avançar o conhecimento dos mecanismos que regulam a foliculogênese *in vivo.* Apesar dos avanços, a obtenção de oócitos bovinos competentes a partir de folículos pré-antrais continua sem sucesso, principalmente devido à ausência de sistemas de cultivo capazes de reproduzir adequadamente os estímulos bioquímicos e biomecânicos críticos para o desenvolvimento folicular. Paralelamente, o estresse oxidativo ainda constitui um obstáculo persistente, comprometendo a qualidade folicular pós-cultivo e demandando estratégias adicionais de controle redox *in vitro*.

A recente conscientização de que estímulos bioquímicos e biomecânicos fornecidos pela matriz extracelular influenciam fortemente a foliculogênese por meio de interações coordenadas bidirecionais vem destacando que os atuais modelos de cultivo *in vitro* precisam considerar mecanismos que vão além da própria unidade folicular. Portanto, caracterizar as mudanças estruturais da matriz extracelular durante o cultivo *in vitro* de tecido ovariano bovino é crítico para orientar o design de plataformas que sustentem a remodelação coordenada da matriz necessária para o desenvolvimento folicular *ex vivo*. Além disso, fornecer um microambiente tridimensional para o cultivo de folículos secundários isolados utilizando andaimes derivados de matriz extracelular descelularizada pode ser uma estratégia interessante, dado que o tecido ovariano após a descelularização retém as pistas bioquímicas e biomecânicas do tecido nativo, críticas para o crescimento folicular. Complementarmente, a suplementação do meio de cultivo com resveratrol encapsulado em nanopartículas poliméricas pode favorecer o equilíbrio redox do microambiente *in vitro*, considerando a potente atividade antioxidante desse composto fenólico, cuja biodisponibilidade, naturalmente limitada, pode ser significativamente aprimorada por meio da nanotecnologia.

4 HIPÓTESES CIENTÍFICAS

Fase 1:

- O cultivo *in vitro* de tecido ovariano bovino induz alterações estruturais na matriz extracelular, compromete a integridade folicular e reduz a capacidade antioxidante enzimática do tecido.

- A suplementação com ácido ascórbico e resveratrol durante o cultivo *in vitro* de tecido ovariano bovino mitiga o estresse oxidativo, preserva a integridade da matriz extracelular e favorece a sobrevivência celular e o desenvolvimento folicular**.**

Fase 2:

- A descelularização de tecido ovariano bovino produz bioandaimes com características bioarquitetônicas semelhantes ao tecido ovariano nativo;

- Andaimes de matriz extracelular ovariana descelularizada fornecem um microambiente tridimensional favorável ao cultivo *in vitro* de folículos secundários bovinos, promovendo maior viabilidade e preservação da ultraestrutura celular em relação ao sistema bidimensional;

- A suplementação com resveratrol nanoencapsulado favorece a viabilidade folicular, preserva a ultraestrutura celular e reduz o estresse oxidativo em folículos secundários bovinos cultivados *in vitro* em andaimes ovarianos descelularizados.

5 OBJETIVOS

5.1 Objetivo geral

Investigar os efeitos do cultivo *in vitro* e da suplementação dos meios com substâncias antioxidantes na integridade estrutural da matriz extracelular e na viabilidade folicular em tecido ovariano bovino e avaliar a eficiência de *scaffolds* ovarianos descelularizados e do resveratrol nanoencapsulado em nanopartículas poliméricas no cultivo *in vitro* de folículos secundários isolados do ovário bovino.

5.2 Objetivos específicos

Fase 1:

- Caracterizar as alterações na integridade estrutural do colágeno e dos glicosaminoglicanos da matriz extracelular induzidas pelo cultivo *in vitro* de tecido ovariano bovino, bem como investigar os efeitos da suplementação com 50 μg/ml de ácido ascórbico e/ou 20 μM de resveratrol na preservação e remodelação desses componentes.

- Investigar o efeito do cultivo *in vitro* do tecido ovariano bovino e da suplementação com 50 μg/ml de ácido ascórbico e/ou 20 μM de resveratrol na morfologia e ativação foliculares, sobrevivência das células do estroma e células da granulosa de folículos primordiais e primários.

- Determinar o perfil redox do tecido ovariano cultivado *in vitro* com e sem suplementação com 50 μg/ml de ácido ascórbicos e/ou 20 μM de resveratrol**,** por meio da quantificação dos níveis de tiol e da expressão gênica e atividade das enzimas antioxidantes *CAT, SOD, GPX1, PRDX6.*

Fase 2:

- Desenvolver e caracterizar bioandaimes descelularizados a partir de tecido ovariano bovino, avaliando a eficiência da descelularização e a preservação da ultraestrutura e dos componentes da matriz extracelular;

- Comparar a viabilidade e a integridade ultraestrutural de folículos secundários bovinos cultivados *in vitro* em um sistema bidimensional ou tridimensional em suporte de bioandaimes ovarianos descelularizados;

-Avaliar o impacto da suplementação do meio de cultivo com nanopartículas poliméricas contendo resveratrol (0,02, 0,2 ou 2μM) ou resveratrol livre (2μM) sobre a viabilidade e a preservação ultraestrutural de folículos ovarianos bovinos cultivados *in vitro* em sistema bidimensional e tridimensional.

- Investigar a modulação da expressão gênica de enzimas antioxidantes (CAT, SOD, GPX1, PRDX6 e NRF2) em folículos bovinos cultivados *in vitro* em suporte de bioandaimes ovarianos descelularizados, em meio suplementado com resveratrol livre 2μM e nanoencapsulado (0,02, 0,2 ou 2μM).

6 CAPÍTULO 2

Ascorbic acid and resveratrol improve the structural integrity of the extracellular matrix and enhance follicular survival in cultured bovine ovarian tissue

[O ácido ascórbico e o resveratrol melhoram a integridade estrutural da matriz extracelular e aumentam a sobrevivência folicular em tecido ovariano bovino cultivado]

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Ascorbic acid and resveratrol improve the structural integrity of the extracellular matrix and enhance follicular survival in cultured bovine ovarian tissue

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Abstract

This study aimed to investigate the changes induced by the culture system and the effect of ascorbic acid and resveratrol on collagen fibers, stromal cells, follicle growth and survival, as well as antioxidant enzyme activity in cultured bovine ovarian tissues. In experiment 1, bovine ovarian fragments were cultured in α-minimum essential medium (α-MEM$^+$) for 6 days. Before and after culturing, the fragments were fixed and processed to assess follicular morphology and diameters, stromal cell survival, collagen fibers, and glycosaminoglycans (GAGs). Uncultured and cultured tissues were also used to measure mRNA expression for superoxide dismutase (*SOD*), catalase (*CAT*), glutathione peroxidase (*GPX*), and peroxiredoxin (*PRDX*). Thiol levels and activity of CAT, SOD, and GPX enzymes were also investigated. In experiment 2, bovine ovarian fragments were cultured in α-MEM$^+$ alone or supplemented with 50 μg/mL ascorbic acid or both 50 μg/mL ascorbic acid and 20 μM resveratrol for 6 days. In experiment 1, cultured tissues had higher percentages of growing follicles, but higher percentage of degenerated follicles than uncultured slices ($P<0.05$). Additionally, the collagen and GAGs network became disorganized, with reduced deposition around primordial and primary follicles ($P<0.05$). The number of stromal and granulosa cells, as well as follicular and oocyte diameters were reduced in both follicular categories compared to uncultured tissue ($P<0.05$). Expression of mRNA for *CAT, SOD, GPX*, and *PRDX* was downregulated in 6-day cultured tissues ($P<0.05$). Similarly,

thiol levels and CAT activity were also reduced (P<0.05). In experiment 2, ascorbic acid or both ascorbic acid and resveratrol increased the rate of follicular diameters and survival, and the number of granulosa and stromal cells compared to tissues cultured in the control medium (P<0.05). Both ascorbic acid and resveratrol improved collagen density and preserved the GAG network, as well as increased thiol levels and CAT activity (P<0.05). In conclusion, *in vitro* culture of ovarian tissue favored follicular activation, but reduced the proportion of normal follicles, collagen, GAG network, stromal cell numbers, and tissue antioxidant protection. Ascorbic acid alone or in association with resveratrol improved the preservation of extracellular matrix components and enhanced follicular survival.

Keywords: *In vitro* culture; Follicular growth; Extracellular matrix; Ovarian stroma; Oxidative stress; Antioxidant.

1 Introduction

Ovarian tissue culture represents a valuable tool to study the factors that regulate primordial follicle development and to promote their growth *in vitro* in a controlled environment [1,2,3]. Because ovarian cortical tissue predominantly contains primordial and early-growing follicles, mature oocytes cannot be obtained unless those follicles appropriately resume development and grow *in vitro* to the antral stage [4]. Thus, an ideal culture system needs to support the activation of dormant follicles and enable them to progress growth. After *in vitro* culturing ovarian tissues, various studies have focusing only on follicle growth and survival, but currently, understanding the changes in stromal cells and extracellular matrix is critical to develop efficient culture systems to increase follicle survival and growth in long term culture [5,6]. Considering that the ovarian tissue is dynamic and mechanically responsive, ovarian cells and their microenvironment can have a key role in maintenance of follicle survival and growth *in vitro* [7,8,9,10]. A growing body of evidence has demonstrated that the crosstalk between the extracellular matrix (ECM) and follicles / ovarian stromal cells through integrin binding and other receptors appears critical for supporting proper folliculogenesis [3,11,12].

In ovarian stroma, collagen one and three are the most represented matrisome-related protein, and the matrix is not static during ovarian development [13,14]. The follicles are systematically distributed along a carefully controlled gradient of collagen on the matrix, and ovarian rigidity appears to play a substantial role in both follicular quiescence and activation [12,15]. Beyond collagen, glycosaminoglycans (GAGs) are heteropolysaccharides, not only

involved in ECM structure but also in water retention, and growth factors sequestering [16]. The GAGs can thereby promote cell growth, migration, adhesion, and differentiation [17]. Besides ECM, ovarian stromal cells have a significant role in folliculogenesis, particularly in activating primordial follicles [18, 19]. Within the follicles, granulosa cells (GCs) enhance glycolysis and promote the activation of primordial follicles [20]. Furthermore, GCs affect follicular growth, development, and maturation in an autocrine and paracrine manner [21]. Therefore, characterizing *in vitro* culture-induced changes in stromal cells, ECM, and GCs of early-stage follicles is a necessary field of research.

Several factors are associated with increased oxidative stress during *in vitro* culture, including ovarian fragmentation, exposure to ischemic conditions, manipulation, and excessive light exposure [2]. In recent years, increasing attention has been paid to the role of oxidative stress as a modulator of the dynamics of ovarian stromal components during *in vitro* culture [22,23,24]. The oxidative stress impacts normal cellular signaling, DNA, lipids, and proteins, which impairs the synthesis of ECM components [25]. The oxidative stress damage induced by advanced glycation end products can result in abnormal cross-linking of collagen and elastin in ECM, which can affect the proliferation and survival of granulosa cells [26]. Several biological responses, such as signaling pathways activation and GCs apoptosis, are triggered by oxidative stress. Efficient culture systems need to be able to mitigate these effects. Hence, antioxidant capacity has been considered a critical endpoint for the success of *in vitro* ovarian tissue culture systems [27,28]. Reduced glutathione (GSH) is a non-enzymatic antioxidant that neutralizes peroxides. Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and peroxiredoxin (PRDX) are representative enzymatic antioxidants, and the expression of these enzymes has been used as a marker of the efficiency of *in vitro* culture systems [22,29]. Supplementation of the culture medium with free radical scavengers has been proposed to reduce the overproduction of ROS and maintain the homeostatic balance during *in vitro* ovarian tissue culture [22]. Ascorbic acid, a substance that protects against the damage caused by ROS and a potent free radical scavenger, has been shown to improve follicular survival, and plays an important role in collagen synthesis during follicular growth, at which the extracellular matrix is constantly remodeled [30]. Resveratrol, another free radical scavenger, promoted primordial follicle activation, reducing DNA fragmentation and stimulating granulosa cell proliferation [31].

This study aims to investigate the changes induced by the culture system and the effect of ascorbic acid and resveratrol in the ECM and stromal cells around primordial and primary follicles, follicular morphology and activation, as well as in the antioxidant status of bovine

ovarian tissue. Furthermore, we measured follicular and oocyte diameter and evaluated the survival of granulosa cells in *in vivo* and *in vitro* grown follicles.

2 Material and methods

2.1 Chemicals

The culture media and other chemicals used in the present study were bought from Sigma Chemical Co. (St. Louis, MO, USA), except those noted within the text.

2.2 Source of ovaries and ethical approval

Ovaries from healthy mixed-bred cows (n = 40) at reproductive age were collected in a local slaughterhouse and divided into two experiments (n = 20 per experiment). All experimental procedures were approved by the Ethics and Animal Welfare Committee of the Federal University of Ceará, ruling number UFC-CE, 04/22. Each animal was considered an experimental unity, and ten repetitions were performed per experiment, comprising a total of forty ovaries. Immediately after the collection, the ovaries were washed once in alcohol 70% for approximately 10 s and twice in α-MEM buffered with 20 mM HEPES and supplemented with 100 µg/ml penicillin and 100 µg/ml streptomycin. After washing, the ovaries were transported within 1 h to the laboratory in α-MEM HEPES solution at 4 °C.

2.3 Experimental design

2.3.1 Experiment 1: Effect of in vitro culture of ovarian tissue in base medium on ECM component dynamics, follicular activation and morphology, and redox status

Upon arrival at the laboratory, under sterile conditions, cortical fragments (3x3x1 mm) were removed from each pair of ovaries and then transferred into a dissection medium composed by α-MEM supplemented with penicillin (100µg/mL) and streptomycin (100µg/mL) pre-warmed at 38.5 ºC. For uncultured control, of each pair of ovaries, four cortical fragments were fixed in paraformaldehyde (4%) for 24 hours at room temperature for histological and histochemical analyses, eight fragments were used to assess thiol levels and activity of

antioxidant enzymes (CAT, SOD, and GPx), and two fragments for quantification of mRNA for *CAT, SOD, PRDX6,* and *GPX1*. The remaining fragments (n = 14 per animal) were cultured *in vitro* in 24-well culture dishes. The culture medium consisted of α-MEM (pH 7,2 – 7,4) supplemented with 1.25 mg/mL of bovine serum albumin, 10 µg/mL insulin, 5.5 µg/mL transferrin, 5 ng/mL selenium, 2mM glutamine, 2mM hypoxanthine, 100 UI/mL penicillin and 100 µg/mL streptomycin (α-MEM$^+$). The fragments (2 per well) were cultured in 500 µL of α-MEM$^+$ for 6 days, at 38.5 ºC, 5% CO_2 in a humidified incubator. Every two days, 250 µL of culture medium was replaced by fresh medium [32]. After culture, part of the fragments was destined for histological and histochemical analysis, while the remaining fragments were stored at -80 ºC for subsequent quantification of mRNA for *CAT, SOD, PRDX6,* and *GPX1* or to evaluate of thiol levels and activity of antioxidant enzymes (CAT, SOD, and GPx)

2.3.2 Experiment 2: Effect of ascorbic acid and resveratrol on ECM components dynamics, follicular activation and morphology, and redox status

Ovarian tissue fragments (n = 36 per animal) were cultured *in vitro* in 500 µL of α-MEM$^+$ alone (control medium) or supplemented with 50µg/mL of ascorbic acid [33] or both 50µg/mL of ascorbic acid and 20 µM of resveratrol [31]. The culture conditions were the same as previously defined. After culturing, part of the fragments was used for histological and histochemical analysis as described in Experiment 1, while the remaining fragments were stored at -80 ºC for evaluation of thiol levels and activity of antioxidant enzymes (CAT, SOD, and GPX). This experiment was repeated ten times.

2.4 Histology analysis

Ovarian fragments before and after culture from both experiments were fixed for 24 hours at room temperature in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4). After fixation, the ovarian fragments were dehydrated in ethanol, clarified with xylene, and embedded in paraffin wax. For each piece of ovarian cortex, 6-µm sections were mounted on slides and stained with eosin and hematoxylin (H&E). Follicles with a visible oocyte were counted. Follicles with mostly flattened granulosa cells surrounding the oocyte were considered primordial follicles; follicles with one layer of mainly cuboidal granulosa cells were classified as primary follicles [22]. Secondary and antral follicles were excluded from the count. Follicles

were further classified as normal when an intact oocyte was present, surrounded by granulosa cells that are well organized in one or more layers, and have no eosinophilic nucleus. Degenerated follicles were defined as those with a retracted oocyte, which has an eosinophilic nucleus and/or is surrounded by disorganized granulosa cells [22]. The same slides were used to analyze stromal cell density, granulosa cell survival, and measurement of follicular and oocyte diameter, as described below.

2.5 Histochemical analyses for collagen and glycosaminoglycans

Ovarian sections of 6 µm were incubated in Sirius Red solution (PSR, Abcam kit) (0.1%) for 1 h at room temperature, according to methodology described by Rittié *et al.* [34]. The collagen fibers in the tissue as a whole and around primordial and primary follicles in both experiments were evaluated. To evaluate glycosaminoglycans (GAGs) in the tissue as a whole and around primordial and primary follicles, the ovarian sections were incubated in Alcian Blue solution for 5 minutes at room temperature [35] according to the manufacturer's instructions. For both analyses, images of fifty fields in the tissue as a whole and fifty primordial and fifty primary follicles were obtained using a DS Cooled DS DS-Ri1 camera attached to a microscope (Nikon, Eclipse, TS 100, Tokyo, Japan) with 400× magnification [14]. The Fiji-ImageJ software (Version 1.54f, 2023) was used to quantify the percentage of collagen and GAGs after delimiting a region of interest (ROI) of 100 μm^2 in the tissue as a whole and around each follicle.

2.6 Assessment of stromal cell density, measurement of granulosa cell number, and follicle and oocyte diameter

Stromal cell density, granulosa cell number, as well as follicle and oocyte diameter were evaluated in morphologically normal primordial and primary follicles. A total of 50 primordial follicles and 50 primary follicles with a visible oocyte nucleus in the section were photographed in each treatment in both experiments using a DS Cooled DS DS-Ri1 camera attached to a microscope (Nikon, Eclipse, TS 100, Tokyo, Japan) with 400× magnification. Stromal cells density was evaluated by calculating the cell number in an area of 100 μm^2 around the follicles. The images were assessed sequentially for cell counting using the Fiji-ImageJ (Version 1.54f, 2023). Initially, the images were converted to 8-bit for better visualization of the cells. Then, a ROI of 100 μm^2 area was determined around the follicles, and stromal cells located within that

region were evaluated by counting the cell nucleus using the cell counter function. The granulosa cells were excluded from this count and counted separately. The average of two perpendicular measurements to one another evaluated on the inner side of the basement membrane was reported as follicular diameter. Average oocyte diameter measurements were made based on two perpendicular measurements from the plasma membrane to the plasma membrane of each oocyte [36]. All measurements were made by a single investigator blind to experimental groups using previously calibrated Fiji-ImageJ (Version 1.54f, 2023).

