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BIANCA RÉGIA SILVA

EFEITOS DA MELATONINA DURANTE O CULTIVO *IN VITRO* DE FOLÍCULOS PRÉ-ANTRAIS E ANTRAIS E NA MATURAÇÃO *IN VITRO* DE COMPLEXOS CUMULUS-OÓCITO BOVINOS

FORTALEZA
2023

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Tese apresentada ao Programa de Pós-Graduação em Biotecnologia - RENORBIO, da Universidade Federal do Ceará (UFC), como requisito para obtenção do título de doutor em Biotecnologia. Área de Concentração: Biotecnologia em Agropecuária.

Orientador: Professor Doutor José Roberto Viana Silva
Coorientador: Professor Doutor Francisco Léo Nascimento de Aguiar

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"Hoje, neste tempo que é seu, o futuro está sendo plantado. As escolhas que você procura, os amigos que você cultiva, as leituras que você faz, os valores que você abraça, os amores que você ama, tudo será determinante para a colheita futura."

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RESUMO

Os objetivos do presente estudo foram: 1) Investigar os efeitos da melatonina sozinha ou em associação com o antagonista luzindol ou com o inibidor de mTORC1 rapamicina no tecido ovariano bovino cultivado *in vitro* (**Fase 1**); 2) Avaliar os efeitos de diferentes concentrações de melatonina durante o cultivo de folículos antrais inicias bovinos (**Fase 2**) e 3) Investigar os efeitos da modulação do monofosfato de adenosina cíclico durante a pré-maturação *in vitro* e o papel da melatonina na maturação *in vitro* (MIV) de CCOs bovinos (**Fase 3**). Na fase 1, fragmentos de tecido ovariano foram cultivados por seis dias em α-MEM$^+$ sozinho ou suplementado com melatonina (1000 pM), melatonina e luzindol (1000 pM) ou melatonina e rapamicina (0,16 µg/ml). Na fase 2, folículos antrais iniciais isolados foram cultivados em TCM-199$^+$ sozinho ou suplementado com melatonina 10^{-6}, 10^{-7} ou 10^{-8} M a 38,5°C com 5% de CO_2 durante 8 dias. Após o cultivo, o crescimento folicular, ultraestrutura, configuração da cromatina, viabilidade e os níveis de EROs e RNAm foram investigados. A fase 3 contemplou dois experimentos. No experimento 1, os CCOs foram pré-maturados por 8 h em meio controle ou com 3-isobutil-1-metilxantina (IBMX) e forscolina, IBMX e peptídeo natriurético tipo C (CNP), CNP e forscolina ou IBMX, CNP e forscolina. No experimento 2, os CCOs foram pré-maturados, seguidos de MIV em meio controle sozinho ou com 10^{-6}, 10^{-7} ou 10^{-8} M de melatonina. Após a MIV, foram avaliadas a configuração da cromatina, projeções transzonais (TZPs), espécies reativas de oxigênio, distribuição mitocondrial, ultraestrutura e expressão de RNAm. Para verificação da normalização dos dados o teste Shapiro-Wilk foi utilizado. Os níveis de RNAm foram analisados pelo teste de Kruskal-Wallis e para as demais avaliações, em geral, utilizou-se os testes de ANOVA e Tukey. Na fase 1, os tecidos ovarianos cultivados com melatonina, melatonina e luzindol ou melatonina e rapamicina apresentaram porcentagem significativamente maior de folículos morfologicamente normais comparados ao meio controle. A melatonina reduziu a porcentagem de folículos primordiais, aumentou a porcentagem de folículos em desenvolvimento e os níveis de colágeno. Contudo, esses efeitos foram bloqueados pelo luzindol ou rapamicina ($P<0,05$). Na fase 2, folículos antrais iniciais cultivados com melatonina 10^{-6} M e 10^{-8} M tiveram um aumento progressivo em seus diâmetros durante o período de cultivo ($P <0,05$). Além disso, oócitos de folículos cultivados com 10^{-7} ou 10^{-8} M de melatonina apresentaram fluorescência aumentada para calceína-AM. Folículos cultivados com 10^{-8} M de melatonina apresentaram ultraestrutura bem preservada. Na fase 3, maiores taxas de retomada meiótica foram observadas no tratamento 10^{-8} M de melatonina ($P<0,05$). Os CCOs maturados com 10^{-7} ou 10^{-8} M de melatonina apresentaram maior atividade mitocondrial

(P<0,05), enquanto aqueles maturados com 10^{-6} ou 10^{-8} M apresentaram maiores níveis de TZPs. CCOs maturados com 10^{-8} M de melatonina aumentaram a expressão de RNAm para superóxido dismutase (SOD) e catalase (CAT) (P<0,05), quando comparados com CCOs não cultivados e pré-maturados, respectivamente. Em conclusão, a suplementação de melatonina durante o cultivo do tecido ovariano bovino promove a ativação folicular e aumenta as fibras de colágeno através de seus receptores acoplados à membrana e mTORC1. Além disso, a suplementação de 10^{-8} M de melatonina no cultivo de folículos antrais iniciais melhora a viabilidade do oócito e preserva a ultraestrutura das organelas do oócito e da granulosa. Finalmente, a pré-maturação com CNP e forscolina combinada com a suplementação de 10^{-8} M de melatonina durante a MIV, melhora as taxas de retomada meiótica, preserva TZPs, aumenta a atividade mitocondrial e a expressão relativa de mRNA para SOD e CAT em CCOs.

Palavras-chave: estresse oxidativo; competência oocitária; desenvolvimento folicular; expressão de RNAm.

ABSTRACT

The objectives of the present study were: 1) Investigate the effects of melatonin alone or in association with the antagonist luzindole or the mTORC1 inhibitor rapamycin on bovine ovarian tissue cultured *in vitro* (**Phase 1**); 2) Evaluate the effects of different concentrations of melatonin during the *in vitro* culture of bovine early antral follicles (**Phase 2**) and 3) Investigate the effects of modulation of cyclic adenosine monophosphate during *in vitro* prematuration and the role of melatonin *in vitro* maturation (MIV) of bovine CCOs (**Phase 3**). In phase 1, fragments of ovarian tissue were cultured for six days in α-MEM+ alone or supplemented with melatonin (1000 pM), melatonin and luzindole (1000 pM) or melatonin and rapamycin (0.16 µg/ml). In phase 2, isolated early antral follicles were cultured in TCM-199+ alone or supplemented with 10^{-6}, 10^{-7}, or 10^{-8} M melatonin at 38.5°C with 5% CO_2 for 8 days. After culture, follicular growth, ultrastructure, chromatin configuration, viability and ROS and mRNA levels were investigated. Phase 3 included two experiments. In experiment 1, CCOs were pre-matured for 8 h in control medium or with 3-isobutyl-1-methylxanthine (IBMX) and forskolin, IBMX and C-type natriuretic peptide (CNP), CNP and forskolin or IBMX, CNP and forskolin. In experiment 2, CCOs were pre-matured, followed by IVM in control medium alone or with 10^{-6}, 10^{-7} or 10^{-8} M melatonin. After IVM, chromatin configuration, transzonal projections (TZPs), reactive oxygen species, mitochondrial distribution, ultrastructure and mRNA expression were evaluated. To verify data normalization, the Shapiro-Wilk test was used. The mRNA levels were analyzed using the Kruskal-Wallis test and for other assessments, in general, the ANOVA and Tukey tests were used. In phase 1, ovarian tissues cultured with melatonin, melatonin and luzindole or melatonin and rapamycin showed a significantly higher percentage of morphologically normal follicles compared to the control medium. Melatonin reduced the percentage of primordial follicles, increased the percentage of developing follicles and collagen levels. However, these effects were blocked by luzindole or rapamycin ($P<0.05$). In phase 2, early antral follicles cultured with 10^{-6} M and 10^{-8} M melatonin had a progressive increase in their diameters during the culture period ($P < 0.05$). Furthermore, oocytes from follicles cultured with 10^{-7} or 10^{-8} M melatonin showed increased fluorescence for calcein-AM. Follicles cultured with 10^{-8} M melatonin showed well-preserved ultrastructure. In phase 3, higher rates of meiotic resumption were observed in the 10^{-8} M melatonin treatment ($P<0.05$). CCOs matured with 10^{-7} or 10^{-8} M melatonin showed greater mitochondrial activity ($P<0.05$), while those matured with 10^{-6} or 10^{-8} M showed higher levels of TZPs. CCOs matured with 10^{-8} M melatonin increased mRNA expression for superoxide dismutase (SOD) and catalase

(CAT) ($P<0.05$), when compared to uncultured and pre-matured CCOs, respectively. In conclusion, melatonin supplementation during bovine ovarian tissue culture promotes follicular activation and increases collagen fibers through its membrane-coupled receptors and mTORC1. Furthermore, supplementation of 10^{-8} M melatonin in the culture of early antral follicles improves oocyte viability and preserves the ultrastructure of the organelles in oocyte and granulosa cell membranes. Finally, prematuration with CNP and forskolin combined with supplementation of 10^{-8} M melatonin during IVM, improves meiotic resumption rates, preserves TZPs, increases mitochondrial activity and relative mRNA expression for SOD and CAT in CCOs.

Keywords: oxidative stress; oocyte competence; follicular development; mRNA stock.

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LISTA DE ABREVIATURAS E SIGLAS

AI	Anáfase I
AKT	Proteína quinase B
AMH	Hormônio antimülleriano
AMPc	Adenosina 3',5'-monofosfato cíclico
AREG	Anfiregulina
ATP	Adenosina trifosfato
CAT	Catalase
CCNB1	Ciclina B1
CCO	Complexo cumulus-oócito
CGO	Complexo granulosa-oócito
CNP	Peptídeo natriurético tipo C
CDK	Quinase dependente de ciclina
CGP	Células germinativas primordiais
c-KIT	Receptor do *kit* ligante
DNA	Ácido desoxirribonucleico
EGF	Fator de crescimento epidermal
EGFR	Receptor do fator de crescimento epidermal
eIF4E	Fator de iniciação da tradução
ERO	Espécie reativa de oxigênio
ERN	Espécie reativa de nitrogênio
FGF-2	Fator de crescimento fibroblástico-2
FIV	Fertilização *in vitro*
FOXO3	Fator de transcrição *forkhead box 3*[a]
FSH	Hormônio folículo estimulante
GDF-9	Fator de crescimento e diferenciação-9
GnRH	Hormônio liberador de gonadotrofina
GPX	Glutationa peroxidase
GR	Glutationa redutase
GSH	Glutationa reduzida
GSSG	Glutationa oxidada
GVDB	Degradação da vesícula germinativa

H_2O_2	Peróxido de hidrogênio
HDAC6	Histona desacetilase 6
IBMX	3-Isobutil-1-Metilxantina
IL-6	Interleucina-6
IGF-1	Fator de crescimento semelhante à insulina-1
KL	*Kit* ligante
LH	Hormônio luteinizante
MI	Metáfase I
MII	Metáfase II
MEC	Matriz extracellular
MEM	Meio essencial mínimo
MOIFOPA	Manipulação de oócitos inclusos em folículos pré-antrais
MT1	Receptor da melatonina do tipo 1
MT2	Receptor da melatonina do tipo 2
MT3	Receptor da melatonina do tipo 3
mTOR	Alvo mamífero da rapamicina
mTORC1	Complexo 1 de rapamicina em mamíferos
NAS	*N*-acetilserotonina
NRF2	Fator nuclear derivado de eritróide 2
$O_2^{-\bullet}$	Radical superóxido
$OH^{\bullet}$	Radical hidroxila
OPU	*Ovum pick up*
PDK1	Quinase-1 dependente de PI3K
PI3K	Fosfatidilinositol-3-quinase
PIP2	Fosfatidilinositol-4,5-bifosfato
PIP3	Fosfatidilinositol-3,4,5-trifosfato
PIVE	Produção *in vitro* de embriões
PRDX6	Peroxiredoxina-6
PTEN	Fosfatase homóloga à tensina
QR2	Enzima citosólica quinona redutase 2
rpS6	Proteína ribossomal S6
GVBD	Rompimento da vesícula germinativa
SOD	Superóxido dismutase

TI	Telófase I
TCM-199	Meio de cultivo de tecido-199
TSC1	Proteína 1 de Esclerose Tuberosa ou Hamartina
TSC2	Proteína 2 de Esclerose Tuberosa
UFC	Universidade Federal do Ceará
VG	Vesícula Germinativa
4E-BP1	Proteínas de ligação do fator 4E

LISTA DE SÍMBOLOS

>	Maior
%	Porcentagem
µg	Micrograma
µM	Micromolar
kg	Kilograma
mg	Miligrama
mm	Milímetro
ml	Mililitro
pH	Potencial hidrogeniônico

SUMÁRIO

1 INTRODUÇÃO

Com o aumento acentuado da população humana, torna-se necessário uma maior produção de alimentos, evidenciando à importância do aprimoramento de tecnologias que proporcionem avanços na sustentabilidade agrícola e na pecuária (BALBOULA *et al*., 2022). Nesse contexto, pesquisas que visem à otimização de biotécnicas reprodutivas como o cultivo de folículos ovarianos, bem como a maturação *in vitro* (MIV) de oócitos, podem fornecer um maior aproveitamento dos gametas femininos para a produção *in vitro* de embriões (PIVE) e consequentemente, maximizar o potencial reprodutivo de bovinos, aumentando a produção de proteínas de origem animal (VIANA; FIGUEIREDO; SIQUEIRA, 2017).

O cultivo *in vitro* de folículos ovarianos seguido de maturação dos oócitos e fertilização *in vitro*, resultou na produção de embriões e descendentes saudáveis em camundongos (EPPIG; O'BRIEN 1996; CARROL, GOSDEN 1993). No entanto, em bovinos, os resultados limitam-se a formação da cavidade antral a partir de folículos pré-antrais cultivados *in vitro* (PAULINO *et al*., 2022; NASCIMENTO *et al*., 2022). Nesse cenário, o cultivo de folículos ovarianos pré-antrais inclusos em tecido ovariano pode ser utilizado no avanço da compreensão dos mecanismos envolvidos na ativação folicular para o aumento do *pool* de folículos em desenvolvimento e maior disponibilidade de oócitos competentes (DEVOS; GROSBOIS; DEMEESTERE, 2020; VO; KAWAMURA, 2021).

Outro sistema de cultivo *in vitro* que tem sido realizado se dá pela utilização de folículos antrais iniciais isolados (CORDEIRO *et al*., 2023; DE SÁ *et al.,* 2020). Em bovinos, o cultivo de folículos antrais iniciais suplementado com N-acetilcisteína manteve a viabilidade celular do complexo granulosa-oócito (CGO) (CORDEIRO *et al*., 2023). Considerando o potencial dessa categoria folicular, estudos que viabilizem uma maior obtenção de oócitos oriundos desses folículos para utilização na MIV bovina tornam-se de grande interesse científico, além de possuir potencial de rentabilidade financeira. A MIV também é uma importante etapa na obtenção de oócitos competentes aptos a serem fertilizados *in vitro* (MERTON *et al*., 2012; JIANG *et al*., 2023). Entretanto, um dos entraves já evidenciados na MIV é o fato de que os complexos cumulus-oócito (CCO) recuperados artificialmente, retomam espontaneamente a meiose, ocasionando assincronia entre a maturação nuclear e citoplasmática (PINCUS; ENZMANN, 1935; BLONDIN *et al*., 1997; SOARES *et al*., 2020). Portanto, a utilização de uma etapa de pré-maturação com moduladores de adenosina 3',5'-monofosfato cíclico (AMPc), têm demonstrado melhorar substancialmente a competência de desenvolvimento do oócito (SUGIMURA *et al*. 2018; SOTO-HERAS *et al*., 2019; MEDINA-CHÁVEZ *et al*., 2021). Outro

entrave que dificulta a eficiência dos sistemas de cultivo em geral, incluindo a MIV, é o estresse oxidativo (YOUSEFIAN *et al*., 2021; SOTO-HERAS; PARAMIO *et al*., 2020), ocasionado pelo aumento excessivo de espécies reativas de oxigênio (ERO) (PIZZINO *et al*., 2017). Como consequência, danos na atividade mitocondrial, na formação do fuso meiótico e na configuração cromossômica têm sido evidenciados (HE *et al*., 2016). Nesse sentido, a suplementação dos meios de cultivo com substâncias antioxidantes, como a melatonina, tem sido recomendada (COSTA *et al*., 2022; SILVA *et al*., 2023).

A melatonina (N-acetil-5-metoxitriptamina) é um hormônio produzido principalmente pela glândula pineal, sendo encontrado também em oócitos (TAMURA *et al*., 2020). Parte das suas ações são mediadas através da ligação com seus receptores MT1 e MT2 pertencentes à classe de receptores acoplados a proteína G (DUBOCOVICH e MARKOWSKA, 2005). A melatonina também pode se ligar a um terceiro receptor, denominado de enzima citosólica quinona redutase 2 e eliminar as ERO e espécies reativas de nitrogênio (ERN) (ČERNYŠIOV *et al*., 2015; BOUTIN *et al*., 2016). Além disso, devido a sua natureza anfifílica, elimina de forma direta diversas espécies reativas (EROS e ERN) (TAN *et al*., 2016; REITER *et al*., 2016). Estudos *in vitro* demonstraram que a melatonina promove a sobrevivência e ativação folicular (HAO *et al*., 2020; BARBERINO *et al*., 2022) e mantém a densidade de células estromais no tecido cortical ovariano bovino (CAVALCANTE *et al*., 2019). Durante a MIV, a melatonina melhora a qualidade dos oócitos bovinos (CUNHA *et al*., 2016). No entanto, em bovinos os mecanismos de ação da melatonina durante a ativação folicular, bem como, seus potenciais efeitos no cultivo de folículos antrais iniciais e na maturação *in vitro* ainda não foram elucidados.

Para uma melhor compreensão da relevância desta tese, a revisão de literatura a seguir abordará aspectos relacionados à segurança alimentar e agropecuária, reprodução de fêmeas bovinas, biotécnicas aplicadas a reprodução animal, incluindo o cultivo *in vitro* do tecido ovariano e de folículos antrais iniciais e a maturação *in vitro*. Além disso, serão abordados fatores relacionados ao estresse oxidativo durante essas biotécnicas. Finalmente, será evidenciada a ação de antioxidantes enzimáticos e não enzimáticos, com ênfase na melatonina.