2.7 Real-time quantitative PCR

Real-time quantitative PCR was used to evaluate the levels of messenger RNAs (mRNA) for *CAT, SOD, GPX1*, and *PRDX6* in Experiment 1, following the protocol outlined by Silva *et al.* [22]. Briefly, the total RNA was isolated in uncultured and cultured ovarian tissue using Trizol reagent (Invitrogen, São Paulo, Brazil) according to the manufacturer's instructions. The total mRNA concentration was assessed by a nanodrop (Biodrop, Cambridge, England), and 50 ng/µL of mRNA was used for reverse transcription. The mRNA quantification was performed using SYBR Green, in a Step One Plus instrument (Applied Biosystems, Foster City, CA, USA). Each real-time reaction (15 µl) contained 7.5 µl of SYBR Green Master Mix (PE Applied Biosystems, Foster City, CA), 5.5 µl of ultra-pure water, 1 µl of cDNA and 0.5 µM of each primer (Table 1). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene expression was used to normalize mRNA levels. The 2^-ΔΔCt method was used to transform the Ct values into normalized relative expression levels [37].

2.8 Biochemical assays to evaluate redox status

Ovarian tissue fragments (100 mg) in both experiments were macerated using potassium phosphate buffer (KH2PO4 and K2HPO4; P9791 and P3786; Sigma-Aldrich; 1:9) in the presence of protease inhibitor (5 mg/mL of aprotinin, A6103; Sigma-Aldrich) and 34.8 mg/mL of phenylmethanesulfonyl fluoride (P76626, Sigma-Aldrich), pH 7.5. Then, the homogenates were centrifuged at 1500G for 10 min at 4°C, and the supernatant was collected for use in the spectrophotometric (Genesis 10s UV-vis; Thermo Scientific) assays as described below, using quartz cuvettes [38]. The obtained samples were destined to evaluate pro-oxidant status based on thiol content and determination of SOD, CAT, and GPx activity.

2.8.1 Determination of total proteins (Bradford method)

Coomassie blue (Quick start/Bradford; Catalogue No. 500–0205; Bio-Rad) was used to determine the total concentration of proteins in each sample according to Bradford [39]. The absorbance was evaluated spectrophotometrically at a wavelength of 595 nm. Total protein concentration was determined using a standard curve constructed using bovine albumin as a standard (0, 2.5, 5, 10, 15, 25, 35, and 50 mg/mL). The standard curve was used to standardize the levels of pro-oxidants (thiol) and antioxidants (SOD, CAT, and GPx), as described below.

2.8.2 Determination of pro-oxidant activity based on thiol content

Total thiol content was determined using 5,5`-dithiobis 2-nitrobenzoic acid (DTNB; Dinâmica, São Paulo, Brazil D8130) as an index of reduced thiol molecules. Thiol residues react with DTNB (10 mM), cleaving the disulfide bond to form a 2-nitro-5-thiobenzoate anion (NTB^{2-}) at a neutral pH. The quantification of NTB^{2-} was determined in a spectrophotometer by measuring absorbance at 412 nm. Results were expressed as nMol of reduced DTNB per milligram of protein [40].

2.8.3 Determination of SOD, CAT, and GPX activity

The SOD activity was measured as the inhibition of adrenaline auto-oxidation [41]. Adrenaline oxidation, in the presence of CAT in the basic medium, leads to the formation of the O_2^- radical, which SOD reacts with, slowing ('inhibiting') the oxidation of adrenaline. The CAT solution (0.048 mg/mL;) was performed by adding (7:3) to glycine buffer, pH 10.2 (Dinâmica, São Paulo, Brazil). Three different volumes (10, 20, or 40 mL) of ovarian tissue homogenate were then added to the solution, and the adrenaline (0.218 mg/mL;) was added to start oxidation. Oxidation was measured at 480 nm every 10 s for 180 s. The CAT activity was determined as the consumption of H_2O_2 as a substrate at 240 nm [42]. A solution of H_2O_2 (PH09717RA; Êxodo Científica, São Paulo, Brazil) and phosphate-buffered saline (PBS; pH 7.4) was mixed in a quartz cuvette at room temperature, and then 50 mg of the ovarian tissue homogenate were added. Every 30 s, the consumption of H_2O_2 was measured twice. The GPX activity was determined by NADPH oxidation. NADPH is consumed by glutathione reductase to convert GSSG to GSH. In the presence of H_2O_2, GPX oxidizes GSH to GSSG and reduces peroxides to alcohols and water. The consumption of NADPH is directly proportional to the

consumption of H_2O_2 and, consequently, GPx activity [43]. The reaction was prepared by mixing 500 mL potassium phosphate buffer (100 mM), composed of 13.6 g/L potassium phosphate monobasic plus 1.86 g/L EDTA and 38 mg/mL GR, 3 mg/mL GSH and 100 mg ovarian tissue homogenate for 10 min at room temperature. Thus, the GPx cysteine could come into contact with GR and GSH. Then, 100 mL NADPH was added to the mixture followed by 100 mL H_2O_2 120 s later. The oxidation of NADPH was measured as a decrease in NAPDH absorbance at 340 nm and was measured every 10 s for 300 s.

Table 7. Oligonucleotide primers used for polymerase chain reaction analysis

Target gene	Primer sequences	GenBank Accession nº.	Amplicon
GAPDH	Sense TGTTTGTGATGGGCGTGAACCA Antisense ATGGCGCGTGGACAGTGGTCATAA	GI: 402744670	183
PRDX6	Sense GCACCTCCTCTTACTTCCCG Antisense GATGCGGCCGATGGTAGTAT	GI: 59858298	105
GPX1	Sense AACGTAGCATCGCTCTGAGG Antisense GATGCCCAAACTGGTTGCAG	GI: 156602645	121
SOD	Sense GTGAACAACCTCAACGTCGC Antisense GGGTTCTCCACCACCGTTAG	GI: 31341527	165
CAT	Sense AAGTTCTGCATCGCCACTCA Antisense GGGGCCCTACTGTCAGACTA	GI: 402693375	165

2.9 Statistical analysis

Statistical analysis was performed using GraphPad Prism 9 software. The Chi-square test was used to analyze the percentage of normal follicles as well as of follicles in each stage of development in both experiments [22]. For the other analyses, the Kolmogorov-Smirnov test was used to test the normality of the data [44]. Analysis of variance (ANOVA) followed by a Tukey test was applied to compare more than two groups with each other. When only two groups were compared, unpaired t-test was applied. Unless stated otherwise, quantitative data were reported as mean ± S.E.M. The P value < 0.05 was considered statistically significant, and significance was defined as *$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$.

3 Results

3.1 Experiment 1: Effect of in vitro culture of ovarian tissue in base medium on ECM component dynamics, follicular activation and morphology, and redox status

3.1.1 In vitro culture of ovarian cortex promotes structural remodeling of ECM

In uncultured tissues, primordial and primary follicles were surrounded by a network of well-organized and evenly distributed collagen fibers (Fig. 1-A: a and c) and GAGs (Fig. 2-A: a and c). On the other hand, the collagen network around the follicles after culture exhibited a great degree of disorganization characterized by the presence of gaps and apparently more curved and fragmented collagen fibers (Fig. 1-A: b and d) while the GAGs network appeared more dispersed and presented gaps around the follicles (Fig. 2-A: b and d). The levels of collagen (Fig. 1-B) and GAGs (Fig. 2-B) surrounding primordial and primary follicles in 6-day cultured tissues were significantly reduced when compared to uncultured tissues ($P < 0.0001$). No differences in collagen and GAGs around cultured primordial and primary follicles were seen. Furthermore, changes in collagen and GAGs were seen beyond the area adjacent to early follicles. In general, the cultured tissue as a whole had a reduction in collagen and GAGs area when compared to uncultured tissues ($P < 0.0001$) (Fig. 3).

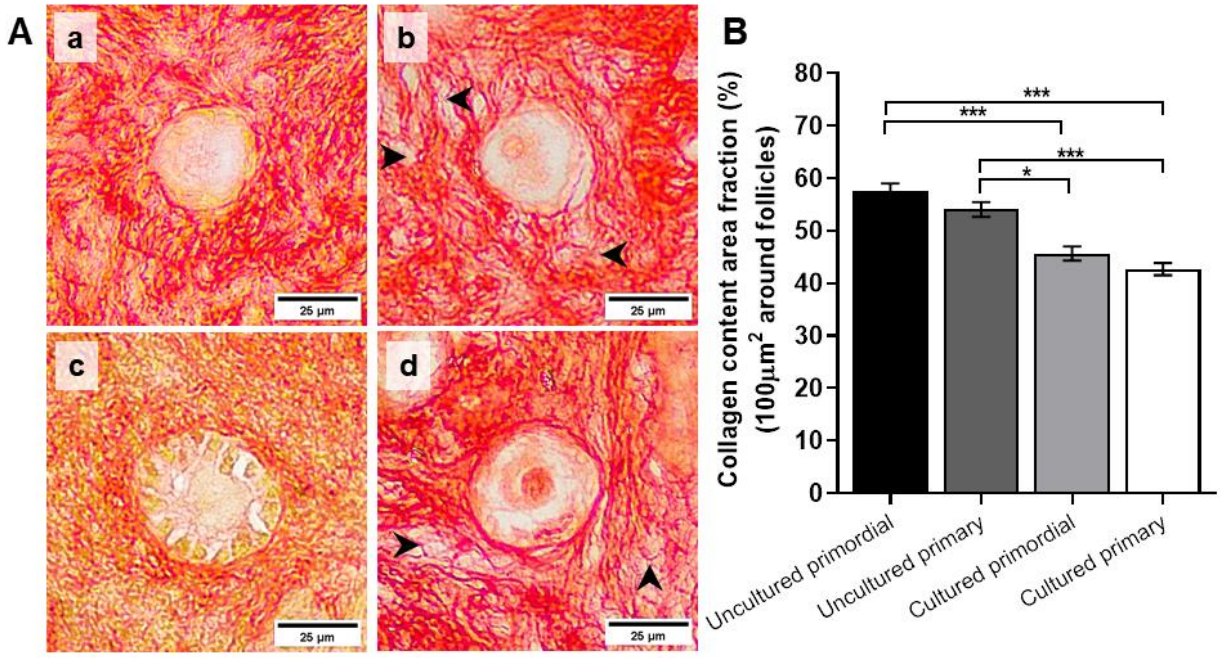

Figure 9. (A) Representative images of the collagen fibers around primordial follicle before (a) and after culture (b) and primary follicle before (c) and after culture (d). Black arrowheads show gaps in collagen network after *in vitro* culture. Magnification = 400x. Scale bars = 25 µm. **(B)** Quantitative measurements of collagen density in an area of 100 μm^2 around the primordial and primary follicles before and after culture. *Asterisks indicate statistically significant difference between treatment groups: $^{*}P < 0.05$, $^{***}P < 0.0001$.

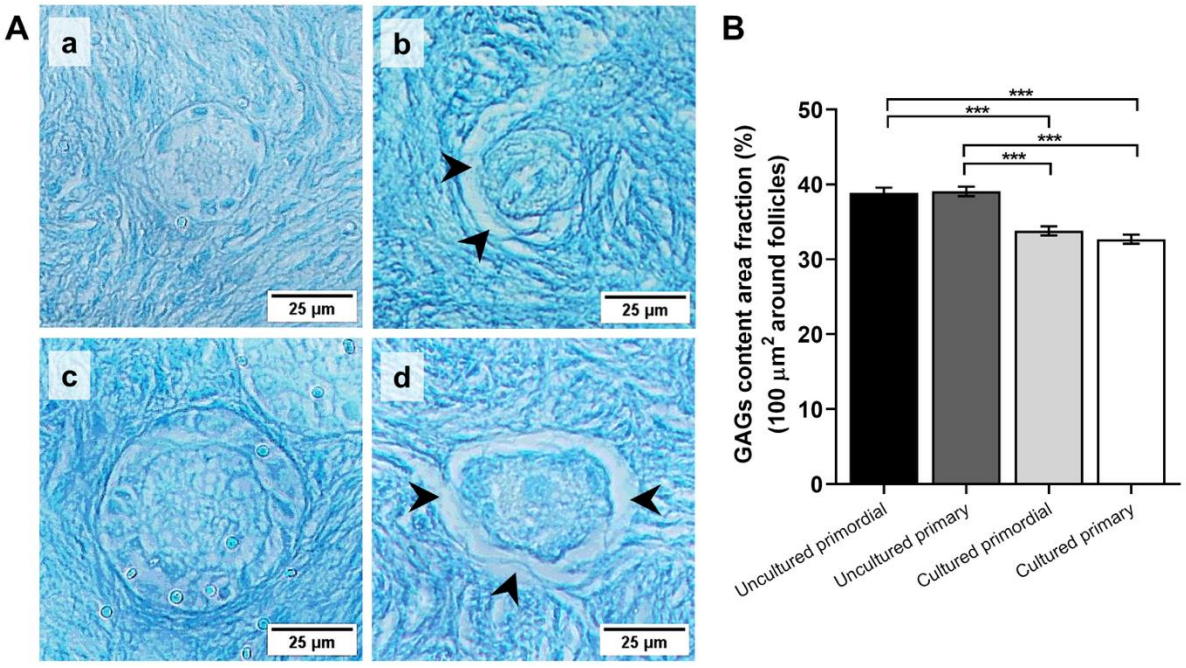

Figure 10. (A) Representative images of the GAGs network around primordial follicle before (a) and after culture (b) and primary follicle before (c) and after culture (d). Black arrowheads show gaps in GAGs network after *in vitro* culture. Magnification = 400x. Scale bars = 25 µm. **(B)** Quantitative measurements of GAGs density in an area of 100 μm^2 around the primordial and primary follicles before and after culture. *Asterisks indicate statistically significant difference between treatment groups: $^{***}P < 0.0001$.

3.1.2 In vitro culture promotes follicular activation and reduces the proportion of morphologically normal follicles

The cultured ovarian tissues had reduced percentages of primordial follicles and a concomitant increase of activated follicles when compared with uncultured control slices (P<0.0001) (Fig. 4). However, cultured tissues had higher percentage of degenerated follicles than those seen in uncultured fragments (P<0.0001) (Fig. 4).

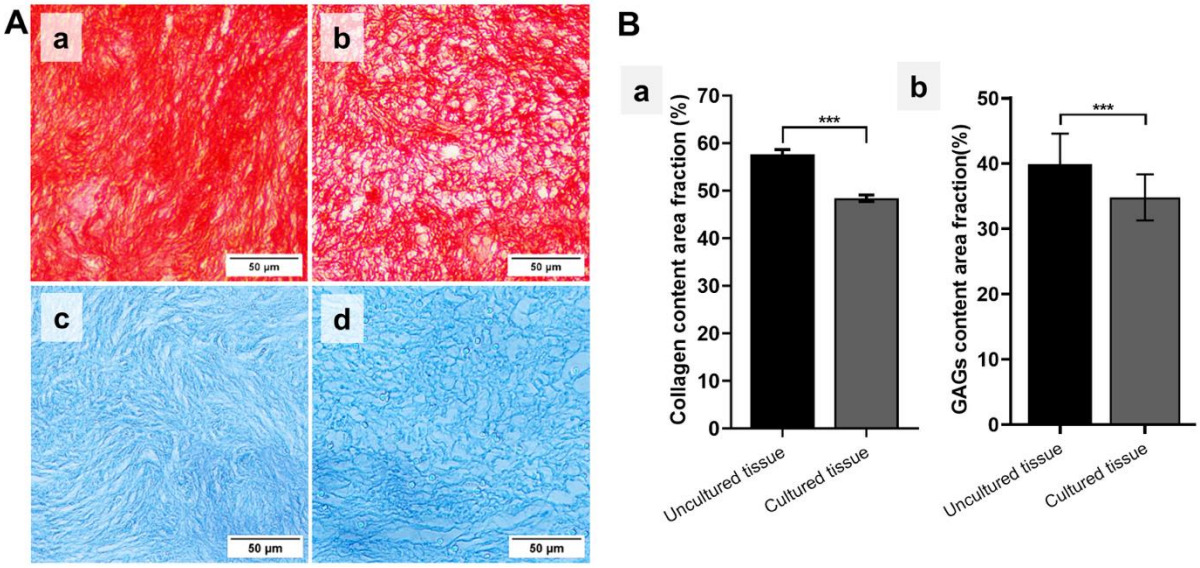

Figure 11. (A) Structural remodeling of the collagen (a – b) and GAGs (c – d) network in the tissue as a whole. Magnification = 400x. Scale bars = 50 µm. **(B)** Quantitative measurements of collagen (a) and GAGs (b) density in tissue before and after culture. *Asterisks indicate statistically significant difference between treatment groups: $^{***}P < 0.0001$.