2 REVISÃO DE LITERATURA

2.1 Segurança alimentar e agropecuária

A população humana vem crescendo de forma muito rápida nos últimos anos e, consequentemente, há uma necessidade contínua da produção de alimentos em consonância com o princípio da sustentabilidade agrícola. Neste cenário, a pecuária tem subsidiando 40% do valor da produção agrícola mundial e apoia os meios de subsistência e segurança alimentar de mais de 1,3 bilhões de pessoas (FAO, 2018). Mesmo com essas contribuições, estima-se que a nação precisa aumentar substancialmente sua produção de carne até 2050, a fim de atender aos requerimentos nutricionais da população em rápido crescimento (HUNTER *et al*., 2017; FAO, 2018). Portanto, abordagens eficientes e sustentáveis para a pecuária são essenciais para atender as expectativas de sistemas agrícolas sustentáveis (LARDY; CATON, 2012; REYNOLDS *et al*., 2015).

A viabilidade econômica da pecuária depende fortemente de um bom desempenho reprodutivo, que é afetado por fatores como genética, idade, peso corporal, nutrição (D'OCCHIO *et al*., 2019), estresse (CIAMPI *et al*., 2020), qualidade do embrião (FONSECA *et al*., 2017) e taxa de concepção (SHAN *et al*., 2020). Deste modo, a bovinocultura apoia a otimização de novas biotécnicas, como aspiração oocitária (*ovum pick* up - OPU) e produção *in vitro* de embriões (PIVE), como forma de maximizar o melhoramento genético de embriões bovinos (BALBOULA *et al*., 2022). Embora avanços significativos na biotecnologia de OPU tenham ocorrido nas últimas décadas, a taxa de produção de blastocistos *in vitro* ainda pode ser considerada baixa (DEB *et al*., 2011). Isto se deve a uma taxa reduzida na competência de desenvolvimento de oócitos advindos de OPU, com subsequente MIV, comparados com oócitos maturados *in vivo* (NAGANO *et al*., 2019). Uma das explicações para essa discrepância na MIV é ausência da sincronização da maturação de oócitos cultivados *in vitro*, sendo este um desafio a ser contornado. Essa maturação é caracterizada por eventos citoplasmaticos e nucleares, sendo ambos cruciais para o sucesso da fertilização e para o desenvolvimento embrionário (SOARES *et al*., 2020). Outro desafio é a ocorrêcia de estresse oxidativo *in vitro*, sendo considerado um dos fatores que comprometem a capacidade de desenvolvimento de oócitos e embriões (YOUSEFIAN *et al*., 2021). Portanto, melhorar as condições de cultivo é de suma importância para aumentar a qualidade e a competência de desenvolvimento dos oócitos aptos a serem utilizados na PIVE, vizando maximizar a potencial multiplicação de patrimônios genéticos de animais de alto valor financeiro.

Recentemente, avanços nas técnicas genômicas permitiram o melhoramento genético, aumentando a precisão da seleção para características reprodutivas hereditárias (BERRY *et al*., 2014; GUTIERREZ-REINOSO *et al*., 2021). Sabe-se que os resultados da reprodução dependem dos níveis de fertilidade tanto dos machos quanto das fêmeas e suas interações potenciais (CHEN *et al*., 2021). Portanto, determinar os benefícios da seleção genômica de características relacionadas à fertilidade em raças leiteiras, tem sido um verdadeiro obstáculo a ser superado, uma vez que tais características estão entre as mais complexas (LIMA *et al*., 2020).

2.2 Aspectos da reprodução de fêmeas bovinas

Sabe-se que a qualidade dos folículos e oócitos desenvolvidos *in vivo* ou *in vitro*, apresentam implicações diretas na qualidade embrionária (RAMOS-IBEAS *et al*., 2020). Com relação às fêmeas bovinas, foco do nosso estudo, devemos considerar a complexidade do maquinário reprodutor feminino, contendo dois ovários que possuem função gametogênica e endócrina. Assim, a função ovariana normal visa salvaguardar a funcionalidade reprodutiva, fornecendo gametas femininos e um ambiente endócrino estável. É no ovário onde ocorrem os dois principais eventos que garantem a eficiência reprodutiva, sendo eles: a oogênese (formação dos oócitos) e a foliculogênese (inclusão destes oócitos em um ambiente folicular para subsequente desenvolvimento). Em conjunto, a oogênese e a foliculogênese promovem mudanças morfológicas e funcionais nos folículos ovarianos, assim como no desenvolvimento e maturação dos oócitos (PORRAS-GÓMEZ; MORENO-MENDOZA, 2017; SÁNCHEZ; SMITZ, 2012).

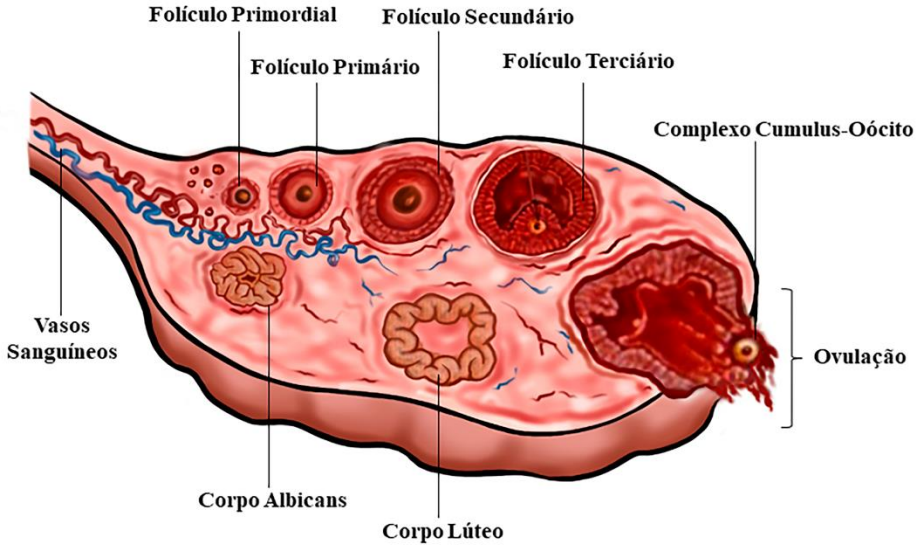
A oogênese tem início ainda na fase embrionária, quando as células germinativas primordiais migram do epiblasto para o saco vitelino (MCLAREN, 2000). Na fase fetal, ainda na gônada primitiva, as células germinativas se multiplicam por mitose e se diferenciam, sendo denominadas de oogônias. As oogônias por sua vez, passam para o estágio de oócitos primários em que o oócito inicia a meiose e para no estágio de prófase I. Já na fase adulta, com o aparecimento da puberdade (estimulação gonadotrófica), os oócitos retomarão seu desenvolvimento e, finalmente, atingirão o estágio de oocítos secundários, quando ocorre a extrusão do primeiro corpúsculo polar. O processo é finalizado com a fecundação do oócito maturo e a liberação do segundo corpúsculo polar (GROSSMAN; SHALGI, 2016).

Já o desenvolvimento folicular ou foliculogênese é um processo gradual, caracterizado por mudanças morfológicas dos folículos que compreendem sua ativação, crescimento e

desenvolvimento até a maturação. A foliculogênese ocorre em duas fases distintas: a pré-antral e a antral (ADONA *et al*., 2015). Na fase pré-antral, folículos com diferentes estágios de desenvolvimento possuem em comum a ausência de cavidade antral, sendo classificados em primordial, primário e secundário. Os folículos primordiais são constituídos por uma única camada de células somáticas pavimentosas (pré-granulosa) (GOUGEON; BUSSO, 2000; GROSSMAN; SHALGI, 2016). Após a ativação dos folículos primordiais, ocorre um aumento no volume do citoplasma e as células da granulosa passam a ter morfologia cúbica, caracterizando o estágio de folículo primário. Além disso, nota-se uma maior quantidade de mitocôndrias e início de formação da zona pelúcida (BASSO; ESPER, 2002). No estágio de folículos secundários, as células da granulosa sofrem intensa multiplicação, dando origem a mais de uma camada de células e ocorre um aumento do diâmetro folicular e oocitário. Além disso, a presença da zona pelúcida, mitocôndrias alongadas e a presença de grânulos corticais é evidenciada (BASSO; ESPER, 2002). Com o desenvolvimento dos folículos secundários ocorre a formação de uma cavidade repleta de líquido folicular denominada de antro. Os folículos antrais são classificados como terciários e pré-ovulatórios ou folículos de Graaf (FIGUEIREDO *et al.,* 2008). Nos folículos terciários, dá-se início à diferenciação das células da granulosa em células do cumulus (circundam o oócito) e células da granulosa murais (formam a parede folicular). A formação da parede folicular e a camada de células do cumulus que circundam o oócito se tornam mais evidentes à medida em que o folículo se desenvolve para o estágio pré-ovulatório (GERSHON; DEKEL, 2020).

Na espécie bovina o desenvolvimento folicular ocorre através de sucessivas ondas de crescimento que possuem três fases distintas (BARUSELLI *et al*., 2007). Na primeira fase, denominada de recrutamento, vários folículos iniciam o crescimento sob controle das gonadotrofinas. Na fase seguinte, ocorre a seleção e dominância, na qual um folículo irá crescer mais com relação aos demais, tornando-se dominante. Durante essa fase, o crescimento folicular está vinculado a ação do FSH e LH. Além disso, o folículo dominante produzirá elevada quantidade de estrógeno. A terceira fase é marcada pelo processo de ovulação, que acontece em consequência ao pico elevado de LH liberado pela hipófise. Outro fator importante para a ocorrência da ovulação é a redução dos níveis de progesterona. Após o período de ovulação, os folículos que não ovularem serão perdidos pelo processo fisiológico de atresia (OLIVEIRA *et al.*, 2014). A figura 1 ilustra didaticamente a estrutura do ovário com os diferentes estágios de folículos pré-antrais e antrais, destacando suas principais características.

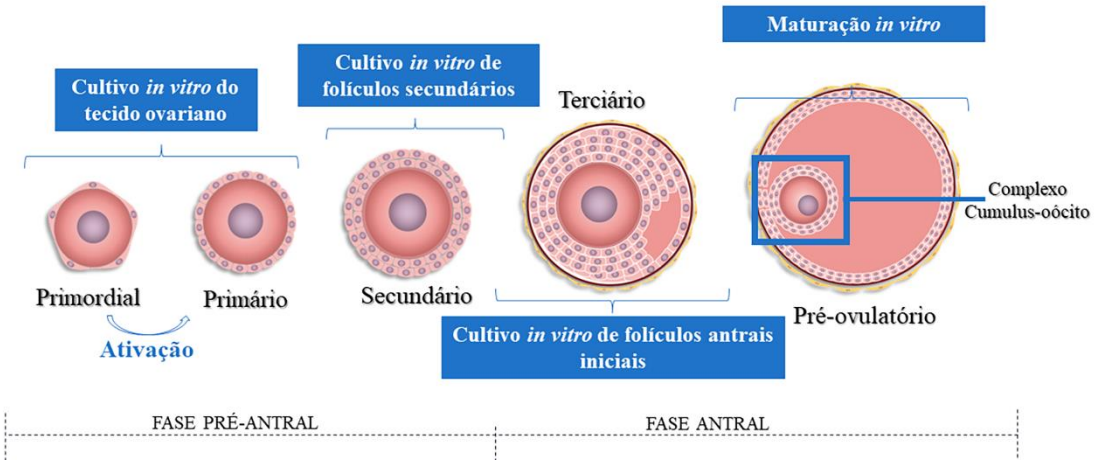
Figura 1. Representação ilustrativa do ovário mamífero com presença de folículos ovarianos em diferentes estágios de desenvolvimento.



Fonte: Elaborada pela autora.

Importante salientar que os folículos pré-antrais não são dependentes das gonadotrofinas, embora sejam responsivos. Assim, os folículos pré-antrais dependem de fatores de crescimento parácrinos para serem ativados e progredirem no seu desenvolvimento. Na fase inicial de crescimento dos folículos primordiais, também conhecida como ativação, ocorre uma transição de folículos quiescentes oriundos do pool de reserva para subsequente crescimento folicular (ver subtópico sobre ativação folicular) (PEDERSEN, 1970; HSUEH *et al*., 2015; ZHANG *et al*., 2015; MONGET *et al*., 2021). Há um consenso científico acerca do fato de que o *pool* de folículos primordiais não é renovável e o folículo primordial não pode retornar ao seu estado quiescente uma vez que inicia seu crescimento (ZHANG *et al*., 2015; ZHANG; LIU, 2015; KALLEN *et al*., 2018). Contudo, este dogma tem sido alvo de discussões com o advento da teoria da neofoliculogênese, que defende uma renovação desta população folicular (NA *et al.,* 2012). Ao nascer, estima-se que a população folicular ovariana bovina seja de aproximadamente 235.000 folículos por ovário (ERICKSON *et al*., 1966; SILVA-SANTOS *et al*., 2011) sendo que desse total, os folículos pré-antrais representam 90% dessa população (SILVA-SANTOS *et al*., 2011). Considerando essa vasta população folicular, diversas biotécnicas estão sendo desenvolvidas afim de subsidiar o aproveitamento desses folículos e oócitos, evitando a atresia. A figura 2 ilustra as diferentes categorias foliculares durante o processo de foliculogênese e as biotécnicas reprodutivas que podem ser empregadas para o aumento do potencial genético de fêmeas de produção ou na cliníca (espécie humana).

Figura 2. Representação ilustrativa do processo de foliculogênese com as diferentes categorias foliculares e as biotécnicas que podem ser empregadas para o aumento da biodisponibilidade dos gametas femininos.



Fonte: Elaborada pela autora.

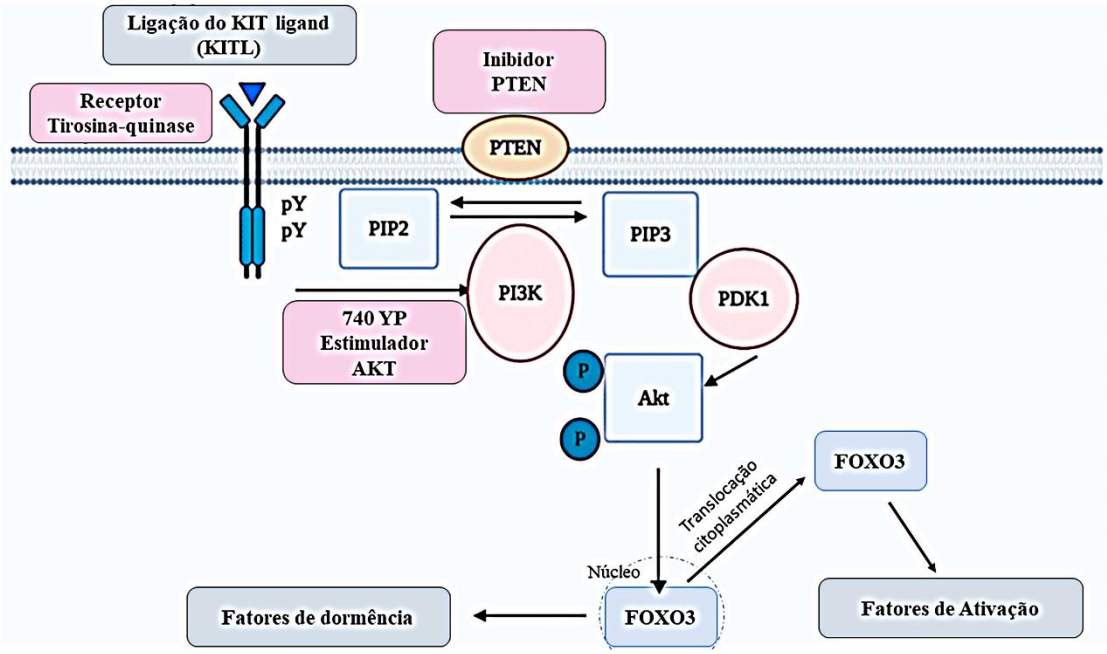
2.2.1. Ativação Folicular

A ativação folicular representa um processo crítico do desenvolvimento folicular. Ela é desencadeada pela supressão de fatores inibitórios e mantenedores da quiescência folicular, que uma vez silenciados, permitem que um *pool* de folículos primordiais passe a se desenvolver (REITER *et al*., 2010). Este evento fisiológico caracteriza-se por alterações morfológicas foliculares, como mudanças nas células da granulosa de morfologia pavimentosa, que passam a ser cuboides com aumento do volume do ooplasma (KALLEN *et al*., 2018). Portanto, os folículos primordiais terão três potenciais destinos fisiológicos: 1) manter-se no pool dos folículos de reserva, permanecendo em quiescência folicular; 2) sofrer ativação folicular, seguido de desenvolvimento e maturação, mas eventualmente morrendo pelo processo natural de atresia folicular e 3) serem ativados, crescerem, desenvolverem-se completamente e serem ovulados após a maturação. Entretanto, apenas uma população reduzida dos oócitos (cerca de 0,1%) será efetivamente ovulada (FIGUREREIDO *et al*., 2018). Desta forma, o restante desta população gametogênica representa uma valiosa fonte de material genético a ser otimizado por meio de biotécnicas reprodutivas.

Embora os mecanismos precisos que resultam na ativação do folículo primordial ainda não sejam totalmente conhecidos (WANG *et al.*, 2017), vários hormônios, fatores de crescimento e vias de sinalização específicas desempenham papéis essenciais nesse processo

(REITER *et al*., 2010; YANG *et al*., 2017). Dentre as vias relacionadas à ativação folicular, a via de sinalização PI3K/AKT/FOXO3 é ativada em resposta à sinalização extracelular e resulta em ativação, crescimento e sobrevivência de folículos primordias (HEMMINGS;RESTUCCIA, 2012). O processo de ativação se inicia quando um fator de crescimento, por exemplo, ligante KIT (KL) interage com o receptor transmenbranar tipo tirosina quinase, denominado proto-oncogene KIT (c-Kit), induzindo uma cascata de segundos mensageiros intracelulares, que por sua vez, fosforilam e ativam a proteína quinase B, também conhecida como AKT. A AKT ativada inibe a atividade do fator de transcrição FOXO3a (*Forkhead Box O3*) no núcleo, levando à translocação de FOXO3a para o citoplasma celular, eventualmente, desencadeando a ativação. Em camundongos, a superexpressão de FOXO3 leva ao aumento da capacidade reprodutiva (PELOSI *et al*., 2013). Outro componente importante dessa via é a fosfatase homóloga à tensina deletada no cromossomo dez (PTEN), que atua como uma fosfatase de fosfatidilinositol-tri-fosfato (PIP3) antagonizando a atividade da PI3K por meio da desfosforilação de PIP3 para PIP2 (MAEHAMA; DIXON, 1998). Na espécie bovina, a ativação do folículo primordial *in vitro* está associada à inibição de PTEN e à translocação citoplasmática de FOXO3a (Figura 3) (BROMFIELD; SHELDON, 2013).