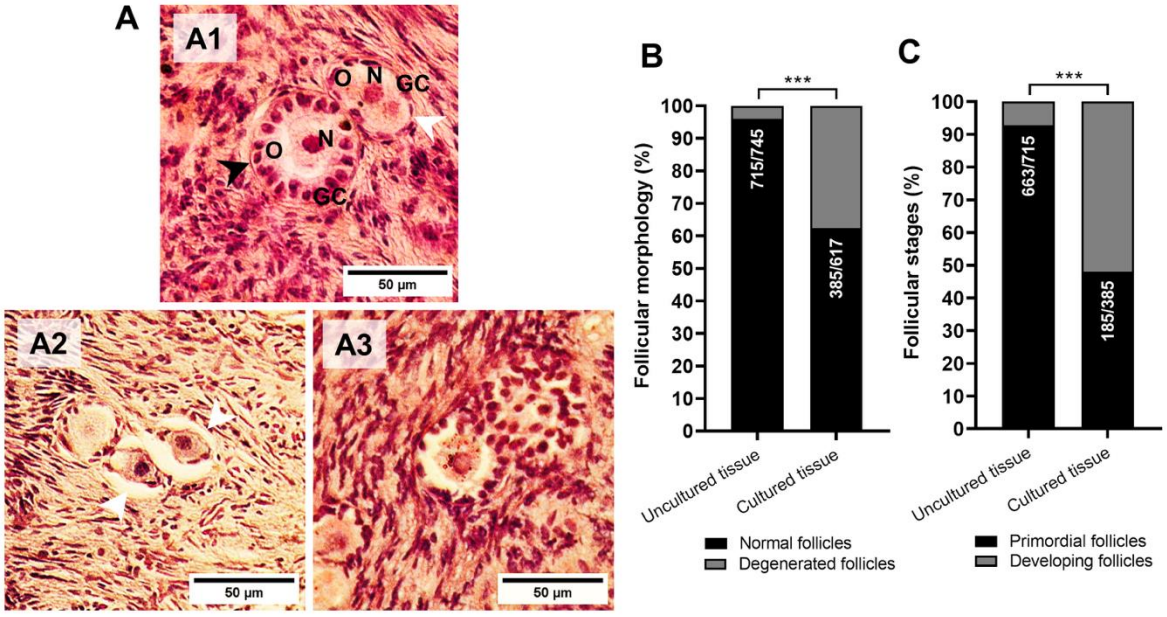

Figure 12. (A) Representative images of ovarian tissue sections stained with hematoxylin and eosin showing follicular histological characterization. A1: Normal primordial (white arrow head) and primary (black arrow head); A2: degenerated primordial follicles (white arrow head); A3: degenerated primary follicle. Magnification = 400x. Scale bars = 50 µm. **(B)** Percentages of morphologically normal follicles in uncultured tissues, and in tissue cultured in α-MEM^{+}. **(C)** Percentages of primordial and primary follicles in uncultured tissues, and tissues cultured in α-MEM+. *Asterisks indicate statistically significant difference between treatment groups: $^{***}P < 0.0001$. O: oocyte; N: oocyte nucleus; CG: granulosa cell.

3.1.3 In vitro culture reduces the survival of stromal and granulosa cell and decreases follicular and oocyte diameter

Representative images of stromal cell density around the primordial and primary follicles are shown in Fig. 5-A. The quantitative measurements (Fig.5-B) demonstrated that there was a decrease in stromal cell number surrounding the primordial and primary follicles in 6-day cultured tissues when compared to uncultured tissues ($P < 0.05$ and $P < 0.0001$, respectively). Furthermore, when the cultured groups were compared to each other, it was found that the stromal cell numbers around primary follicles were lower than in primordial follicles ($P < 0.0001$). It was also observed that the density of stromal cells in the tissue as a whole was significantly decreased after *in vitro* culture (Fig. 6). The changes in granulosa cell number and in follicular and oocyte diameter in primordial and primary follicles are shown in Table 2. After 6 days of culture, the granulosa cell average number, as well as the follicular and oocyte diameters was significantly reduced in both primordial and primary follicles when compared to uncultured tissue ($P < 0.0001$).

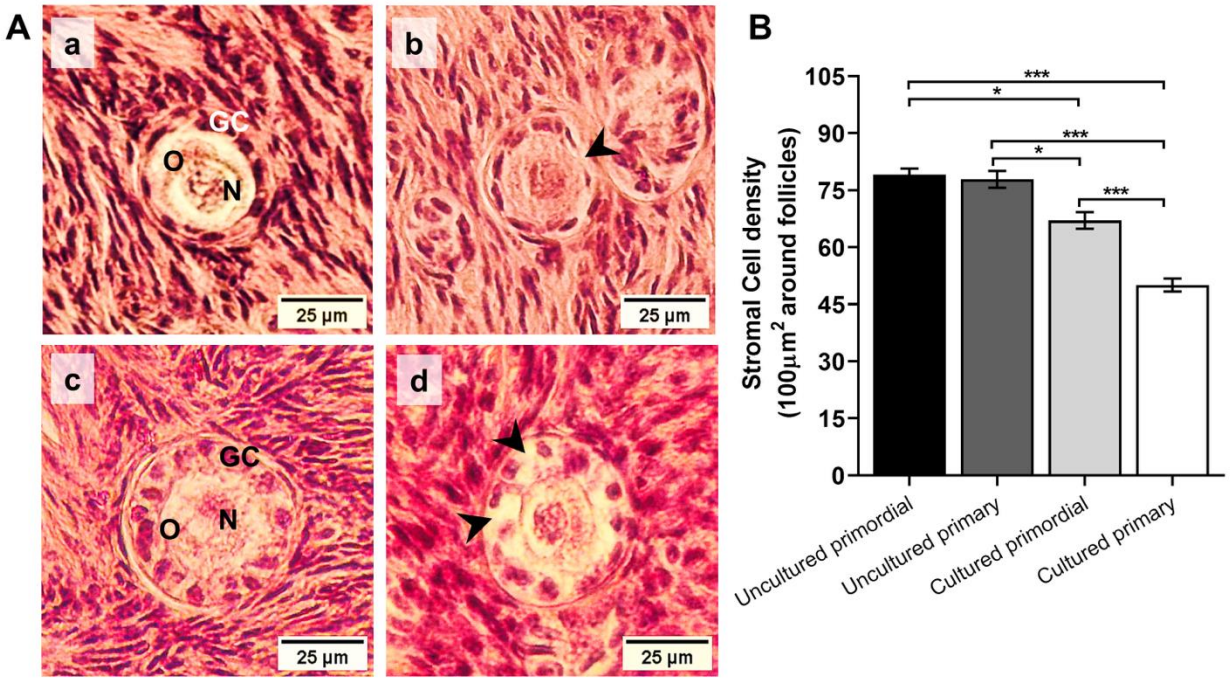

Figure 13. (A) Representative images of the stromal cell density around primordial follicle before (a) and after culture (b) and primary follicle before (a) and after culture (d). Black arrowheads show granulosa cell absent after *in vitro* culture. Magnification = 400x. Scale bars = 25 µm. The **(B)** image shows the quantitative measurements of stromal cells in an area of 100 μm^2 around the primordial and primary follicles before and after culture. *Asterisks indicate statistically significant difference between treatment groups: $^{*}P < 0.05$, $^{***}P < 0.0001$.

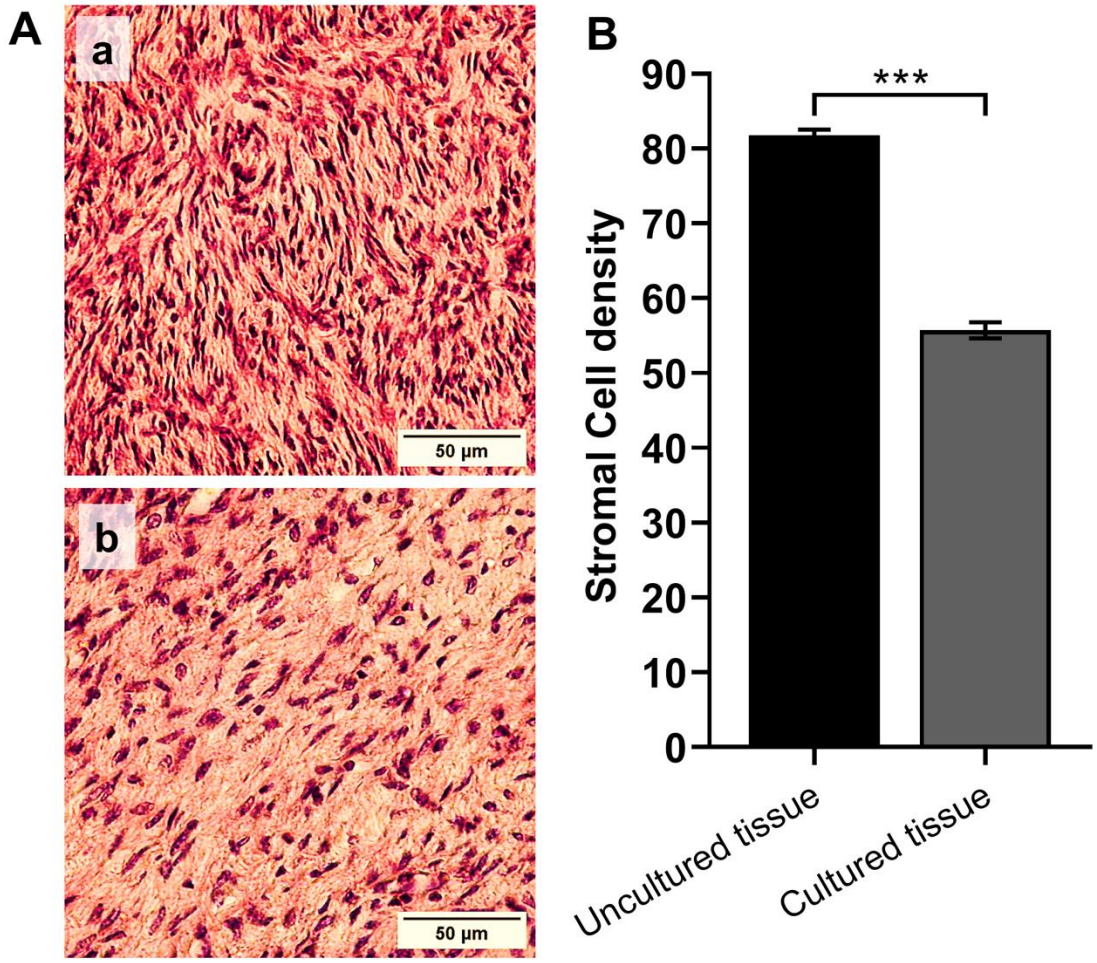

Figure 14. (A) Representative images of the stromal cells distribution within the ovarian cortex as a whole before (a) and after culture (b). Magnification = 400x. Scale bars = 50 µm. **(B)** Quantitative measurement of stromal cells density before and after culture. *Asterisks indicate statistically significant difference between treatment groups: $^{***}P < 0.0001$.

Table 8. Follicular and oocyte diameter and granulosa cell number in primordial and primary follicles before and after *in vitro* culture (experiment 1).

	Diameter				**N. GC**	
	Follicle		***Oocyte***			
	Primordial	Primary	Primordial	Primary	Primordial	Primary
Uncultured control	32.90 ± 0.5***	47.04 ± 1.0***	23.08 ± 0.5***	31.61 ± 0.6***	10.4 ± 0.2***	16.7 ± 0.3***
Cultured	28.63 ± 0.7	35.64 ± 0.9	18.26 ± 0.4	22.21 ± 0.7	8.3 ± 0.2	11.6 ± 0.3

GC: granulosa cell. Values referring to follicular and oocyte diameter are expressed in µm. *Asterisks indicate statistically significant differences between treatment groups: ***P < 0.0001.

3.1.4 In vitro culture reduces the levels of mRNA for CAT, SOD, PRDX6, and GPX1

The relative expression of mRNA for *CAT, SOD, PRDX6*, and *GPX1* is shown in Fig. 7 (A-D). This figure demonstrates that 6-day *in vitro* culture significantly downregulated of all assessed mRNA when compared to those seen in uncultured tissues for *SOD* ($P < 0.0001$) and for *CAT, PRDX6*, and *GPX1* ($P < 0.001$).

3.1.5 In vitro culture decreases thiol levels and CAT enzymatic activity but not SOD and GPX

The Fig. 8-A shows the levels of reduced DTNB molecules as an index of reduced thiol molecules in uncultured and cultured tissues. The concentration of reduced thiol molecules was significantly decreased in ovarian tissue at the end of 6 days of culture. The Fig. 8-B shows that CAT activity was significantly reduced after culture ($P < 0.05$). When the activity of SOD and GPX was evaluated (Fig. 8-C and D), no statistically significant difference was found between uncultured and cultured tissues ($P = 0.1907$ and $P = 0.9459$, respectively, for SOD and GPX).

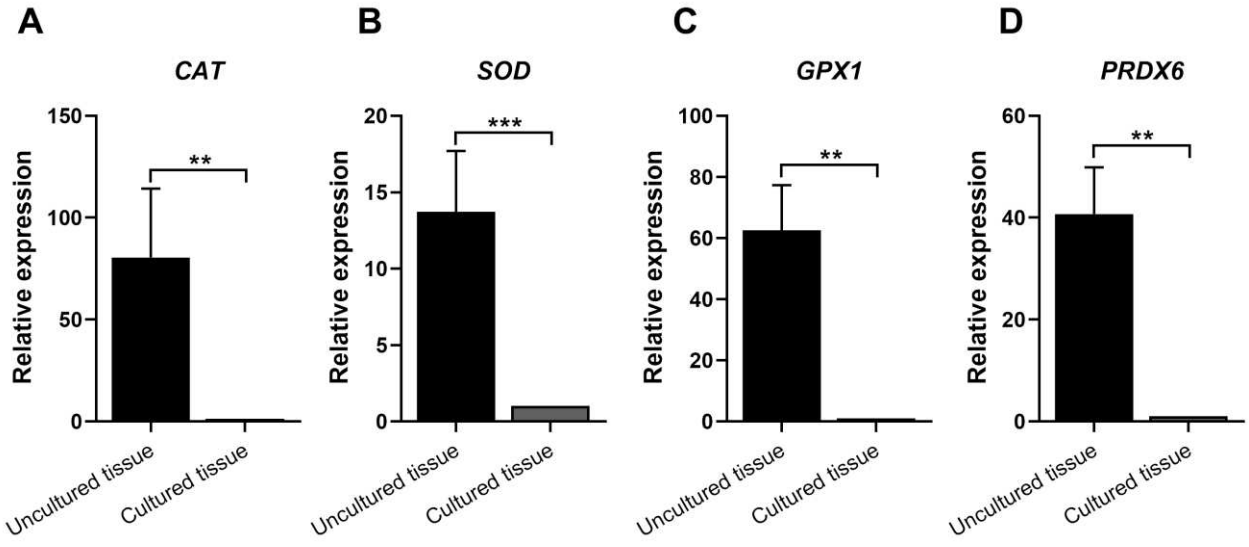

Figure 15. Relative mRNA expression levels for antioxidant enzymes in uncultured and cultured ovarian tissues. *Asterisk indicates statistically significant difference between groups: ***$P < 0.0001$, **$P < 0.001$.

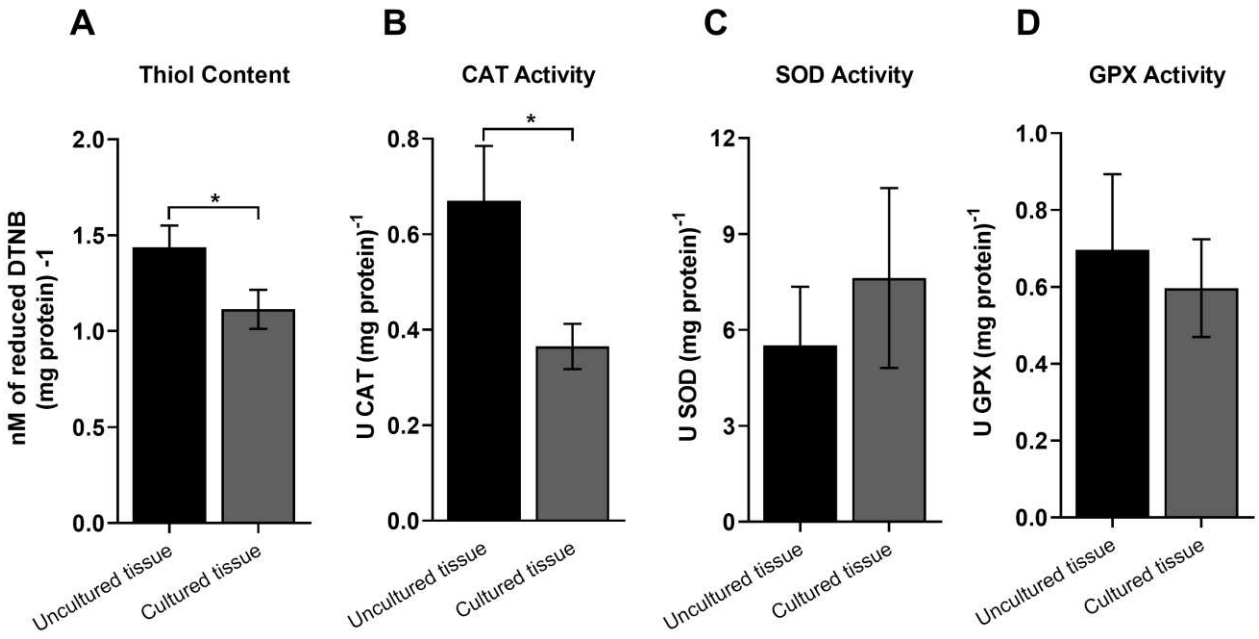

Figure 16. Redox status measured by thiol levels and activity of antioxidant enzymes in uncultured and cultured ovarian tissues. *Asterisk represents significant difference between groups: *P < 0.05.

3.2 Experiment 2: Effect of ascorbic acid and resveratrol on ECM components dynamics, follicular activation and morphology, and redox status

3.2.1 Ascorbic acid combined with resveratrol restores collagen and GAGs in ECM

Fig. 9-A shows representative images of the collagen network (a-h) and GAGs network (i-p) surrounding primordial and primary follicles in uncultured tissue and after culture under different treatment conditions. Cultured ovarian tissues showed a reduction in collagen (Fig. 9-B) and GAGs (Fig. 9-C) density around primordial and primary follicles, as well as in the tissue as a whole (Fig. 10-B and C), compared to the uncultured control ($P < 0.0001$). However, primordial follicles in tissue cultured with both ascorbic acid and resveratrol had a higher density of GAGs than primary follicles cultured in control medium ($P < 0.0001$) (Fig. 9-C). Ascorbic acid alone or in combination with resveratrol did not affect collagen preservation around the follicles (Fig. 9-B) or GAGs density in the whole tissue (Fig. 10-C). However, both ascorbic acid and resveratrol were

effective in maintaining an increased density of collagen throughout the tissue (Fig. 10-B) compared to tissues cultured in control medium ($P < 0.05$).