Figura 3. Representação esquemática da via de sinalização PI3K/AKT/FOXO3. Em resposta à ligação de KL, uma cascata de eventos ocorre para o controle da ativação e crescimento de folículos ovarianos primordiais.

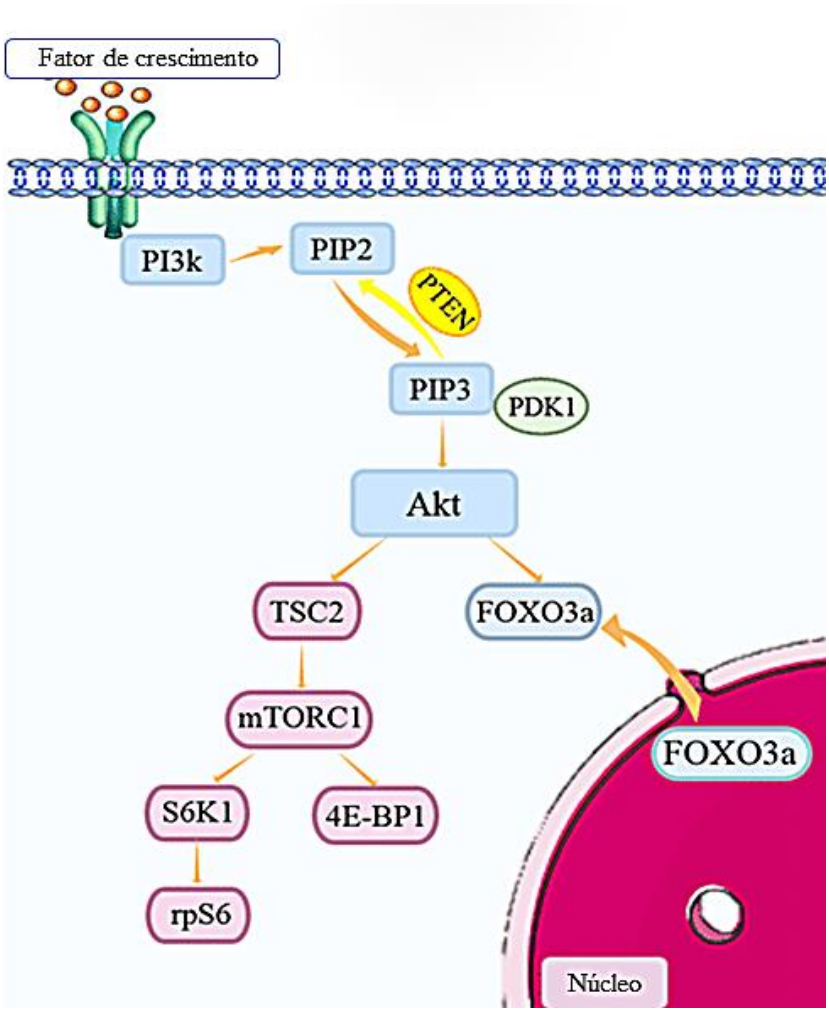


Fonte: Adaptado de VO *et al*. 2021.

Outra via importante no processo de ativação folicular é via relacionada à proteína alvo da rapamicina em mamíferos (mTOR), sendo considerada essencial para a oogênese, desenvolvimento folicular, manutenção da reserva folicular e maturação oocitária (LIU *et al*., 2018; CORREIA *et al*., 2020). O mTOR é uma serina/treonina quinase conservada que interage com diversas proteínas para formar dois complexos distintos denominados MTORC1 e MTORC2. Diversos estudos evidenciaram que o mTORC1 pode estar diretamente relacionado com o controle da ativação de folículos primordiais (ADHIKARI *et al*., 2013; ZHAO *et al*., 2018; TONG *et al*., 2013). Estudos reportaram a relação entre o heterodímero composto pela esclerose tuberosa 1 (TSC1) e esclerose tuberosa 2 (TSC2) na regulação negativa da sinalização mTORC1 (ADHIKARI et al., 2009; SALUSSOLIA *et al.*, 2019). O processo de ativação folicular via PI3K/AKT/mTOR ocorre da seguinte maneira: Com a ligação do KL ao seu receptor c-KIT na superfície do oócito, ocorrerá a ativação da sinalização PI3K/Akt e, em seguida, o AKT fosforila o TSC2, induzindo a inativação do complexo TSC1/TSC2. Como consequência, acontece a ativação de mTORC1. Uma vez ativado, o mTORC1 provome a ativação a jusante da proteína ribossomal S6 quinase (S6K), da proteína de ligação do fator 4E de inibição da tradução eucariótica (4E-BP1) e da proteína ribossomal S6 (rpS6) que promovem o crescimento celular modulando a tradução de proteínas alvo (CASTRILLON *et al*., 2003; REDDY *et al*., 2008; ZHANG *et al*., 2014). Dessa forma, a via de sinalização mTORC1 é regulada pela via de sinalização PI3K-AKT a jusante (MA; BLENIS, 2009; BEN, 2010) (Figura 4). Estudos *in vivo* mostraram que a supressão de mTORC1 mantém a quiescência dos folículos primordiais de camundongos (REDDY *et al*., 2008; ZHOU *et al*. 2017). Além disso, foi relatado que o TSC1 interage com o PTEN e suprime o MTORC1 na ativação de folículos primordiais (ZENG *et al.,* 2012).

A rapamicina, um inibidor específico da mTOR, tem sido usada para a compreensão da ativação folicular, bem como na preservação de folículos ovarianos na síndrome da insuficiência ovariana prematura (DOU *et al*., 2017).

Figura 4. Representação esquemática da via de sinalização PI3K/AKT/mTOR. Uma vez fosforilada, a AKT poderá ativar a FOXO3a ou regular a atividade do complexo TSC1/TSC2 de mTORC1, que ativará a S6K, 4E-BP1 e rpS6, desencadeando a ativação folicular.



Fonte: Adaptado de HUANG et al., 2022.

A via Hippo também está envolvida na ativação e crescimento folicular, através da ação da proteína associada ao YES (*Yes-associated protein* -YAP) e o do motivo de ligação ao PDZ do coativador transcricional (TAZ) (HSUEH *et al.*, 2015). Com a interrupção da via de sinalização Hippo após a fragmentação do córtex ovariano, ocorre a desfosforilação de YAP levando ao 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 CCN e os níveis de fatores inibidores de apoptose – BIRC (*baculoviral inhibitors of apoptosis repeat containing*), estimulando o crescimento celular, sobrevivência e proliferação (PAN, 2007; HOLBOURN-ACHARYA; PERBAL, 2008). Outras moléculas também foram relatadas como envolvidas na ativação de folículos primordiais, incluindo as proteínas quinases ativadas

por mitógeno (MAPK3/1), E-caderina e histona desacetilase 6 (HDAC6) (ZHAO *et al*., 2018; LI *et al*., 2020; ZHAO *et al*., 2020; ZHANG *et al*., 2021).

Não obstante, um fenômeno de ativação folicular massiva, denominado de "*burn-out*" pode ocorrer devido à remoção dos fatores supressores da ativação folicular (exemplo, PTEN, p27, REDDY *et al*., 2010). Como consequência, ocorre um esgotamento severo do *pool* de reserva folicular, e depleção da capacidade reprodutiva da fêmea, resultando na morte de muitos folículos pelo processo fisiológico da atresia folicular. Esta atresia se dá em associação com a remoção de fatores mantenedores da sobrevivência folicular em conjunto com a eliminação dos fatores supressores da ativação (exemplo, PI3K signaling, REDDY *et al*., 2010). Portanto, fica evidente a complexa gama de eventos relacionados à ativação folicular seguida do crescimento aos quais os folículos podem passar, sendo fundamental a realização de pesquisas que visem à otimização das biotécnicas reprodutivas.

2.3 Biotécnicas Aplicadas a Reprodução Animal

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

Os sistemas de cultivo de foliculos pré-antrais *in vitro* visam resgatar os folículos do ambiente ovariano e cultivá-los *in vitro* até a sua completa maturação. Para tanto, a bioteconologia da Manipulação de Oócitos Inclusos em Folículos Ovarianos Pré Antrais (MOIFOPA), também conhecida como Ovário Artificial, vem se destacando nas últimas décadas, como uma ferramenta capaz de elucidar os mecanismos envolvidos na regulação da foliculogênese. Diversas são as potenciais utilizações da MOIFOPA, as quais podemos exemplificar: ensaios iniciais de desenvolvimento de fármacos por meio de estudos *in vitro*, apresentando-se como uma alternativa à experimentação animal; produção de bancos de germoplasma (por meio da criopreservação de gametas e tecidos ovarianos de espécies ameaças de extinção ou de alto valor zootécnico); transplante de tecido ovariano (com a finalidade de reposição hormonal) e a otimização das biotécnicas de reprodução assistida em animais, incluindo seres humanos (FIGUEIREDO *et al*., 2018).