3.2.2 Ascorbic acid or resveratrol does not affect follicular activation but enhanced follicle morphology

Ovarian tissues cultured in all treatments had a decrease in the percentage of primordial follicles and an increase in the percentage of developing follicles compared to uncultured tissue ($P < 0.0001$). Ascorbic acid alone or in combination with resveratrol did not affect follicular activation rates (Fig. 11-A). However, supplementation of medium with ascorbic acid alone or in combination with resveratrol improved the rate of morphologically normal follicles compared to tissue cultured in control medium. (Fig. 11-B)

3.2.3 Ascorbic acid and resveratrol increase stromal cell survival and differentially affect granulosa cell, follicle, and oocyte diameters

Ovarian tissues cultured in all treatments showed a significant reduction in the survival of stromal cells surrounding primordial and primary follicles when compared to the uncultured control ($P < 0.0001$). The number of cells surrounding primary follicles in tissues cultured in control medium was lower than those around primordial follicles ($P < 0.05$) (Fig. 12-A). Ascorbic acid alone or in combination with resveratrol improved the survival of stromal cells around primordial and primary follicles compared to the primary follicles in tissues cultured in control medium ($P < 0.0001$) (Fig. 12-A). Furthermore, both ascorbic acid and resveratrol increased the overall density of stromal cells when compared to in the tissue cultured in α-MEM^{+} alone or supplemented with ascorbic acid ($P < 0.0001$) (Fig 12-B). Medium supplementation with both ascorbic acid and resveratrol enhanced the survival of granulosa cells in primary follicles ($P < 0.05$), but not in primordial follicles. In terms of follicular and oocyte diameter, ascorbic acid or both ascorbic acid and resveratrol increased the diameter of primary follicles ($P < 0.0001$), but not primordial follicles, and increased the diameter of oocytes in primordial follicles ($P < 0.0001$), but not in primary follicles (Table 3).

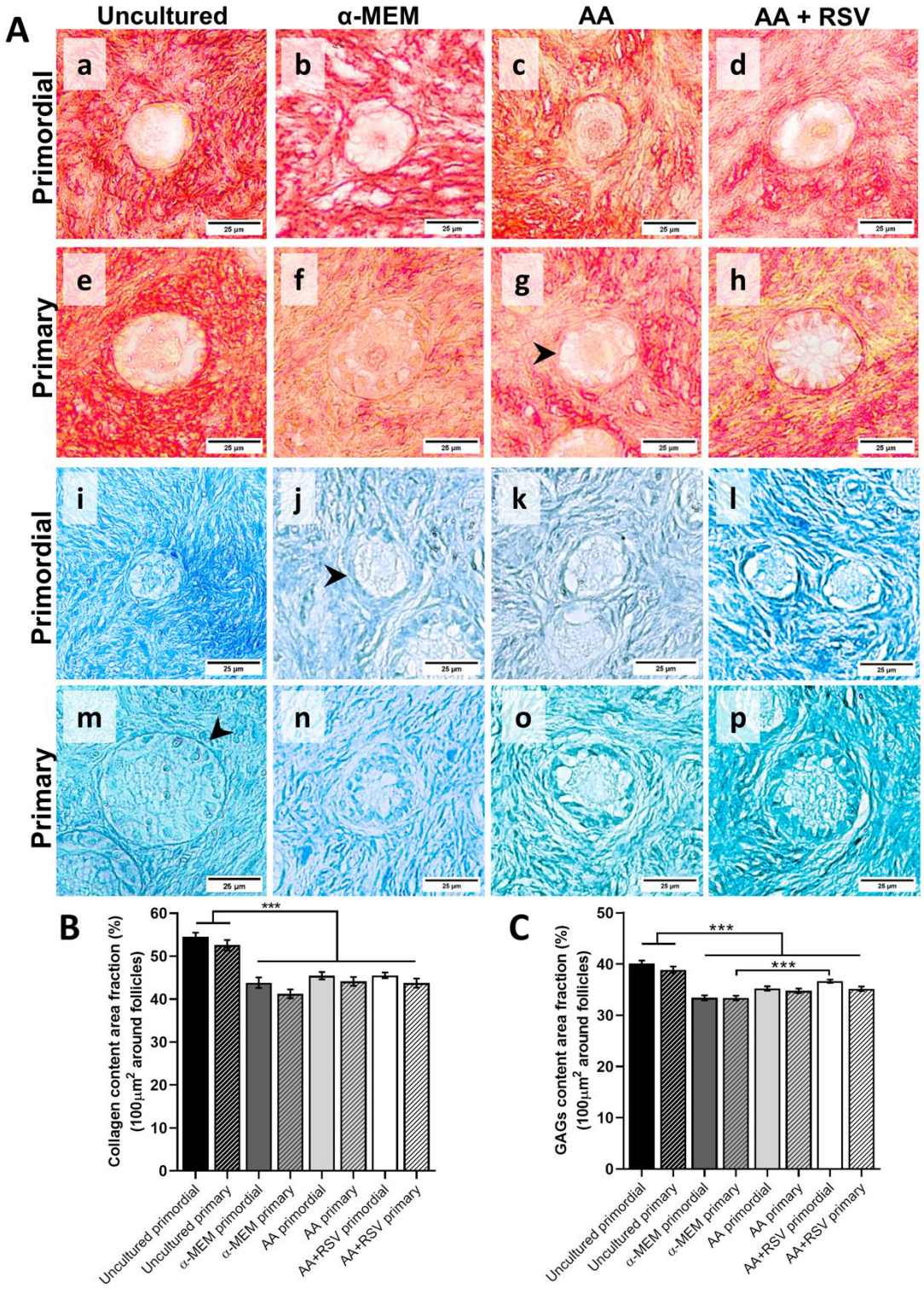

Figure 17. (A) Representative images of the collagen (a - h) and GAGs network (I - p) surrounding primordial and primary follicle in uncultured tissue and after culture under different treatment

conditions: α-MEM (control medium), AA (control medium added with ascorbic acid), AA + RSV (control medium added with ascorbic acid and resveratrol). Black arrowheads show primary follicle in g and m and primordial follicle in j. Magnification = 400x. Scale bars = 25 µm. (**B)** Quantitative measurements of collagen, and **(C)** GAGs density in an area of 100 μm^2 around the primordial and primary follicles in uncultured tissue, and in tissue cultured in α-MEM alone or supplemented with ascorbic acid (AA) or ascorbic acid combined with resveratrol (AA+RSV). *Asterisks indicate statistically significant difference between treatment groups: ***P < 0.0001.

3.2.4 Ascorbic acid combined with resveratrol increases thiol levels and CAT activity

At the end of 6 days of culture, the concentration of reduced thiol molecules (P < 0001) and CAT activity (P < 0.001) were significantly higher in ovarian tissue cultured in medium supplemented with both ascorbic acid and resveratrol compared to tissues cultured with α-MEM^+ or supplemented with ascorbic acid (Fig. 13). In turn, SOD activity was elevated in tissues cultured with both ascorbic acid and resveratrol compared to both the uncultured control (P < 0.05) and cultured with both ascorbic acid and resveratrol (P < 0.0001), which exhibited reduced SOD activity compared to the other groups. No significant effect of antioxidant supplementation on GPX activity was observed (Fig. 13).

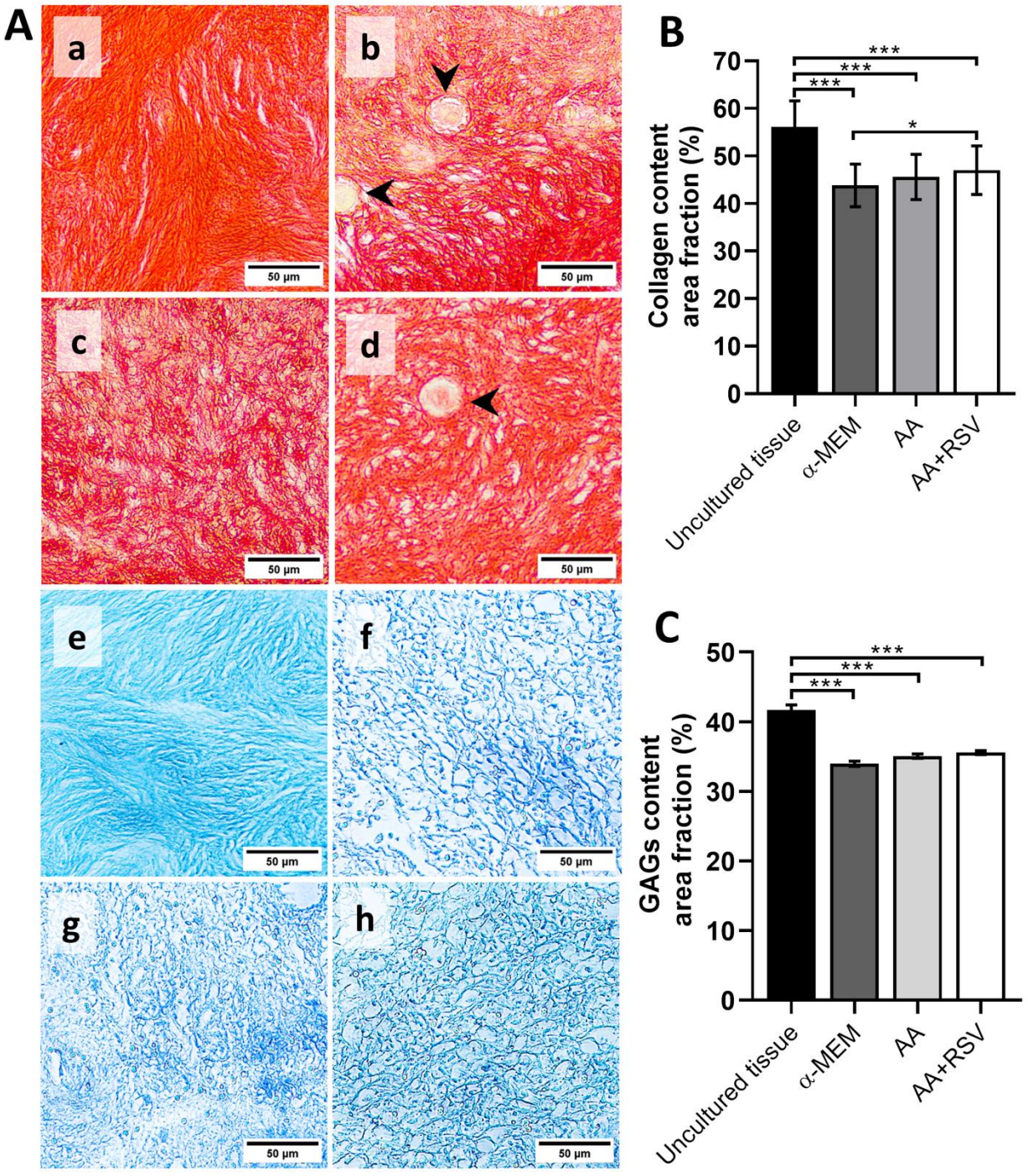

Figure 18. (A) Representative images of the collagen (a - d) and GAGs network (e - h) within the ovarian cortex as a whole in uncultured tissue (a and e), and after culture under different treatment conditions: α-MEM (control medium) (b and f), AA (control medium added with ascorbic acid) (c and g), AA + RSV (control medium added with ascorbic acid and resveratrol) (d and h). Black

arrowheads show primordial follicles in the tissue. Magnification = 400x. Scale bars = 50 µm. **(B)** Quantitative measurements of collagen **(A)**, and **(C)** GAGs density in the overall tissue in uncultured tissue, and in tissue cultured in α-MEM alone or supplemented with ascorbic acid (AA) or ascorbic acid combined with resveratrol (AA+RSV). *Asterisks indicate statistically significant difference between treatment groups: *P < 0.05, ***P < 0.0001.

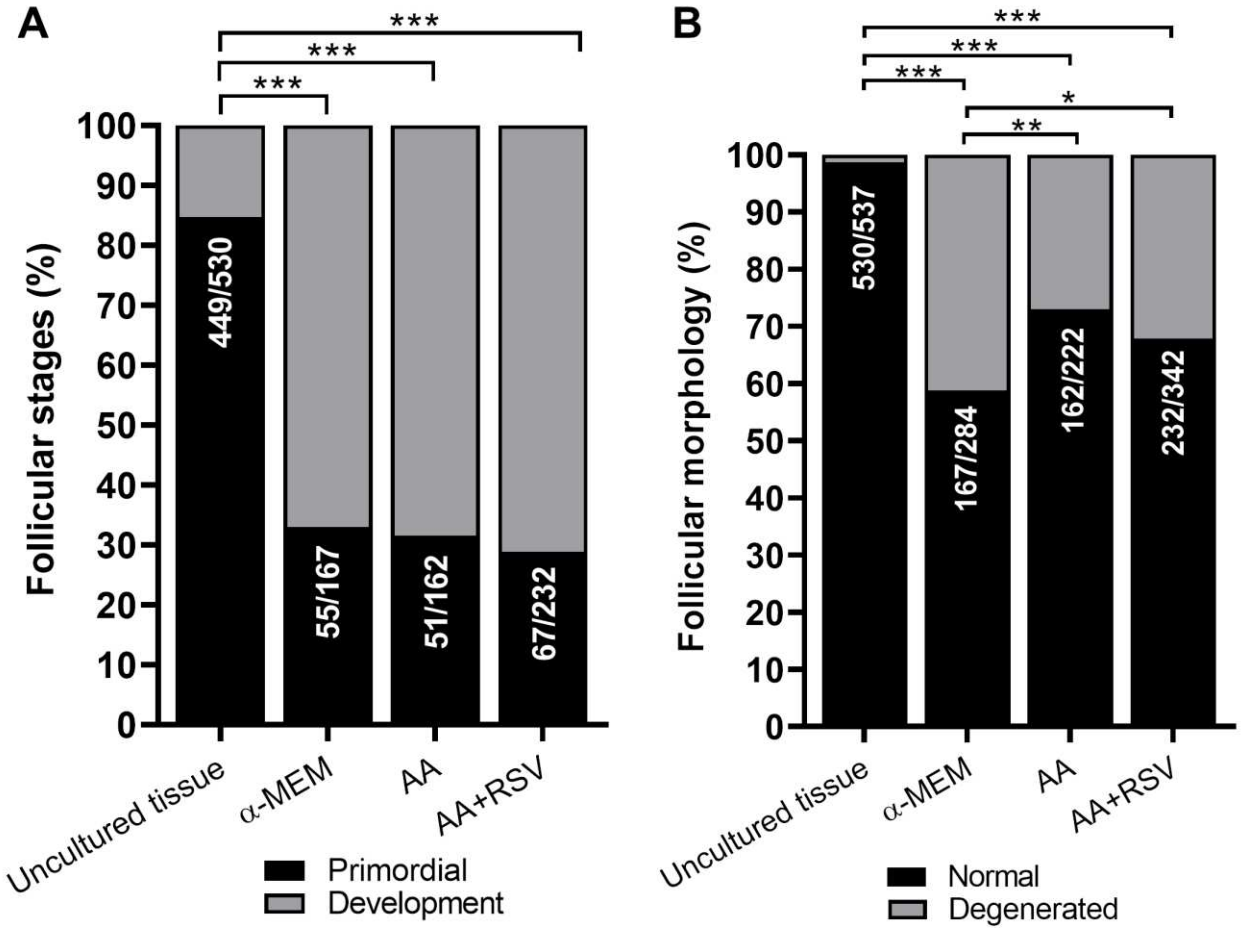

Figure 19. (A) Percentages of primordial and primary follicles in uncultured tissues, and in tissue cultured in α-MEM alone or supplemented with ascorbic acid (AA) or ascorbic acid combined with resveratrol (AA+RSV). **(B)** Percentages of morphologically normal follicles in uncultured tissue, and in tissue cultured in α-MEM alone or supplemented with ascorbic acid (AA) or ascorbic acid combined with resveratrol (AA+RSV). *Asterisks indicate statistically significant difference between treatment groups: *P < 0.05, **P < 0.001, ***P < 0.0001.

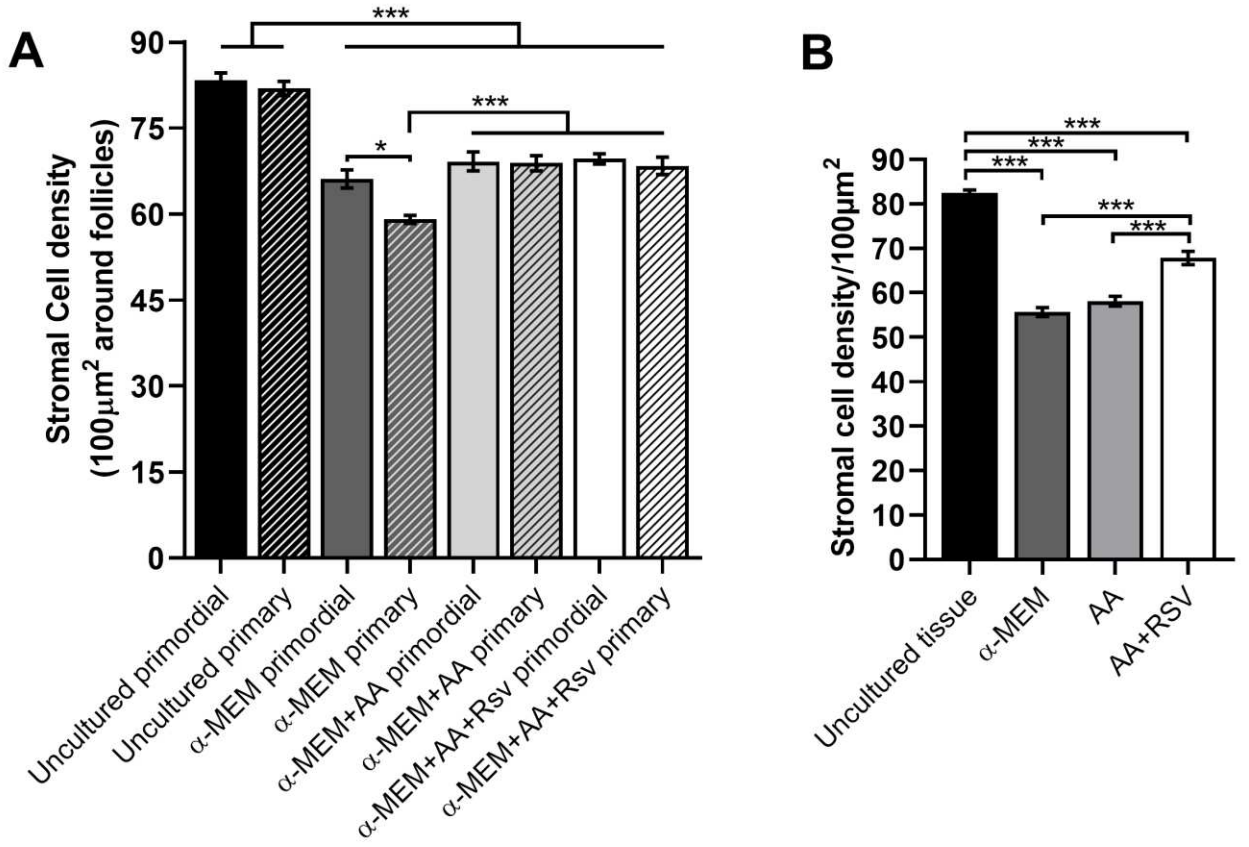

Figure 20. (A) Quantitative measurements of stromal cell density in an area of 100 μm^2 around the primordial and primary follicles in uncultured tissue, and in tissue cultured in α-MEM alone or supplemented with ascorbic acid (AA) or ascorbic acid combined with resveratrol (AA+RSV). **(B)** Quantitative measurements of cell density in the overall tissue in uncultured tissue, and in tissue cultured in α-MEM alone or supplemented with ascorbic acid (AA) or ascorbic acid combined with resveratrol (AA+RSV). *Asterisks indicate statistically significant difference between treatment groups: *$P < 0.05$, ***$P < 0.0001$.

Table 9. Follicular and oocyte diameter and granulosa cell number in primordial and primary follicles before and after *in vitro* culture in different treatment groups (experiment 2)

	Diameter				**N. GC**	
	Follicle		***Oocyte***			
	Primordial	Primary	Primordial	Primary	Primordial	Primary
Uncultured control	33.58 ± 0.5[a]	48.26 ± 1.0[a]	23.68 ± 0.5[a]	32.67 ± 0.4[a]	10.5 ± 0.2[a]	17.0 ± 0.3[a]
α-MEM$^+$	28.76 ± 0.7[b]	35.22 ± 0.8[b]	18.26 ± 0.4[b]	22.71 ± 0.6[b]	8.2 ± 0.2[b]	11.8 ± 0.3[b]
AA	29.70 ± 0.7[b]	39.18 ± 0.6[c]	20.16 ± 0.2[c]	23.39 ± 0.5[b]	8.5 ± 0.1[b]	12.4 ± 0.3[bc]
AA + Rsv	30.90 ± 0.6[b]	41.23 ± 0.7[c]	20.60 ± 0.2[c]	24.17 ± 0.3[b]	8.7 ± 0.1[b]	13.2 ± 0.2[c]

GC: granulosa cell. Values referring to follicular and oocyte diameter are expressed in µm. [a,b,c] Different lowercase letters represent significant differences among tissues cultured in the different treatments.

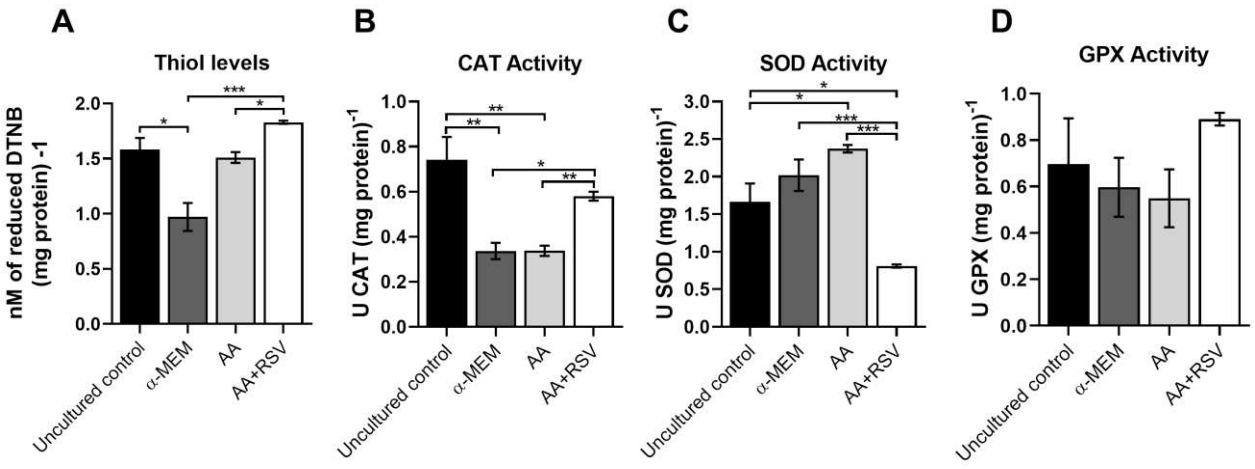

Figure 21. Redox status measured by thiol levels and activity of antioxidant enzymes in uncultured ovarian tissue, and in tissue cultured in α-MEM alone or supplemented with ascorbic acid (AA) or ascorbic acid combined with resveratrol (AA+RSV). *Asterisk represents significant difference between groups: *P < 0.05, **P < 0.001, ***P < 0.0001.

4 Discussion

There is increasing body of evidences recognizing that the ovarian environment strongly influences follicular dormancy and activation in mammals [45, 46, 47]. Therefore, characterizing the structural changes induced by *in vitro* culture and the effect of antioxidant supplementation on the extracellular matrix remodeling and follicular components dynamics after culture may help to overcome the limitations of current cortical tissue culture systems in domestic species, such as cattle. In the present study, we showed that primordial follicle activation occurs concomitantly with a loosening of the ovarian cortex during *in vitro* culture. Grosbois *et al*. [48] reported similar results in human species. However, the proportion of morphologically normal follicles is generally reduced after *in vitro* culture, which is in line with previous studies. Vitale *et al.* [49] cultured bovine ovarian tissue slices (4 x 2 x 1 mm) in 400 µL of culture medium for 8 days and showed 23.75% of follicle degeneration. Additionally, Silva *et al.* [22] found around 40% of atretic follicles after culturing bovine ovarian fragments (3 x 3 x 1 mm) in 1 mL of culture medium for 6 days. In humans, Vitale *et al.* [50] demonstrated that 18.5% of the follicles were degenerated after culturing

ovarian tissues (4 x 2 x 1 mm) for 6 days in 300 µL of culture medium. In our study, although antioxidant supplementation did not affect follicular activation rates, the addition of ascorbic acid or both ascorbic acid and resveratrol resulted in higher rates of morphologically normal follicles. This effect was observed alongside improved structural preservation of the extracellular matrix after culture. It is becoming clear that the growth and survival of early-stage follicles is dependent on physical cues from the ECM [3]. Evidence has shown that a balanced loosening of the ECM appears to be necessary to initiate follicular growth [51], but that unregulated degradation of the ECM negatively affects follicular health [7].

The *in vitro* culture of bovine ovarian tissues promotes a significant reduction in the percentage of collagen and GAGs in the region adjacent to early follicles and also in whole ovarian tissue. Additionally, the remaining collagen and GAGs fibers after *in vitro* culture were more curved and had a greater number of gaps. The effects reported here may be particularly critical for follicular healthy activation and growth under *in vitro* culture. Collagen forms a structurally stable compound, providing follicles with physical support and biochemical cues that modulate tissue homeostasis [52]. Previous studies have demonstrated that the ovary is a mechanoresponsive organ that presents well-regulated collagen gradients necessary to maintain follicular survival and support adequate folliculogenesis [15,53,54]. In turn, GAGs are heteropolysaccharides, not only involved in ECM structure, but also in water retention, and growth factor sequestering [16]. The GAGs can thereby promote cell growth, differentiation, migration, and adhesion [17]. It was reported that in the ovary, GAGs such as hyaluronan inhibits human follicular granulosa cell apoptosis [55], while the reduction of hyaluronan synthesis inhibited follicle growth *in vitro* [56]. The homeostasis of extracellular matrix components is significantly influenced by the presence of antioxidant substances in the culture medium [22, 32], and our study supports these previous findings. Specifically, the addition of ascorbic acid and resveratrol to the culture medium led to a partial improvement in the density of collagen and GAGs after culture.

In our study, stromal cell was decreased around primordial and primary follicles after 6 days of culture. Stromal cell density is an essential indicator of tissue integrity and is likely to play a role in folliculogenesis through cellular mechano-sensing [48]. Among the stromal cells, fibroblast-like cells produce ECM molecules involved in peri-follicle scaffolding and biomechanics; spindle-shaped cells produce steroids, mainly androgens, and participate in follicle modulation [5]. Abir *et al.* [57] reported that isolated human primordial follicles are not activated

in vitro, but spontaneous activation has been observed when follicles are maintained within the ovarian cortex containing stromal cells [48]. The depletion of stromal cell population after *in vitro* culture has been associated with increased oxidative stress during culture and appears to represent an important barrier to efficiency culture ovarian tissues *in vitro* [58]. In the present research, the supplementation with ascorbic acid combined with resveratrol enhanced the survival of stromal cells around the follicles as well as in the tissue as a whole. The results observed in the present study are in line with previously reported experiments performed in other mammals, including humans [22,32,48,59,60].

Our research shows that granulosa cells were also affected by *in vitro* culture. The average number of granulosa cells was reduced after culture in both primordial and primary follicles, and antioxidant supplementation appeared to have different effects on granulosa cell survival in primordial and primary follicles. Addition of ascorbic acid and resveratrol to the culture medium resulted in increased granulosa cell survival in primary follicles, whereas no such effect was observed in primordial follicles. The granulosa cells play key roles in follicular development, by providing nutrients and maturation-enabling factors to maintain oocytes development and to protect them from oxidative stress [61]. Granulosa cell apoptosis can trigger follicular atresia, which is responsible for the reduction in follicle numbers [62]. We also observed that after *in vitro* culture the diameters of primordial and primary follicles and their respective oocytes were smaller than those observed in follicles from uncultured tissue, but the ascorbic acid alone or combined with resveratrol partially improved it. Extensive bidirectional signaling occurs between oocytes and granulosa cells from the primordial stages onwards [63]. In sheep, it has been suggested that follicular granulosa cells from the early preantral stages are involved in the regulation of cell proliferation and protein trafficking. Furthermore, it was reported that early follicular development is accompanied by increased in oocyte diameter concomitant with proliferation of granulosa cells [64].

The current research has shown strong evidences that the *in vitro* environment increased oxidative stress and reduced cellular antioxidant protection in cultured tissues. Additionally, we demonstrated that antioxidant supplementation enhanced redox homeostasis during culture. The thiol levels were significantly lower in 6-days cultured tissues, but the presence of ascorbic acid combined with resveratrol restored thiol levels. Oxidation of GSH by GPX only occurs in the presence of H_2O_2; therefore, a low thiol concentration represents a higher rate of GSH oxidation

[65]. Additionally, we observed that mRNA levels for *CAT, SOD, PRDX*, and *GPX1*, as well as CAT enzyme activity, were downregulated in the cultured tissues in base medium (α-MEM^{+}), but the presence of ascorbic acid combined with resveratrol increased CAT activity. Based on this, it is suggest that the increase in oxidative stress combined with the reduction in antioxidant protection may have favored the death of stromal and granulosa cells and the remodeling of the ECM components. Regarding to collagen, a growing body of evidence has demonstrated that ROS overload can cause unregulated synthesis of matrix metalloproteinases [66,67,68], compromising the delicate balance of the collagen gradient necessary for healthy follicular development. Previous studies have also reported that oxidative stress can deregulate GAGs metabolism, such as abnormal accumulation of hyaluronan fragments in ovarian tissue and directly compromising gamete quality through impaired granulosa cell function [69]. Oxidative stress also inhibits the proliferation and migration of fibroblasts, which is a crucial modulator of ECM [70]. It was also reported that gene suppression for CAT and SOD promotes apoptosis and suppresses the proliferation of bovine granulosa cells [71]. In our study, we also demonstrated that the addition of ascorbic acid and/or resveratrol significantly reduced structural and functional damage to the extracellular matrix and follicular components. The positive effect of antioxidant supplementation on the extracellular matrix and follicular components has also been reported in previous studies [22, 32].

In conclusion, *in vitro* environment induces a reduction of collagen, glycosaminoglycans, and stromal cells around primordial and primary follicles enclosed in cultured of bovine ovarian tissues. Concomitantly, the *in vitro* culture favored follicular activation, but reduced the proportion of morphologically normal follicles, the number of granulosa cells and follicular and oocyte diameters, as well as downregulated the expression of mRNA for *SOD, CAT, GPX*, and *PRDX6;* and decreases CAT activity. However, the supplementation with ascorbic acid and resveratrol partially improved the preservation of extracellular matrix components and enhanced follicular survival after *in vitro* culture, probably by maintaining a more balanced redox environment.

Data availability

The data underlying this article will be shared upon reasonable request to the corresponding author.

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Declaration of competing interest

None of the authors have any conflict of interest to declare.

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7 CAPÍTULO 3

Decellularized ovarian bioscaffolds and resveratrol-loaded polymeric nanoparticles improve *in vitro* development of bovine preantral follicles

[Bioestruturas ovarianas descelularizadas e nanopartículas poliméricas carregadas com resveratrol melhoram o desenvolvimento *in vitro* de folículos pré-antrais bovinos]

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Decellularized ovarian bioscaffolds and resveratrol-loaded polymeric nanoparticles improve *in vitro* development of bovine preantral follicles

Bioscaffolds and nanoparticles in follicle growth

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Abstract

This study evaluated the effects of decellularized ovarian bioscaffolds and resveratrol-loaded polymeric nanoparticles (RLPN) on the *in vitro* development of bovine secondary follicles. Decellularized extracellular matrix (dECM) from cortical fragments was obtained by freeze–thaw cycles and sequential incubation in Triton X-100 and sodium dodecyl sulfate. Decellularization efficiency and extracellular matrix integrity were assessed by hematoxylin-eosin, Hoechst staining, scanning electron microscopy, and quantification of collagen and glycosaminoglycans. Bovine

secondary follicles were isolated and cultured for 12 days in either a two-dimensional (2D) system or in dECM scaffolds in medium supplemented with 0.02, 0.2, or 2.0μM RLNP, blank nanoparticles, or unencapsulated resveratrol. Follicular viability and ultrastructure were evaluated by calcein-AM/ethidium homodimer-1 staining and transmission electron microscopy. Expression of mRNA for catalase, superoxide dismutase, glutathione peroxidase 1, peroxiredoxin 6, and nuclear factor erythroid 2-related factor 2 was assessed by qRT-PCR. Quantitative data were analyzed by unpaired t-tests or one-way ANOVA, followed by Tukey's test ($P < 0.05$). Hematoxylin-eosin and Hoechst staining confirmed effective cell removal, while collagen, glycosaminoglycans, and ECM ultrastructure were preserved. Follicles cultured in the three-dimensional (3D) system showed increased viability, further enhanced by 0.02 or 2.00 μM RLPN. Follicles cultured with 0.02 μM RLPN exhibited well-preserved morphology, including intact zona pellucida, oocyte membrane, and organelles. RLPN downregulated the expression of antioxidant genes. In conclusion, the decellularization protocol effectively removed cellular content and preserved ECM structure and ultrastructure. 3D culture system in combination with medium supplemented with 0.02 μM RLPN supported follicular development and ultrastructure, as well as downregulated antioxidant gene expression.

Keywords: Decellularization; Ovarian follicles; Nanotechnology; Resveratrol; Antioxidant.

1 Introduction

Most of the ovarian follicles are lost due to atresia, which is significantly increased beyond the secondary follicular stage in bovine species (Dey *et al*., 2024a; Dey *et al.,* 2024b). This physiological follicular depletion reinforces the need for improvement of reproductive biotechnologies capable of utilizing the pool of early follicles. The *in vitro* culture (IVC) of secondary follicles isolated from bovine ovaries is a key tool to elucidate their specific requirements for development up to maturation stages and to improve the use of ovarian reserve by assisted reproductive technologies (Ebrahime *et al*., 2025). Despite efforts to establish a suitable IVC system for secondary follicles (Cheng *et al.,* 2024; Jachter *et al.,* 2022), obtaining matured oocytes from isolated bovine secondary follicles cultured *in vitro* remains unsuccessful (Azevedo

et al., 2022; Nascimento *et al.,* 2022; Paulino *et al.,* 2018), underscoring the challenge of reproducing the physiological conditions required for complete follicular development.

Recent evidences have shown that follicles are highly responsive to biomechanical signals originating from the ovarian stroma that act during folliculogenesis (Wang *et al.,* 2025). The coordinated activity of these mechanical cues, along with endocrine and paracrine signaling, shapes individual cells, oocytes, and the entire follicle (Zhao *et al.,* 2023). Thus, after isolation, secondary follicles are challenging to *in vitro* culture without the supporting structure of the ovary (Alshaikh *et al.,* 2020). Therefore, biocompatible scaffolds to encapsulate isolated follicles can provide a suitable environment for their *in vitro* development (Dadashzadeh *et al.,* 2023). Ovarian follicles have been cultured in a three-dimensional culture system using different biomaterials, such as alginate (Zheng *et al.,* 2023), collagen (Joo *et al.,* 2016), fibrin (Chiti *et al.,* 2016), and polyethylene glycol (Ahn *et al.,* 2015). However, none of these materials have demonstrated the ability to fully replicate all the functions of the native ovarian ECM, including cellular support and regulatory roles (León-Félix *et al*., 2024). Several studies have proposed the use of decellularized ovarian extracellular matrix (dECM) as a natural scaffold that closely mimics the native ovarian extracellular matrix (ECM) (Francés-Herrero *et al.,* 2024; Almeida *et al.,* 2023). For instance, Laronda *et al*. (2015) showed that bovine ovarian dECM positively influenced murine primary ovarian cells and stimulated the formation of follicle-like structures *in vitro*. However, the effect of dECM on the *in vitro* development of isolated follicles in bovine species remains unexplored.

In vitro redox dysregulation is also correlated with poor follicular quality after culturing (He *et al.,* 2025; Bezerra *et al.,* 2024; Azevedo *et al.,* 2022). During *in vitro* culture of follicles, various factors, such as ischemia, ovarian fragmentation, manipulation, and light exposure can increase the generation of reactive oxygen species (ROS) that leads to oxidative stress (Costa *et al.,* 2025). Elevated ROS levels are linked with a reduction in follicular viability, lipid peroxidation leading to changes in membrane permeability and selectivity (Gashler *et al.,* 2017), vacuolization and degeneration of mitochondrial cristae and matrix (Wang *et al.,* 2023), endoplasmic reticulum stress (Harada *et al.,* 2021), and zona pellucida (ZP) damage (Wang *et al.,* 2021). Consequently, antioxidant supplementation of the culture medium has been proposed to maintain the redox balance during *in vitro* ovarian follicles culture (Silva *et al.,* 2023). Moreover, the expression levels of enzymatic antioxidants such as catalase (*CAT*), superoxide dismutase (*SOD*), glutathione

peroxidase (*GPX*), peroxiredoxin (*PRDX*), and nuclear factor erythroid 2-related factor 2 (*NRF2*) are frequently used as indicators of the antioxidant capacity of *in vitro* culture systems.