Os folículos pré-antrais podem ser cultivados de duas formas: inclusos no tecido ovariano ou na forma isolada. No que concerne ao sistema de cultivo de folículos inclusos no tecido ovariado, este pode ser cultivado em fragmentos de tecido, ou ainda utilizando-se o ovário inteiro ((FIGUEIREDO *et al.,* 2018). O cultivo de tecido ovariano vem sendo desenvolvido em várias espécies de produção (caprinos: SOUSA *et al.,* 2021; ovinos: MONTE

et al., 2021; bovinos: RIBEIRO *et al.,* 2015; PASSOS *et al.,* 2016). Como vantagens deste sistema de cultivo, destacam-se a manutenção do contato intercelular e a integridade tridimensional dos folículos, fornecendo um complexo sistema de suporte que mimetiza o ovário *in vivo* (ABIR *et al*., 2006). Isto se deve ao fato de que os folículos pré-antrais são cercados por uma abundante matriz extracelular (MEC) presente no córtex ovariano. Trabalhos recentes de Nagamatsu *et al.* (2019) demonstraram que a MEC cortical dos ovários de camundongo fornece estresse mecânico para manter os folículos primordiais em estado dormente.

Em se tratando de sistema de cultivo de folículos na forma isolada, diferentes estratégias têm sido empregadas com sucesso, em que os folículos podem ser cultivados diretamente sobre uma placa ou sobre uma monocamada de células de cultivo (sistema bidimensional - 2D) ou ainda inclusos em uma matriz (sistema tridimensional - 3D). Para esse propósito, vários tipos de matrizes podem ser utlizadas para encapsular os folículos, permitindo que as células somáticas se proliferem e mantendo a estrutura do folículo e as interações célula-célula (BELLI *et al*., 2012). Como vantagens desse sistema, podemos citar o acompanhamento individualizado do crescimento e sobrevivência do folículo ao longo do cultivo, bem como a determinação de requerimentos foliculares específicos (ABIR *et al*., 2006). Pode-se ainda realizar cultivo em dois passos, no qual realiza-se primeiramente o cultivo de tecido ovariano, permitindo a ativação e o desenvolvimento dos folículos primordiais para o estágio secundário, para posterior isolamento folicular e cultivo até o estágio antral (FIGUEIREDO *et al.,* 2018). Conforme relatado por McLaughlin *et al.* (2018) cultivo do tecido cortical ovariano humano seguido do cultivo de folículos secundários isolados produziu oócitos capazes de retomar a meiose após a MIV.

Em geral, no sistema de cultivo de folículos pré-antrais isolados, utilizam-se folículos secundários para o aproveitamento de um maior número de oócitos competentes (suínos: WU; EMERY; CARRELL, 2001; bubalinos: GUPTA *et al.,* 2008; ovinos: ARUNAKUMARI; SHANMUGASUNDARAM; RAO, 2010; caprinos: SARAIVA *et al*., 2010; MAGALHÃES *et al*., 2011; e bovinos: PAULINO *et al*., 2018, 2019; BEZERRA *et al.,* 2019, 2020; VASCONCELOS *et al.,* 2021). Recentemente, a utilização de folículos antrais inicias aplicando o sistema de cultivo na forma isolada tem sido sugerido (DE SÁ *et al*., 2020; CORDEIRO *et al.,* 2023).

2.3.2 Cultivo *in vitro* de folículos antrais iniciais

Embora a reserva ovariana seja constituída majoritariamente por folículos pré-antrais (~90%), uma vez que essa população supere o desafio da transição entre o estágio pré-antral para o estágio antral, os folículos antrais possuem grande potencial para adquirir competência oocitária *in vitro* (CLARKE *et al*., 2018).

Os folículos antrais são caracterizados por possuírem várias camadas de células da granulosa, duas ou mais camadas de células da teca e um espaço preenchido de líquido folicular denominado antro (GERSHON; DEKEL, 2020). O potencial de competência oocitária dessa categoria folicular tem sido relacionado com a capacidade de sintetizar e secretar hormônios esteroides sexuais, como andrógenos e estrógenos a partir das células da granulosa. Além disso, a presença do antro confere suporte nutricional e mecânico ao oócito até a ovulação (HERNÁNDEZ-OCHOA *et al*., 2018). No entanto, durante a foliculogênese, a maioria dos folículos antrais sofrerão atresia devido a fatores inibitórios (estradiol e inibina) liberados no processo de seleção/dominância, resultando na interrupção do crescimento e, consequentemente, em uma perda acentuada dessas população folicular (GARCIA-GUERRA *et al*., 2018; GINTHER *et al*., 2001). Portanto, é premente o desenvolvimento de um sistema de cultivo *in vitro* eficiente que suporte o crescimento de folículos antrais iniciais visando obter um maior número de oócitos recuperados de execelente qualidade para a MIV.

Diversos fatores de crescimento e andrógenos atuam durante a fase folicular antral, desempenhando um papel importante no crescimento e sobrevivência de folículos antrais iniciais. Dentre esses fatores, a ativina e o fator de crescimento semelhante à insulina (IGF-1) desempenham um papel central na aquisição da dependência de FSH no estágio antral inicial do desenvolvimento folicular (PANGAS *et al*., 2006). Os andrógenos derivados da teca ligam-se aos receptores de andrógenos nas células da granulosa (TETSUKA *et al*., 1995), induzindo assim, a expressão de receptores de FSH e o crescimento folicular durante a transição entre o estágio folicular pré-antral para antral (ORISAKA *et al*., 1995; VENDOLA *et al*., 1998; WANG *et al*., 2001).

Peluffo *et al*. 2010 evidenciaram que oócitos de macacos Rhesus oriundos de folículos antrais iniciais cultivados *in vitro* possuem habilidade de concluir a meiose, e serem fertilizados *in vitro*, podendo atingir o estágio de mórula. Em cabras, o cultivo *in vitro* de folículos antrais iniciais suplementado com anetol seguido da maturação *in vitro* permitiu a produção *in vitro* de embriões (DE SÁ *et al.,* 2020). Barro *et al*. (2020) relataram que oócitos oriundos de folículos antrais inicias de ovelhas cultivados *in vitro* com melatonina, apresentaram aumento da

atividade mitocondrial e aquisição da competência meiótica. Recentemente em bovinos, o cultivo de folículos antrais iniciais suplementado com N-acetil-cisteína manteve a viabilidade celular do complexo granulosa-oócito após 8 dias de cultivo (CORDEIRO *et al*., 2023). Portanto, a elaboração de um sistema de cultivo eficiente que suporte o desenvolvimento de folículos antrais iniciais bovinos, poderá maximizar o aproveitamento dos oócitos a serem utilizados em biotecnologias de reprodução assistida como MIV e subsequente FIV.

2.3.3 Maturação *in vitro* (MIV)

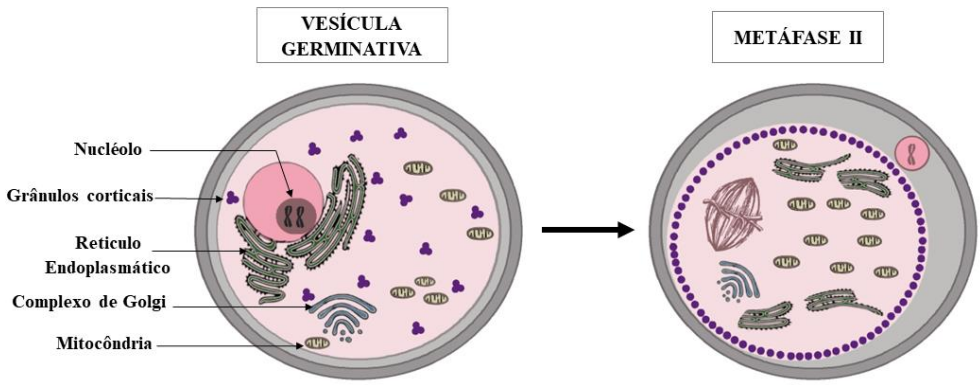
Dentre os principais estágios da PIVE, destaca-se a MIV de oócitos imaturos recuperados de folículos ovarianos. A MIV é um dos processos mais importantes e complexos para a PIVE (SOTO-HERAS; PARAMIO, 2020), e é constituída por eventos nucleares e citoplasmáticos que devem acontecer no oócito de forma orquestrada (BLANCO *et al*., 2011).

A maturação nuclear é caracterizada pela retomada da meiose e progressão para o estágio de metáfase II (MII). O oócito de mamíferos passa por duas divisões celulares sucessivas durante seu processo de maturação (MA *et al*., 2013). Primeiro, o oócito é parado no estágio de diplóteno. Nesta fase, o oócito contém um grande núcleo, denominado de vesícula germinativa (VG). A degradação da vesícula germinativa (GVBD) envolve a condensação da cromatina e a desintegração da membrana nuclear (JAFFE; EGBERT, 2017; GRANADOS-APARICI *et al*., 2019). Após a GVBD, o oócito retoma a meiose e entra na metáfase I (MI) (ALMONACID *et al*., 2019). A primeira meiose termina com a formação de um oócito haploide e a extrusão do primeiro corpúsculo polar. Uma segunda parada ocorre em MII até o momento que o oócito é fertilizado (JAFFE; EGBERT, 2017; CONTI *et al*., 2018). Falhas nesses eventos meióticos podem impedir que os oócitos atinjam a maturação nuclear adequada (ZENG *et al*., 2018; CONTI *et al*., 2018), evidenciando a importância de estudos que subsidiem a otimização desse processo *in vitro* em conjunto com a maturação citoplasmática.