Among the natural phenolic compounds, resveratrol is known for improving follicular development *in vitro* through its anti-apoptotic and antioxidant properties (Costa *et al.,* 2025; Macedo *et al.,* 2017; Bezerra *et al.,* 2018). Although promising results have been reported, its low water solubility (~ 3 mg/100 mL) and rapid metabolism can impact its bioavailability, limiting the extent to which its free form exerts its effects (Freitas *et al.,* 2023; Moreira-Pinto *et al.,* 2021). In this context, encapsulating resveratrol within polymeric nanoparticles is a promising strategy, as it can enhance the effectiveness of its action. The advantages of polymeric nanoparticles include superior biocompatibility compared to other materials, minimal toxicity, capable of sustained drug release, and biodegradability (Freitas *et al.,* 2023).

This study aimed to decellularize and characterize bovine ovarian tissue and to evaluate its effect on the follicle viability and ultrastructure, and expression of mRNA for *CAT, SOD, GPX1, PRDX6*, and *NRF2* in bovine secondary follicles cultured in medium supplemented with different concentration of *in vitro* resveratrol-loaded polymeric nanoparticles.

2 Material e methods

2.1 Chemicals

Culture media and other chemicals used in the present study were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise specified in the text.

2.2 Synthesis and physicochemical characterization of resveratrol-loaded polymeric nanoparticles

Polymeric nanoparticles were prepared using the nanoprecipitation method, as described by de Freitas *et al.* (2023). The mean particle size, polydispersity index (PDI), and zeta potential were evaluated to characterize the physicochemical properties of the formulations. For this purpose, the hydrodynamic size distribution, polydispersity index (PDI), and zeta potential of the nanoparticles were analyzed at 25 °C by dynamic light scattering (DLS). Measurements were

conducted with a Malvern Zetasizer Nano ZS (Malvern Instruments, Malvern, UK), equipped with a 4 mW He-Ne laser at a wavelength of 633 nm at an angle of 90°. Prior to analysis, samples were diluted 1:10 in ultrapure water and vortexed to ensure proper dispersion. All measurements were performed in triplicate, and results were expressed as mean ± standard deviation (SD).

2.3 Bovine ovaries and ethical approval

Ovaries from 50 healthy mixed-breed cows of reproductive age were collected at local slaughterhouses and assigned to two distinct experiments, as detailed below. All procedures performed in this study were approved by the Ethics and Animal Welfare Committee of the Federal University of Ceará under approval number 04/22. Immediately after collection, the ovaries were washed once with 70% alcohol and twice with PBS buffered with 20 mM HEPES and supplemented with 100 µg/ml penicillin and 100 µg/ml streptomycin, then designated for decellularization. The ovaries to be used for follicular isolation were washed twice in TCM199 buffered with 20 mM HEPES and supplemented with the same antibiotics. Then, they were transported to the laboratory within 1 h in the respective washing solution at 4 °C.

2.4 Bovine ovarian tissue decellularization

Upon arrival at the laboratory, the ovaries (n = 10 pairs) were rinsed in PBS solution and stored at -80 ºC for 24 – 48 h. Before use, they were thawed in water bath at 37 ºC for 1 hour. After thawing, antral follicles were punctured with a needle, and cortical slices (2 mm x 2 mm x 1 mm) were taken using a scalpel. Random samples from each ovarian pair were fixed in 4% paraformaldehyde prepared in PBS (pH 7.4) for 24 hours at room temperature for evaluation as native tissue. Two decellularization solutions (DS) were prepared 24 hours prior to use: (DS1) - 1% Triton-X100 (v/v) (T8787-100 ML, Sigma-Aldrich, USA) in distilled water; (DS2) - 0.5% SDS (w/v) (L3771-100 G, Sigma-Aldrich, USA). Initially, samples were immersed in 20 mL DS1 for 9 hours under constant agitation (150 rpm) in an orbital shaker at 37 ºC, followed by 9 hours in 20 mL DS2 under the same conditions. After decellularization, the samples were washed 10 times in 50 mL PBS at 37 ºC with hourly solution changes. The resulting samples were either used to assess the decellularization efficiency or stored at -80°C.

2.5 Histological analysis and DNA staining

Native bovine ovarian tissue and decellularized extracellular matrix (dECM) morphology were analyzed using classical light microscopy. Samples were fixed for 24 hours at room temperature and processed for histological. Sections (6 μm thick) were mounted on glass slides and stained with hematoxylin-eosin (H&E) to assess the remaining cell content after decellularization. Some sections were stained with 20 μM Hoechst 33342 (B2261-100MG, Sigma-Aldrich, USA) for 1 min for DNA fluorescence labeling. For cell counting, random fields from sections were analyzed at 400× magnification using a Nikon Eclipse TS 100 microscope and the ImageJ software (Version 1.54f, 2023). Cells were manually counted in a 100 μm² area, following Silva *et al*. (2024), with all measurements performed by a single operator. Images of sections stained with Hoechst 33342 under constant exposure settings and analyzed with ImageJ. Residual nuclear content was considered proportional to the fluorescence intensity after subtraction of tissue auto fluorescence (Alshaikh *et al*., 2020).

2.6 Histochemical evaluation for collagen and glycosaminoglycans

For collagen assessment, 6 μm-thick sections from both native and decellularized bovine ovarian samples were dewaxed using xylene and stained with 0.1% Sirius Red solution for 1 hour at room temperature, according to the protocol described by Rittié *et al.* (2017). The excess stain was eliminated with a 0.5% acetic acid solution, followed by dehydration of the sections and subsequent mounting on slides. For glycosaminoglycan (GAG) evaluation, the sections were stained with Alcian Blue for 5 minutes at room temperature, following the manufacturer's protocol. After staining, the sections were dehydrated and mounted on slides for analysis. For both collagen and GAG assessments, images from 50 fields (5 per animal) were captured using a DS Cooled DS-Ri1 camera attached to a Nikon Eclipse TS 100 microscope (Tokyo, Japan) at 400x magnification. Quantification of collagen and GAG content was performed using Fiji-ImageJ software (Version 1.54f, 2023).

2.7 Scanning electron microscopy (SEM)

For SEM, both native bovine ovaries and dECM samples were fixed in Karnovsky's solution at 4°C for 24 hours, followed by post-fixation with 2% osmium tetroxide for 1 hour. After washing with distilled water, the samples underwent gradual dehydration through a graded acetone series (30%, 50%, 70%, 90%, and 100%), with each step lasting 15 minutes. Subsequently, the specimens were dried using a critical point dryer, mounted on stubs, coated with colloidal gold, and observed using a scanning electron microscope (Inspect S50-FEI).

2.8 Follicle isolation and *in vitro* culture

A total of 40 bovine ovaries were collected following the previously described procedures. The ovarian cortex was sectioned into 1–2 mm fragments using a sterile scalpel blade and transferred to TCM-199 medium supplemented with 100 µg/ml penicillin, 100 µg/ml streptomycin, and HEPES buffer. Secondary follicles measuring approximately 150–200 µm in diameter were manually dissected from the cortical strips using 26-gauge needles under a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan). Follicles selected for culture exhibited an intact basement membrane, two or more granulosa cell layers, absence of an antral cavity, and a morphologically healthy, round oocyte centrally located within the follicle.

To culture the follicles, the decellularized scaffolds were removed from the -80ºC freezer and thawed for 1 hour in a water bath containing distilled water. Subsequently, the structures were sterilized in a PBS solution with penicillin and streptomycin, then placed in TCM-199 medium supplemented with penicillin (100 µg/ml), streptomycin (100 µg/ml) and HEPES solution, and stored in an incubator at 38.5 ºC with 5% CO2 for 4 hours prior to use. The base medium used for follicle culture was TCM-199 (pH 7.2–7.4), enriched with 3.0 mg/ml bovine serum albumin (BSA), 2 mM glutamine, 2 mM hypoxanthine, 100 IU/ml penicillin-streptomycin, 10 µg/ml insulin, 5.5 µg/ml transferrin, 5 ng/ml selenium (ITS), 50 µg/ml ascorbic acid, and 100 ng/ml equine chorionic gonadotropin (eCG) (TCM-199^{+}). Isolated secondary follicles were randomly distributed and cultured in two systems: (I) two-dimensional (2D) system, i.e., the follicles were cultured in Petri dishes (60 ×15 mm; Corning, USA) in drops of 100 µL TCM-199^{+} (TCM199^{+}2D); (II) three-dimensional (3D) system, i.e., the follicles were inserted in dECM scaffolds and cultured in 24-well culture dishes (2 scaffolds per well) in 500 µL TCM-199^{+} alone (TCM199^{+}3D) or

supplemented with 0.02, 0.2, or 2.0 µM resveratrol-loaded polymeric nanoparticles (RLNP-3D groups), 2.0 µM blank nanoparticles BLNP (2µM-3D); or 2.0µM non-encapsulated resveratrol RSV (2µM-3D). Follicles were cultured for 12 days in a humidified incubator at 38.5°C with 5% CO_2 in air. Half of the culture medium was refreshed every second day of culture.

2.9 Assessment of follicular viability by fluorescence microscopy

Following the culture period, follicles (n = 30 per treatment) were manually retrieved from the dECM scaffolds using 26-gauge needles. They were then incubated for 15 minutes at 37°C with 5% CO_2 in 100 µL droplets of TCM-199 medium containing 4 µM calcein-AM and 2 µM ethidium homodimer-1 (EthD-1) (Molecular Probes - L3224, Invitrogen, Karlsruhe, Germany) to assess esterase activity in the cytoplasm and the labeling of nucleic acids in non-viable cells following membrane disruption. After staining, follicles were washed three times in TCM-199 medium and analyzed under a fluorescence microscope (Nikon, Eclipse TS100, Japan) as described by Paulino *et al.* (2018). Oocytes and granulosa cells with preserved viability displayed green fluorescence from calcein-AM, whereas non-viable cells exhibited red fluorescence due to EthD-1 staining. Fluorescence intensity was quantified using ImageJ software (Version 1.54f, 2023). The mean pixel intensity within the follicular region was measured after background correction to determine staining levels. Non-cultured follicles were used as the reference for relative fluorescence quantification, following the approach described by Rocha-Frigoni *et al.* (2016).

2.10 Morphological and ultrastructural assessments of dECM, and cultured follicles

To assess the structural stability of collagen and glycosaminoglycan (GAG) networks throughout the culture period, dECM scaffolds were stained with Picrosirius red and Alcian blue, respectively, on days 0, 2, 4, 6, 8, 10, 12, and 14. Additionally, scaffold ultrastructure was examined by scanning electron microscopy (SEM) on day 12 of culture. Histochemical and SEM processing were performed using standard methods already described previously. To analyze cell morphology and organelle organization in oocytes and GCs, transmission electron microscopy (TEM) was performed on follicles cultured in the 2D system or in the 3D system in medium supplemented with 0.02, 0.2, or 2.0µM RLNP, Blank-NP, or RSV. After culture, scaffolds containing follicles (6–10 per treatment) were fixed in Karnovsky's solution for 4 hours at room temperature (~25 °C),

embedded in 4% low-melting agarose droplets, and kept in sodium cacodylate buffer. As no significant differences in viability were found among different concentrations of RLNP, follicles treated with the lowest concentration (0.02 µM) were selected for ultrastructural analysis. The specimens were post-fixed with 1% osmium tetroxide, 0.8% potassium ferricyanide, and 5 mM calcium chloride. After dehydration in acetone, samples were embedded in epoxy resin (Epoxy Embedding Kit, Fluka Chemika). Semithin sections (2 µm) were stained with toluidine blue and examined under light microscopy at 400x magnification, while ultrathin sections (70 nm) were counterstained with uranyl acetate and lead citrate for examination under a Morgani-FEI transmission electron microscope.

2.11 Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Real-time PCR was used to evaluate the levels of mRNAs for *CAT, SOD, GPX1*, *PRDX6*, and *NRF2* in follicles cultured follicles 2D system or in 3D system in medium supplemented with 0.02 µM RLNP, Blank-NP or RSV, according to Azevedo *et al*. (2022). After culture, follicles were carefully removed from dECM scaffolds, and total mRNA was extracted using the Trizol purification kit (Invitrogen, São Paulo, Brazil), according to the manufacturer's guidelines. The total mRNA concentration was measured using a nanodrop (Biodrop, Cambridge, England), and 50 ng/µL of mRNA was used for reverse transcription. Quantification of mRNA was conducted using SYBR Green on a StepOnePlus instrument (Applied Biosystems, Foster City, CA, USA). Each quantitative PCR reaction (15 µL total volume) was prepared with 7.5 µL of SYBR Green Master Mix (PE Applied Biosystems, Foster City, CA), 5.5 µL of ultrapure water, 1 µL of cDNA, and 0.5 µM of each primer (Table 1). The relative expression levels of all target genes were normalized to the endogenous control gene Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Kussano *et al.,* 2024) calculated using the 2^-ΔΔCt method, as described by Cao *et al.* (2021).

2.12 Statistical analysis

Statistical analyses were performed using GraphPad Prism version 9.0. An unpaired t-test was applied to compare cell remnants and the percentage of collagen and GAGs between native and decellularized tissues. For treatment group comparisons, data with normal distribution were

evaluated using one-way ANOVA followed by Tukey's post hoc test, while non-normally distributed variables were assessed with the Kruskal–Wallis test and Dunn's multiple comparisons. Results are presented as mean ± standard error of the mean (SEM), unless otherwise specified. Differences were considered statistically significant when $p < 0.05$.

Table 10. Oligonucleotide primers used for polymerase chain reaction analysis

Target gene	Primer sequence (5′ ➔ 3′)	Sense (S), anti-sense (As)	GenBank accession no.
GAPDH	TGTTTGTGATGGGCGTGAACCA	S	GI: 402744670
	ATGGCGCGTGGACAGTGGTCATAA	As	
CAT	AAGTTCTGCATCGCCACTCA	S	GI: 402693375
	GGGGCCCTACTGTCAGACTA	As	
SOD	GTGAACAACCTCAACGTCGC	S	GI: 31341527
	GGGTTCTCCACCACCGTTAG	As	
GPX1	AACGTAGCATCGCTCTGAGG	S	GI: 156602645
	GATGCCCAAACTGGTTGCAG	As	
PRDX6	GCACCTCCTCTTACTTCCCG	S	GI: 59858298
	GATGCGGCCGATGGTAGTAT	As	
NRF2	GACCCAGTCCAACCTTTGTC	S	GI: 0304941
	GACCCGGACTTACAGGTACT	As	

3 Results

3.1 Physicochemical characterization confirms successful synthesis of RLNP

Table 2 shows the physicochemical characterization of resveratrol-loaded polymeric nanoparticles (RLNP) and blank nanoparticles (BLNP). The synthesis method employed is robust and effective in producing nanoparticles with sizes below 150 nm. RLNP showed a PDI of 0.26, while BLNP presented a PDI of 0.16, indicating moderate size distribution uniformity. Both

formulations exhibited a negative surface charge, with zeta potential values close to -6 mV, reflecting low surface charge intensity.

Table 11. Physicochemical properties of the formulations, including particle size, polydispersity index (PDI), and zeta potential.

	Size (nm) ± SD	**PDI ± SD**	**Zeta potential (mV) ± SD**
RLNP	130.87 ± 36.36	0.26 ± 0.01	-5.80 ± 1.26
BLNP	142.23 ± 1.55	0.16 ± 0.01	-5.65 ± 1.85

3.2 Decellularization eliminates cells and maintains tissue macro- and microarchitecture

Macroscopic analysis showed that decellularized tissues changed color from red to white and maintained their shape and homogeneity without any signs of deformation (Figure 1A, B). The SEM revealed that ECM fiber integrity was preserved after decellularization. The fiber appearance and organization in the dECM closely resembled those of the native ovarian tissue. The SEM also confirmed the preservation of both dense and thin collagen fibers, indicating that the resulting scaffolds retained their native three-dimensional architecture (Figure 2A-C). Histological analysis revealed that the resulting ECM-based scaffolds were devoid of cells and basophilic staining was absent in the decellularized scaffolds, whereas cell nuclei were distinctly visible in native tissues, which served as the control (Figure 1C, D). Cell density analysis demonstrated absence of nuclei in the ECM-based scaffolds compared to the untreated tissues ($P < 0.05$) (Figure 1G). Additionally, the existence of DNA was verified through Hoechst 33342 staining (Figure 1E, F) indicating absence of DNA in decellularized tissues ($P < 0.05$) (Figure 1H).

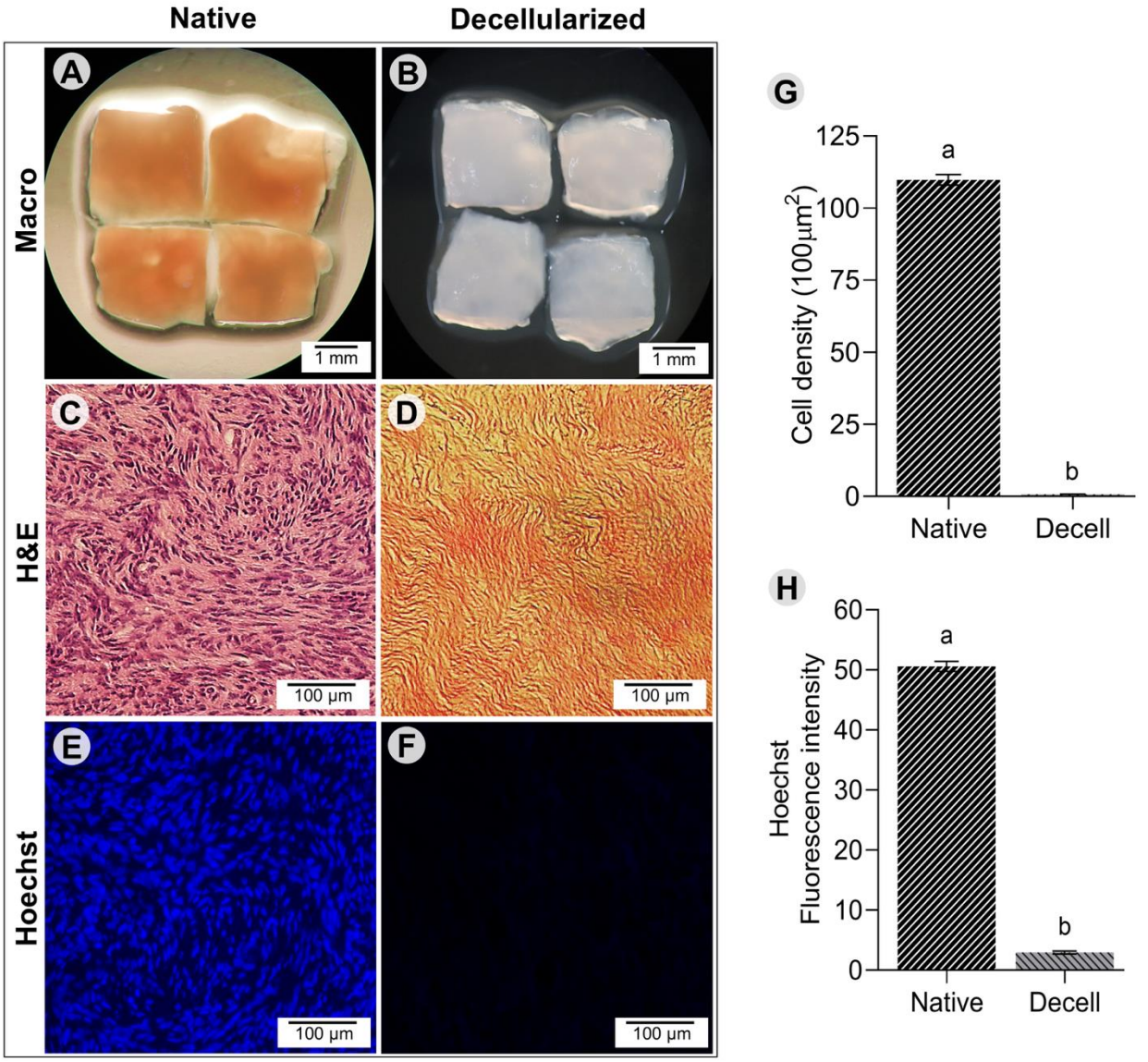

Figure 22. Macroscopic images of native (control group) and decellularized bovine ovarian tissue (dECM group) (A, B) (Magnification = 25×; scale bar = 1mm). Corresponding hematoxylin-eosin staining (C, D), and Hoechst staining (E, F) (Magnification = 400×; scale bar = 100 µm). Quantification of stromal cell density (G), and Hoechst fluorescence intensity (H) in native and dECM ovarian tissues. a, b, c: Different lowercase letters indicate statistically significant differences between treatments ($P < 0.05$).

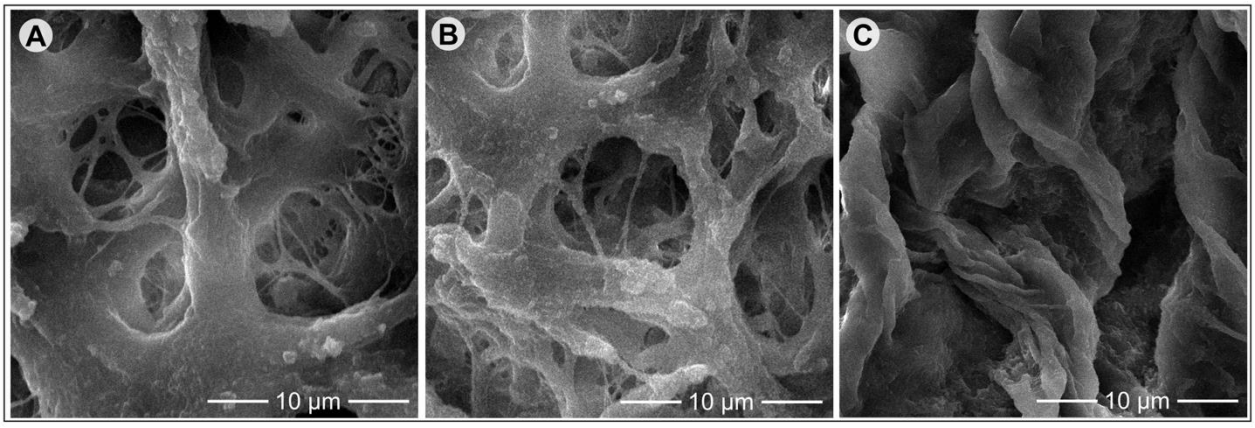

Figure 23. Scanning electron micrographs showing the microarchitecture of native ovarian tissue (A), dECM immediately after decellularization (B), and after 12 days in culture (C). Magnification = 5,000×; scale bar = 10 µm.

3.3 Decellularization preserves ovarian extracellular matrix components

Histochemical analyses confirmed the preservation of key ECM components after tissue decellularization. Picrosirius red and Alcian blue staining revealed the persistence of collagen (Figure 3A, B) and GAGs (Figure 3C, D), displaying a comparable distribution between ECM-based scaffolds and native tissues. These morphological findings were confirmed by stereological quantifications, which demonstrated no differences in collagen (Figure 3E), and GAGs content (Figure 3F) between ECM-based scaffold and native tissues ($P < 0.05$). The mean of collagen and GAGs in the decellularized ovarian tissue was about 95% and 93%, respectively, compared with native tissue collagen and GAGs content prior to decellularization.

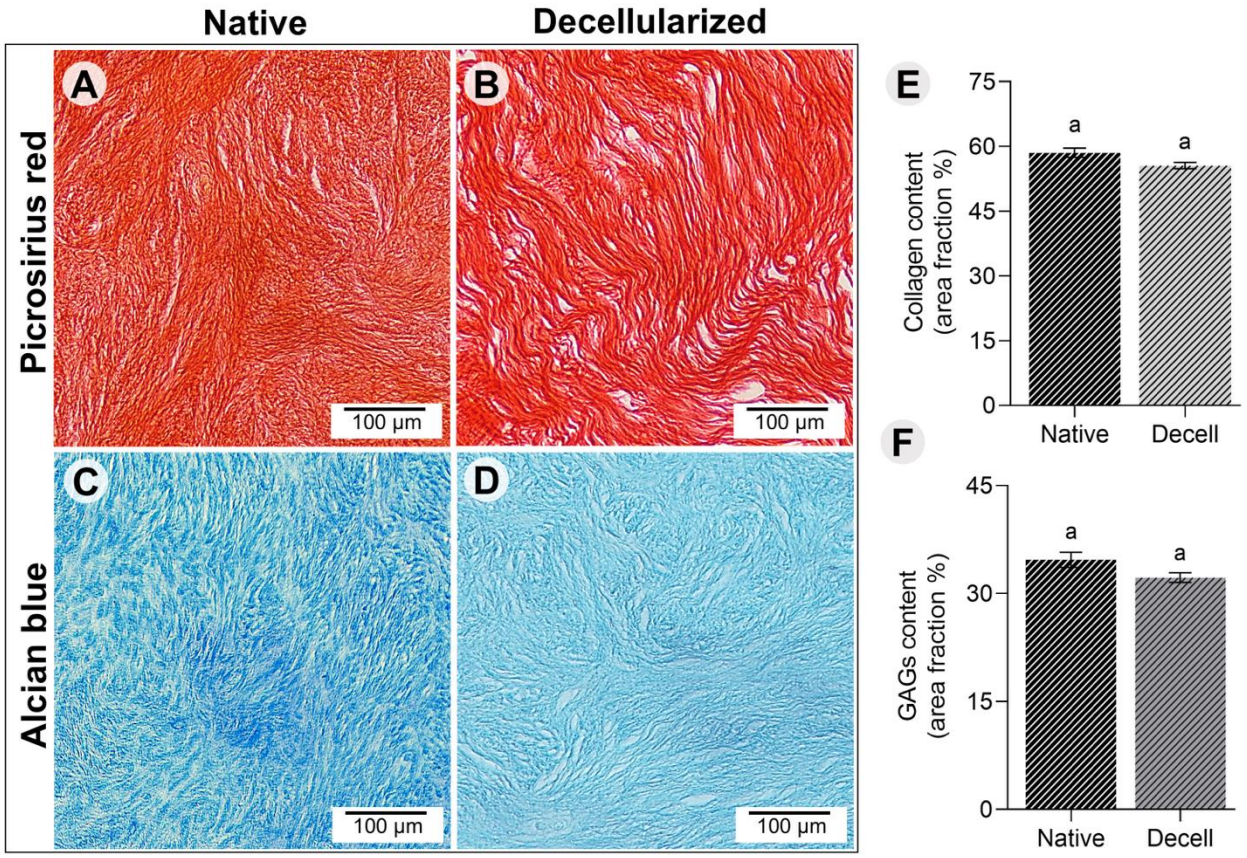

Figure 24. Histochemical staining of native (control group) and decellularized bovine ovarian tissue (dECM group). Picrosirius red staining for collagen (A, B), and Alcian blue staining for glycosaminoglycans (C, D) (magnification: 400×; scale bar = 100 µm). Quantification of collagen content (E), and glycosaminoglycans (F). a, b, c: Different lowercase letters indicate statistically significant differences between treatments ($P < 0.05$).

3.4 The dECM undergoes time-dependent structural changes *in vitro*

Picrosirius red staining showed that the total collagen content remained stable until day 12 of *in vitro* culture, with no apparent disarray in the collagen fiber bundles of the decellularized scaffolds. However, by day 14, a greater degree of fiber disorganization became evident, characterized by the presence of gaps, increased fiber fragmentation, and a significant reduction in fiber density within the scaffolds (Figure 4A-G and O). Similarly, GAGs stained with Alcian blue remained quantitatively stable until day 10 of culture, although the GAG network appeared straighter at this stage. From day 12 onward, a significant decline in GAG density was observed,

accompanied by a more dispersed network with an increased presence of gaps (Figure 4H-N, and P). The SEM analysis corroborated the histochemical findings, revealing alterations in the ultrastructural integrity of the dECM after 12 days in culture. The finer fibers present in the native tissue and immediately after decellularization were no longer observed. Additionally, the porous architecture present shortly after decellularization was no longer apparent after 12 days of culture (Figure 2C).

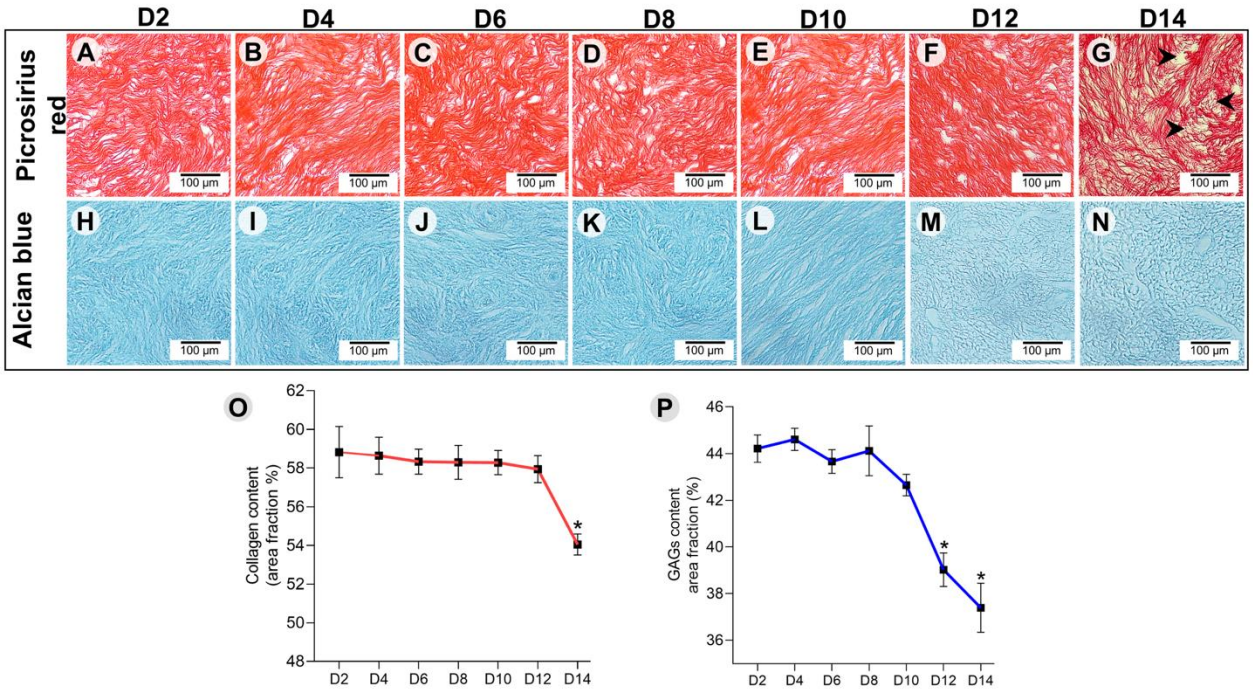

Figure 25. Structural remodeling of collagen (A–G) and GAGs (H–N) in decellularized ovarian scaffolds over 14 days of *in vitro* culture. Quantitative analysis of collagen (O) and GAG content (P) throughout the culture period. Black arrowheads indicate collagen fiber disruption points on day 14. Magnification = 400×; scale bar = 100 µm. Asterisks indicate statistically significant differences between time points ($P < 0.05$).

3.5 The dECM and RLNP enhance follicular viability

After culture, follicles in all treatments and culture conditions exhibited a significant reduction in calcein-AM fluorescence intensity and an increase in EthD-1 fluorescence compared to the fresh control (Figure 5A-X)**.** Follicles cultured in the 3D groups, except those supplemented with BLNP, exhibited higher calcein-AM fluorescence intensity compared to the 2D group. Meanwhile, lower EthD-1 fluorescence intensity was observed in all the 3D groups relative to the 2D group ($P < 0.05$), (Figure 5Y, Z). In follicles cultured in 3D system, supplementation of the culture medium with RLNP at all tested concentrations further increased calcein-AM fluorescence intensity compared to the other treatments (Figure 5Y). However, this supplementation did not significantly affect EthD-1 fluorescence intensity ($P > 0.05$) (Figure 5Z).

3.6 The dECM and RLNP improve follicular ultrastructure

Follicles cultured in the 2D system exhibited a ruptured ZP, with the space originally occupied by the oocyte filled by granulosa cells. However, these cells were well preserved, displaying reticulum and mitochondria, although mitochondrial cristae were difficult to visualize (Figure 6A, B). In contrast, follicles cultured in the 3D system in medium supplemented with 0.02μM RLNP exhibited oocytes with an intact ZP and visible microvilli. Moreover, mitochondria with preserved cristae were observed. Although some granulosa cells showed gaps between them, they remained well preserved, with numerous mitochondria featuring clearly visible cristae, along with the presence of endoplasmic reticulum (Figure 6C, D). In follicles cultured in the 3D system in medium supplemented with 2.0 μM BLNP, although remnants of an irregular ZP were visible, no viable oocyte was identified. Granulosa cells of these follicles displayed intense vacuolization, an extremely heterochromatic nucleus, and heterogeneous nucleoli. Furthermore, an intact plasma membrane separating the cells was not observed (Figure 6E, F). Finally, in follicles cultured in the 3D system with 2.0 μM RSV, no intact oocyte was identified, with only fragments of the ZP remaining. However, granulosa cells had preserved morphology, with a well-defined nucleus, mitochondria with reasonably preserved cristae, and structurally intact endoplasmic reticulum (Figure 6G-H).

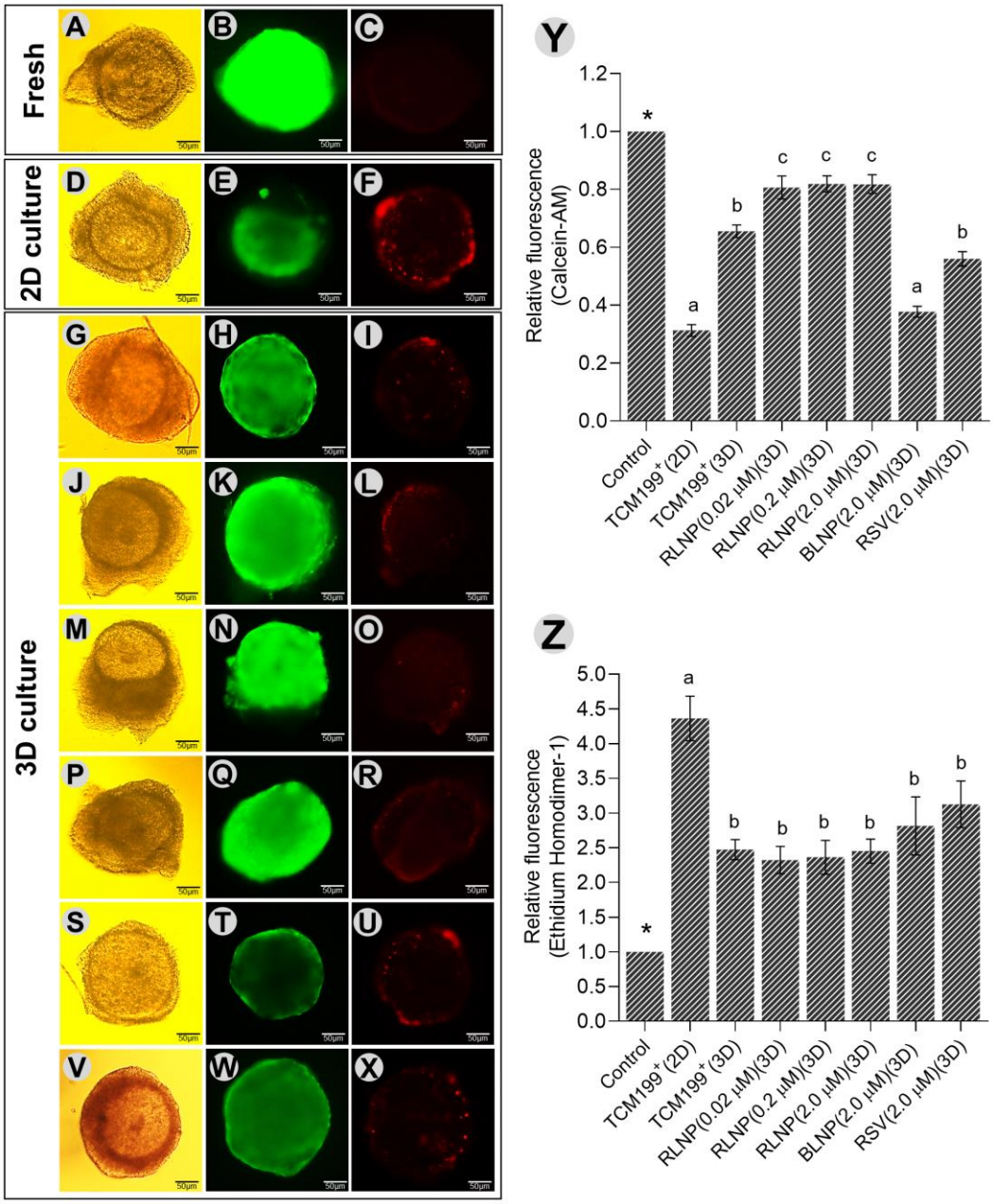

Figure 26. Representative images of secondary follicles in bright field (left column), stained with calcein-AM (middle column), or EthD-1 (right column): fresh control (A–C); 2D system with TCM-199 alone (D–F); 3D system with TCM-199 alone (G–I); or supplemented with 0.02 (J–L), 0.2 (M–O), or 2.0 µM (P–R) resveratrol-loaded nanoparticles, blank nanoparticles (S–U), or unencapsulated resveratrol (V–X). Quantification of calcein-AM (Y) and EthD-1 (Z) fluorescence. Scale bar = 50 µm.