A maturação citoplasmática envolve eventos relacionados à capacitação citoplasmática do oócito (FERREIRA *et al*., 2009) incluindo a redistribuição de organelas, mudanças no citoesqueleto, aparelho de Golgi, liberação de cálcio e armazenamento de RNAm, proteínas e fatores de transcrição (MAO *et al*., 2014; BLANCO *et al*., 2011, VAN DEN HURK; ZHAO, 2005; CROCOMO, 2015). O processo de armazenamento de proteínas e de RNAm está relacionado especificamente com a maturação molecular do oócito que é por sua vez, essencial para a recomposição genômica, ativação e envio de mensagens indispensáveis para regulação da embriogênese. Assim, o oócito é o principal responsável por assegurar o desenvolvimento

embrionário inicial, principalmente devido à presença de grande quantidade de RNAm e proteínas que foram acumulados durante o crescimento do oócito (FAIR *et al*., 2007, HAMATANI *et al*., 2008). Além disso, muitas organelas são redistribuídas durante o processo da maturação citoplasmática. As mitocôndrias, através da fosforilação oxidativa no metabolismo dos carboidratos e dos ácidos graxos, são as responsáveis pela produção de grande parte da energia celular em forma de ATP. Em oócitos na fase de VG, as mitocôndrias encontram-se na zona cortical do citoplasma, com organização heterogênea, enquanto que em oócitos nos estágios de MI e MII, estas são muito mais numerosas e encontram-se distribuídas por todo o citoplasma, com organização homogênea (KATSKA *et al*., 2011; MAO *et al*., 2014). (Figura 5). A eficiência desses eventos citoplasmáticos e nucleares irá garantir o sucesso e a competência de oócitos aptos a serem fertilizados e o subsequente desenvolvimento embrionário *in vitro.*

Figura 5. Representação ilustrativa do processo de maturação oocitária. O oócito sofre uma série de alterações coordenadas que afetam o núcleo e o citoplasma. Durante a maturação nuclear, o oócito haplóide em metáfase I expulsa metade de seu material genético e faz a transição para um gameta haplóide em metáfase II. Já na maturação citoplasmática, ocorrem mudanças nas organelas. Antes da quebra da vesícula germinativa, as mitocôndrias possuem distribuição heterogênea e a vesícula germinativa (VG) e o retículo endoplasmático se apresentam intactos. Em metáfase II, as mitocôndrias estão distribuídas de forma homogênea, e o corpúsculo polar é extrusado. Além disso, os grânulos corticais migram para a periferia do oócito.



Fonte: Elaborada pela autora.

É sabido que a PIVE de embriões utilizando oócitos oriundos de MIV ainda apresenta limitações, considerando que nem todos os oócitos têm a capacidade de se desenvolver em um embrião viável após a MIV (RIZOS *et al*., 2002). Uma das causas deste insucesso está relacionada à diminuição da qualidade dos gametas provindos dos sistemas de cultivo *in vitro* (ROCHA *et al*., 2016). Em bovinos, por exemplo, a taxa de produção de embriões oriundos da MIV permanece em 30 a 40% (LONERGAN *et al*., 2016; SUDANO *et al*., 2019), percentuais esses que podem ser significativamente otimizados.

Um passo fundamental para superar as baixas taxas de sucesso da MIV é identificar um ambiente de cultivo ideal, que mimetize ou supere as condições de desenvolvimento oocitário *in vivo*. Atualmente, novos tipos de sistemas de MIV vêm sendo desenvolvidos para permitir o crescimento e a competência de oócitos *in vitro* (BAHRAMI *et al*., 2019, SCHEFER *et al*., 2021). Diversos fatores podem influenciar na qualidade e competência do oócito, como por exemplo, o diâmetro folicular no qual o CCO é recuperado. Folículos pré-ovulatórios bovinos com diâmetros entre 15 a 25 mm geralmente contêm oócitos competentes. Por outro lado, oócitos oriundos de folículos pequenos ou médios (1-6 mm) possuem oócitos com diâmetro de 100 a 110 μm e, nesse tamanho, os oócitos ainda atingirão gradativamente a competência para completar a maturação nuclear e citoplasmática (HYTTEL *et al*., 1939). De fato, os CCOs recuperados para a MIV iniciam espontaneamente a meiose, uma vez removidos artificialmente dos folículos. Este fenômeno resulta na retomada prematura da meiose e consequentemente, extrusão precoce do primeiro corpúsculo polar, levando a uma assincronia na maturação citoplasmática (RIZOS *et al*., 2002; SUTTON *et al*., 2003). Dessa forma, estudos que promovam a sincronização dos eventos nucleares e citoplasmáticos nos oócito são prementes.

Estudos prévios tem demonstrado que a modulação dos níveis de AMPc em CCOs de mamíferos durante a MIV poderia melhorar substancialmente a competência de desenvolvimento do oócito em diferentes espécies (bovinos: LUCIANO *et al*., 2011; ALBUZ *et al.,* 2010; SUGIMURA *et al.,* 2018; camundongos: ALBUZ *et al.,* 2010; ZENG *et al*., 2014; suínos: TANGA *et al.,* 2023; humanos: NOGUEIRA *et al.,* 2006; VANHOUTTE *et al.,* 2009; e caprinos, SOTO-HERAS *et al.,* 2019). O AMPc desempenha um papel fundamental na maturação nuclear, atuando na parada e retomada da meiose que está associada a uma maior competência de desenvolvimento (MEDINA-CHÁVEZ *et al.,* 2021). Os níveis de AMPc intraoocitário são regulados pela fosfodiesterase (PDE) e pela adenilato ciclase (AC), que desempenham papéis na degradação e síntese de AMPc, respectivamente (ALBUZ *et al*., 2010; ZHANG *et al.,* 2010). Um aumento nos níveis de AMPc ativa a proteína quinase A dependente

de AMPc, resultando em parada meiótica através da inibição do fator promotor de maturação (MPF) e das MAPKs (ROSE *et al*., 2013). Além disso, o AMPc se comunica com a via GMPc para o controle da retomada da meiose. O GMPc das células da granulosa é transferido através de junções comunicantes e mantém altos níveis de AMPc pela inibição da PDE3 nos oócitos (NORRIS *et al.,* 2009). Esses achados evidenciam que elevados níveis de AMPc controlam várias vias para inibição da retomada meiótica.

Afim de controlar o AMPc durante a MIV, protocolos em duas etapas ou sistemas de pré-maturação *in vitro* vêm sendo desenvolvidos. Esses sistemas baseiam-se na utilização de uma etapa inicial de pré-maturação que mantém o oócito em parada meiótica, prolongando assim, a maturação nuclear para que uma adequada maturação citoplasmática aconteça (ALBUZ *et al.,* 2010; FRANCIOSI *et al .,* 2014). Entre os sistemas que foram desenvolvidos, os mais promissores são aqueles que inibem ou retardam farmacologicamente a retomada meiótica, elevando a concentração de AMPc no oócito e mantendo a funcionalidade das projeções tranzonais (ALBUZ *et al*., 2010; LUCIANO *et al*., 2004). Elevados níveis de AMPc intracelular podem ser mantidos por inibidores de PDE, como o 3-isobutil-1-metilxantina (IBMX) ou o peptídeo natriurético do tipo C (CNP), para interromper a degradação de AMPc, ou ainda por meio da ativação contínua de AC induzida pela forscolina (ALBUZ *et al*., 2010). Em bovinos e camundongos, o tratamento com modulador de AMPc durante a pré-MIV melhorou o rendimento embrionário e o desenvolvimento subsequente, incluindo o rendimento fetal, peso e implantação (ALBUZ *et al*., 2010; LI *et al*., 2016). Com a utilização da fase de pré-MIV, o tempo de cultivo *in vitro* aumenta, podendo levar a um aumento da produção de ERO, resultando em estresse oxidativo (ZHENWEI *et al*., 2019). Dessa forma, o controle das ERO nos sistemas de cultivo *in vitro* é crucial para garantir a sua eficiência.

2.4 Estresse oxidativo durante o cultivo *in vitro* de folículos ovarianos e maturação *in vitro*

Os folículos e os oócitos requeridos para cultivo *in vitro* necessitam de uma regulação cuidadosa do ambiente redox para um desenvolvimento eficiente *in vitro*. Sabe-se que o estado redox de uma célula depende da proporção de moléculas oxidadas e reduzidas (SCHAFER *et al*., 2010) que atuam na manutenção da função celular normal (SIES *et al*., 1970; 2019; 2020). Um desequilíbrio redox, também chamado de estresse oxidativo, ocorre quando a produção e acúmulo de ERO ou ERN em sistemas biológicos estejam acima da capacidade das células e tecidos de neutralizar a um nível seguro essas espécies reativas (PIZZINO *et al*., 2017).