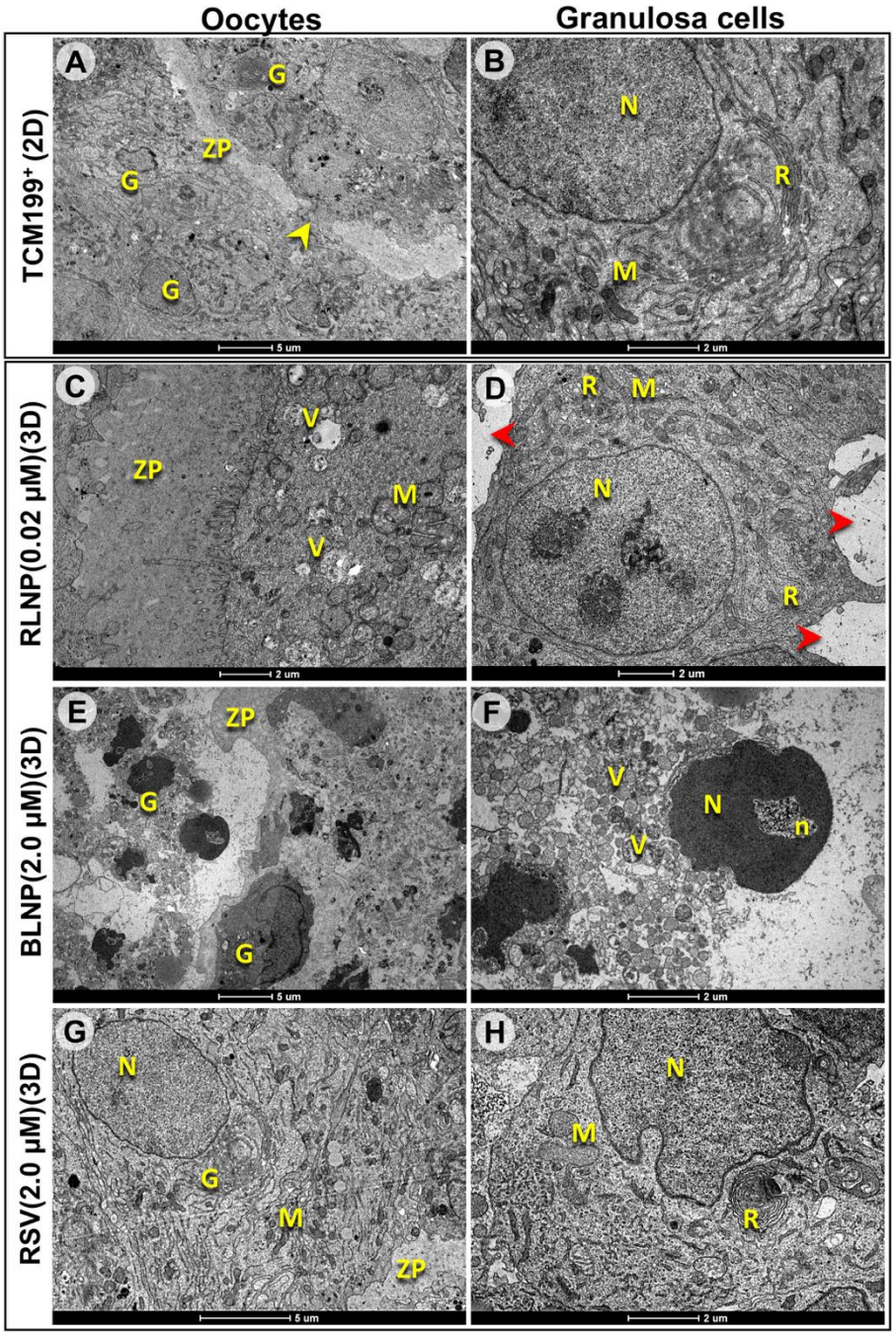

Figure 27. Electron micrograph of oocyte (left column), and granulosa cells (right column) from secondary follicles cultured in 2D system in TCM-199^{+} (A, B) or in 3D system in TCM-199^{+} supplemented with resveratrol-loaded nanoparticles 0.02 µM (C, D), blank nanoparticles (E, F) or nonencapsulated resveratrol (G, H). Red arrowheads indicate gaps between adjacent granulosa cells. Scale bar = 5 µm (A, E, and G); 2 µm (B, C, D, F, and H). Symbols: ZP, zona pellucida; G, granulosa cells; N, nucleus; M, mitochondria; R, reticulum; V, vacuoles; n, nucleolus.

3.7 The RLNP downregulated mRNA expression of antioxidant enzymes

After 12 days of culture, follicles cultured with 0.02 μM RLNP showed reduced mRNA expression of *CAT* ($P < 0.01$), *SOD* ($P < 0.0001$), *PRDX6* ($P < 0.0001$), and *NRF2* ($P < 0.05$) compared to the TCM199^{+} control group ($P < 0.05$), although *SOD* levels were similar to those observed in follicles cultured with unencapsulated resveratrol (Figure 7). No significant differences in *GPX1* expression were observed among treatments ($P > 0.05$).

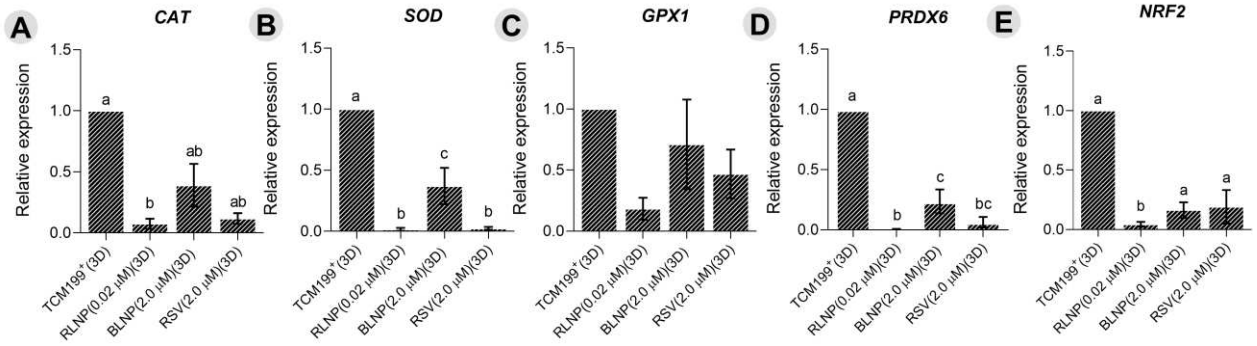

Figure 28. Relative mRNA expression levels of *CAT* (A), *SOD* (B), *GPX1* (C), *PRDX6* (D), or *NRF2* (E) in secondary follicles cultured in dECM for 12 days in TCM199^{+} alone or supplemented with 0.02 μM resveratrol-loaded nanoparticles, 0.2 μM blank nanoparticles or 2 μM nonencapsulated resveratrol. a, b, c: Different lowercase letters indicate statistically significant differences between treatments ($P < 0.05$).

4 Discussion

This study demonstrated that culturing secondary follicles in dECM-based bioscaffolds associated with medium supplemented with RLNP represents a promising strategy for *in vitro* development of culture systems for bovine early follicles. Maintaining ECM integrity during the decellularization process is particularly crucial for functional ovarian tissue engineering, where ECM–follicle interactions play a key role (Wang *et al.,* 2025; Vasse *et al.,* 2024; Fiorentino *et al.,* 2023; McInnes *et al.,* 2022). In our study, freeze-thaw cycles followed by 9-hour incubations in

Triton X-100 and SDS effectively removed cells and residual DNA, and yielded scaffolds with preserved macro- and microarchitecture, resembling native tissue. Alongside effective cell removal, the preservation of ovarian microstructures and ECM components is a critical determinant of bioscaffold quality following decellularization (Léon-Félix *et al.,* 2025). The GAGs and collagen levels remained comparable to controls and stable during *in vitro* culture for 10 and 12 days, respectively, despite minor microarchitectural changes. Moreover, follicle viability was maintained throughout the culture period, indicating no apparent cytotoxicity**.** Nevertheless, it is important to highlight that decellularization protocols may still induce the loss or denaturation of specific ECM proteins (Almeida *et al* 2023). Therefore, further proteomic analyses are essential to better characterize potential alterations in the ovarian matrisome.

Due to its preserved porous architecture, decellularized extracellular matrix scaffolds are considered suitable for *in vitro* culture, as it enables efficient diffusion of culture media and promotes nutrient exchange (Nikniaz *et al*., 2021). Follicles cultured in the 3D system exhibited greater intracellular esterase activity, and reduced membrane damage compared to those maintained in the 2D system under identical conditions. These findings indicate that the 3D system alone already provides improved conditions for follicular maintenance, irrespective of culture medium supplementation. Silva *et al.* (2024) demonstrated a correlation between non-specific esterase activity and both viability and growth of ovarian follicles in bovine. Accumulating evidence indicates that follicular development relies on dynamic and reciprocal interactions between the follicle and its surrounding microenvironment (Wang *et al.,* 2025). Thus, dECM scaffolds may offer a supportive mechanical microenvironment by conveying biomechanical cues essential for promoting follicular development (Alshaikh *et al.,* 2020). Further, GAGs and other specific ECM domains have a strong binding affinity for growth factors that can be *in vitro* released and influence follicular development (Francés-Herrero *et al.,* 2024; López-Martínez *et al.,* 2021; Yan *et al.,* 2018). The bioactivity of bovine ovarian dECM has already been demonstrated by Laronda *et al*. (2015), who reported estradiol secretion by primary mouse ovarian cells cultured on dECM scaffolds. Similarly, Alaee *et al*. (2020) showed that preantral follicles cultured in dECM scaffolds exhibited increased diameter, enhanced antral cavity formation, and higher estradiol and progesterone secretion after 12 days of *in vitro* culture compared to 2D systems. In addition, porcine ovarian cells seeded onto dECM scaffolds expressed key granulosa cell markers, including

STAR, CYP11A1, CYP19A1, AMH, FSHR, and LHR (Pennarossa *et al*., 2021), indicating the ability of ECM-based scaffolds ability to drive follicular development *in vitro*.

In this study, the physicochemical characterization of the nanoparticles demonstrated mean sizes below 150 nm for both formulations, a feature commonly associated with efficient cellular uptake (Foroozandeh and Aziz, 2018). The PDI values ranged from 0.16 for blank nanoparticles to 0.26 for resveratrol-loaded nanoparticles, indicating moderate size distribution uniformity, which is considered acceptable for drug delivery systems (Danaei *et al.,* 2018). Both formulations exhibited negative surface charges, with zeta potential values close to -6 mV. Although low zeta potential magnitudes typically indicate reduced colloidal stability, Freitas *et al.* (2023) reported that these formulations maintained colloidal stability for up to 90 days.

Notably, in our study, RLNP supplementation of culture medium in the 3D system enhanced follicular viability. Transmission electron microscopy revealed that oocytes from follicles cultured with RLNP had a well-preserved zona pellucida, while granulosa cells showed intact mitochondria and endoplasmic reticulum, with no evident ultrastructural damage. Several studies have reported that resveratrol enhances ATP production and mitochondrial biogenesis in mammalian granulosa cells, including those from aged cows, thereby improving mitochondrial function and supporting oocyte development *in vitro* (Nishigaki *et al*., 2021; Sugiyama *et al*., 2015). As a known Sirtuin-1 (SIRT1) activator, resveratrol upregulates SIRT1 expression and increases ATP levels in bovine oocytes, resulting in improved fertilization outcomes (Takeo *et al*., 2014). In rats, it also promotes TZP synthesis by increasing cytosolic calcium, activating Calcium/Calmodulin Dependent Protein Kinase II Beta (CaMKIIβ), and releasing actin monomers (Chen *et al*., 2022). In our study, the enhanced effect of resveratrol when encapsulated in polymeric nanoparticles strongly suggests that employing this drug delivery system is advantageous, since it can improve the aqueous solubility and bioavailability of resveratrol, enhance its physicochemical stability, and enable targeted and controlled drug release (Freitas *et al.,* 2023; Summerlin *et al.,* 2015).

Resveratrol has been widely reported in the literature as exhibiting a potent antioxidant effect in the ovary (Jiang *et al.,* 2024; Saber *et al.,* 2024). *After 12 days of culture,* follicles cultured with 0.02 µM RLNP showed reduced mRNA expression of *CAT*, *SOD*, *PRDX6*, and *NRF2* compared to follicles cultured in control medium, although *SOD* levels were similar to those follicles cultured with unencapsulated resveratrol. Overall, ROS generation modulates

transcriptional antioxidant responses by activating signaling pathways and enzymes involved in redox homeostasis and ROS elimination (Ngo *et al*., 2022). The NRF2 is a key regulator of antioxidant gene expression and, upon ROS exposure, translocates to the nucleus to induce the transcription of antioxidant enzymes (Espinosa-Diez *et al*., 2015). In our study, it is plausible that RLNP exerted a direct free radical-scavenging effect, contributing to a less oxidative microenvironment, reducing the activation of endogenous antioxidant defenses. As a direct antioxidant agent, resveratrol neutralizes various ROS through hydrogen atom transfer and sequential proton loss electron transfer mechanisms, thereby protecting cellular biomolecules from oxidative damage (Truong *et al*., 2018). The ROS-scavenging properties of resveratrol have been consistently demonstrated in recent studies. Cai *et al*. (2023) showed that resveratrol alleviates oxidative stress in rat granulosa cells (GCs) by decreasing intracellular ROS levels, which was accompanied by a reduction in malondialdehyde content. Similarly, Jiang *et al*. (2024) observed this antioxidative effect in *Bos grunniens* granulosa cells, along with a concomitant increase in intracellular glutathione levels.

In conclusion, the decellularized bovine ovarian tissue exhibited minimal residual cellular content and well-preserved ECM ultrastructure. The 3D culture system provided a more favorable environment for follicular development compared to the 2D system, especially when supplemented with RLNP. At 0.02 μM, RLNP preserved follicular ultrastructure, maintained essential features such as the zona pellucida, granulosa cells, and intracellular organelles. Moreover, 0.02 μM, RLNP downregulated the expression of *CAT, SOD, GPX1, PRDX6*, and *NRF2*.

Data availability

All data produced or analyzed during this study are included in this article and can be shared upon reasonable request to the corresponding author.

Author contribution statement

FCC, BRS, RPR, and JRVS designed research; FCC, EITA, DRN, MFLN, AAS, VANA, MAMD, and AVFR performed research; JOE provided critical reagents; FCC, and JRVS analyzed data;

FCC, BRS wrote the manuscript; JRVS, and JOE critically analyzed the manuscript. All authors edited and approved the final manuscript.

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Declaration of competing of interest

The authors declare that they have no conflicts of interest.

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8 CONCLUSÕES GERAIS

- O microambiente *in vitro* induz uma redução de colágeno, glicosaminoglicanos e células estromais ao redor dos folículos primordiais e primários contidos em cultura de tecidos ovarianos bovinos.

- O cultivo *in vitro* de tecido ovariano bovino favorece a ativação folicular, mas reduz a proporção de folículos morfologicamente normais, o número de células da granulosa e os diâmetros folicular e oocitário, bem como diminuiu a expressão de mRNA para SOD, CAT, GPX e PRDX6; e diminuiu a atividade da CAT.

- A suplementação do meio de cultivo de tecido ovariano bovino com ácido ascórbico e resveratrol melhora parcialmente a preservação dos componentes da matriz extracelular e aumenta a sobrevivência folicular *in vitro*, provavelmente pela manutenção de um ambiente redox mais equilibrado.

- A descelularização o tecido ovariano bovino descelularizado produz *scaffolds* com conteúdo celular residual mínimo e ultraestrutura da matriz extracelular remanescente bem preservada.

- O cultivo de folículos ovarianos secundários isolados bovinos em sistema de cultura tridimensional proporcionou um ambiente mais favorável ao desenvolvimento folicular, especialmente quando suplementado com resveratrol encapsulado em nanopartículas poliméricas.

- A suplementação do meio de cultivo de folículos secundários isolados bovinos com 0,02 µM de resveratrol nanoencapsulado preservou a ultraestrutura folicular, mantendo a zona pelúcida, células da granulosa e organelas, além de reduzir a expressão de genes antioxidantes

9 PERSPECTIVAS

Os achados deste estudo oferecem subsídios relevantes para o aprimoramento das estratégias de cultivo *in vitro* de tecido ovariano bovino, evidenciando a matriz extracelular como um elemento-chave cuja preservação é essencial para manter a integridade estrutural e funcional do microambiente folicular ao longo do cultivo. Os resultados obtidos abrem novas perspectivas para a compreensão das interações bidirecionais entre os folículos e seu microambiente, com o objetivo de avançar no desenvolvimento de abordagens capazes de modular estrategicamente os sinais biomecânicos que regulam o crescimento folicular durante o cultivo de tecido ovariano na espécie bovina. No contexto da bioengenharia ovariana, a combinação de *scaffolds* ovarianos descelularizados com nanopartículas poliméricas contendo resveratrol representa um avanço promissor na construção de um ovário artificial funcional, capaz de fornecer um microambiente tridimensional biomimético para o suporte ao crescimento de folículos isolados na espécie bovina. No entanto, a capacidade desses *scaffolds* em sustentar a recelularização com outras populações celulares, como células estromais ou células-tronco, ainda precisa ser aprofundada. O desenvolvimento de estratégias que viabilizem a reconstrução de um nicho ovariano mais completo, funcional e capaz de manter o desenvolvimento folicular *in vitro* por períodos prolongados pode trazer grandes benefícios para a reprodução assistida.

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