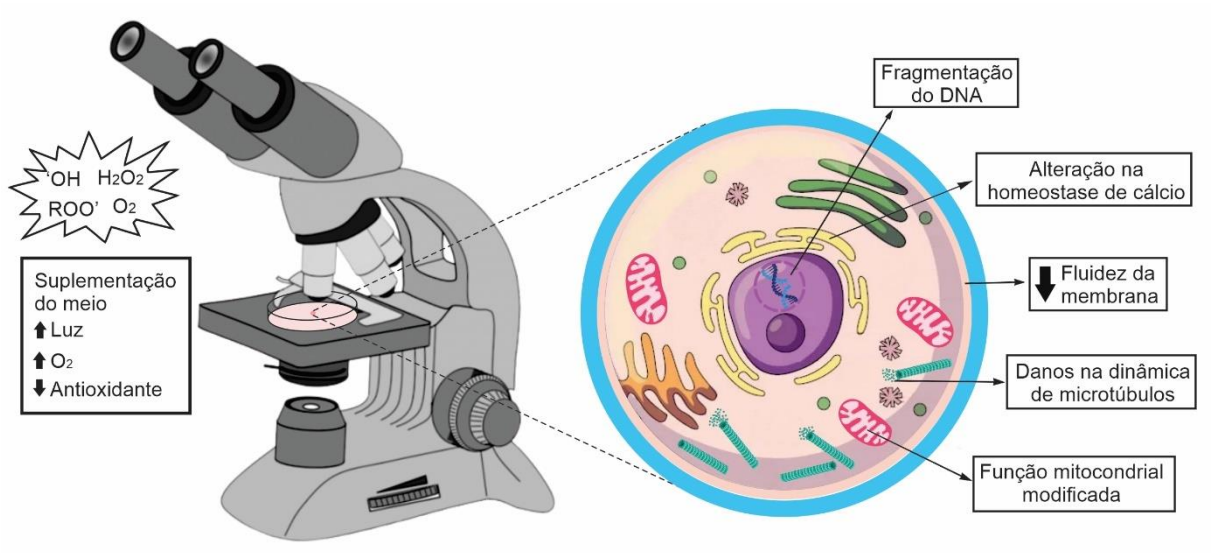
As ERO são encontradas em maior escala em comparação com as ERN, sendo constituídas por moléculas de oxigênio que são altamente reativas e aceptoras de elétrons (AGARWAL *et al*., 2006; FREITAS *et al*., 2017). Em uma condição de equilíbrio, 2 elétrons solitários no orbital mais externo do oxigênio molecular são compartilhados, afim de garantir um mesmo número quântico de elétrons no último orbital. Contudo, ele só pode aceitar um elétron de cada vez, pois é reduzido a H_2O (MAILLOUX *et al*., 2014). Isso resulta na produção de uma série de intermediários, como o ânion superóxido de radical livre (O_2^{-}), o peróxido de hidrogênio não radical (H_2O_2) e o íon hidroxila de radical livre ($OH^{-}\cdot$) (MAILLOUX *et al*., 2014). Portanto, concentrações suprafisiológicas de ERO resultam em estresse oxidativo (PISOSCHI *et al*., 2015) que por sua vez, pode levar a uma grande variedade de disfunções do mecanismo celular. Como consequência, ocorrem danos em lipídios, DNA e nas organelas, alterando a função enzimática e desencadeando parada do crescimento e morte celular prematura (HE *et al*., 2021; RYTER *et al*., 2007; DI *et al*., 2016; PANDAY *et al*., 2015; LETTIERI *et al*., 2020). Além disso, níveis elevados de ERO levam a ocorrência de peroxidação lipídica, induzindo à síntese de produtos altamente reativos e mutagênicos, como o malondialdeído (MDA), um importante marcador molecular indireto de estresse oxidativo (ESTERBAUER *et al*., 1993; GENTILE *et al*., 2017). Outro indicador do ambiente oxidativo é o tiol (-SH), presente na glutationa reduzida (GSH). A oxidação de GSH por GPx ocorre apenas na presença de H_2O_2. Assim, um baixo teor de tiol indica uma maior taxa de oxidação de GSH, enquanto um alto teor de tiol indica uma menor taxa de oxidação (RADAK *et al.,* 2013; FURTADO *et al*., 2021).

Durante o cultivo ou maturação *in vitro*, o estresse oxidativo pode ser originado de diversas fontes como pH, temperatura, suplementação do meio, níveis de oxigênio, luz, dentre outros (CASTILLO-MARTÍN *et al*., 2014, LI *et al*., 2016, ISPADA *et al*., 2018). Um dos parâmetros cruciais é o pH externo (pHe) do meio de cultivo. O pHe em meio de cultivo tamponado com bicarbonato somente é alcançado após o equilíbrio em uma incubadora a 37°C (CONAGHAN *et al.,* 2014). Oócitos desnudos não possuem habilidade de manter o pH interno (pHi) em 7,1 (LANE *et al*., 1999; SWAIN *et al*., 2010), evidenciando a importância de calibrar o pHe com base em um pHi ligeiramente superior entre 7,2 e 7,3 (LANE *et al*., 2000; SWAIN *et al*., 2010; HENTEMANN *et al*., 2011). Outro aspecto relevante é a temperatura das incubadoras de CO_2. Corriqueiramente essa temperatura é ajustada para 37°C a fim de mimetizar as condições *in vivo* (BAAK *et al*., 2019).

Já foi relatado que a incidência luminosa durante o período de cultivo é prejudicial, causando foto-oxidação do meio de cultivo (BOGNAR *et al*., 2019; WALE *et al*., 2016). Isso

ocorre pelo fato de que comprimentos de onda específicos são absorvidos por enzimas da cadeia de transporte de elétrons (AGARWAL *et al*., 1978), resultando em aumento da geração de ERO. Os componentes do meio também podem causar estresse oxidativo. Já foi demostrado que uma alta concentração de glicose (> 20 mM) durante a MIV aumenta os níveis de ERO intraoocitário, reduz a glutationa, prejudicando assim a competência oocitária (HASHIMOTO *et al.*, 2000). Além disso, a osmolalidade do meio tem sido analisada. Deng *et al*. (2015) verificaram que o meio hiperosmótico (310-330 mOsm/Kg) prejudica o desenvolvimento embrionário (SWAIN *et al*., 2012) e pode induzir alta produção de ERO no ambiente *in vitro* (DENG *et al.,* 2015). Portanto, muitos fatores podem culminar em estresse oxidativo, levando oócitos e células foliculares a acionar suas defesas antioxidantes para remover produtos nocivos, e equilibrar os níveis de ERO dentro de limites fisiológicos (LUSHCHAK 2014; LEI *et al*., 2016; MENEGON *et al*., 2016). A figura 6 ilustra o estresse oxidativo que ocorre durante o cultivo *in vitro*.

Figura 6. Representação ilustrativa do estresse oxidativo no cultivo *in vitro*. O equilíbrio entre a produção e eliminação de espécies reativas de oxigênio (ERO) é perdido devido à exposição a fatores oxidativos e falta de antioxidantes foliculares. Isso leva a danos por estresse oxidativo causados por radicais de íon superóxido (O_2^-) radicais hidroxila (OH^-), radical peroxil (ROO^-) e peróxido de hidrogênio (H_2O_2), que afetam diferentes componentes celulares e vias biológicas.



Fonte: Elaborada pela autora.

2.5 Antioxidantes enzimáticos no controle do estresse oxidativo

Existem diferentes níveis de defesas antioxidantes em organismos vivos para eliminar radicais ou reparar danos causados pelo estresse oxidativo, incluindo os antioxidantes enzimáticos. Em geral, os antioxidantes podem atuar como doadores de hidrogênio ou elétrons, neutralizando processos de oxidação, reagindo com compostos que podem danificar moléculas e sequestrando esses compostos (ATTA, MOHAMED, ABDELGAWAD, 2017).

Os antioxidantes podem ser classificados em: enzimáticos ou não enzimáticos. Compreendem os antioxidantes enzimáticos aqueles já presentes nas células e que atuam na primeira linha de defesa celular. São eles as enzimas: superóxido dismutase (SOD), catalase (CAT), a glutationa peroxidase (GPx) e as peroxirredoxinas (PRDX) (NIMSE; PAL, 2015; STECKIEWICZ *et al*., 2019; JIA *et al.,* 2023). A SOD é uma das primeiras enzimas a converter O_2- em oxigênio e H_2O_2 (CARRILLO-GONZÁLEZ *et al*., 2021). Há diferentes tipos de SOD, a Cu/Zn-SOD que está localizada no citoplasma e no núcleo, a Mn-SOD, encontrada nas mitocôndrias e a EC-SOD, presente no espaço extracelular (WANG *et al.,* 2017; MARKLUND, 1982; WEISIGER; FRIDOVICH; 1973). Já a CAT é detectada principalmente nos peroxissomos (ALI *et al*., 2020) e atua catalisando o H_2O_2 em H_2O (WANG *et al.,* 2017; CHANCE *et al.*, 1979). A GPX atua diretamente na remoção de H_2O_2. Há alguns tipos de GPX já relatadas sendo elas: a GPX1, que é a forma citosólica; a GPX-GI, uma forma gastrointestinal; a GPX-P, que é uma forma secretada no plasma e a GPX-EP, secretada no epidídimo (CARRILLO-GONZÁLEZ *et al*., 2021). Dentre as peroxirredoxinas, a PRDX-6 apresenta atividade crucial na redução do estresse oxidativo. A PRDX6 é responsável pela eliminação de peróxidos, incluindo H_2O_2, hidróxidos de cadeia curta e hidroperóxido fosfolipídico e atua no reparo da peroxidação lipídica (FISHER 2017, 2018; JIANG *et al*., 2023).

Já os antioxidantes não-enzimáticos incluem os oligoelementos (cobre, zinco e selênio), vitaminas (vitaminas C e E, caroteno e betacaroteno) e outros produtos naturais, como o resveratrol e a melatonina (YAN *et al*., 2022; SILVA *et al*., 2023). Esses podem atuar por mecanismos de ação distintos. Desta forma, os antioxidantes enzimáticos e não enzimáticos podem fornecer a célula um ambiente adequado para a sobrevivência, reduzindo o estresse oxidativo (SOTO-HERAS, PARAMIO *et al*., 2020). Para isso, esses antioxidantes atuam em fases diferentes. Os antioxidantes de defesa de primeira linha neutralizam rapidamente quaisquer radicais livres que induziriam a produção de outros radicais ou moléculas que poderiam se tornar radicais livres. As três principais enzimas nesta categoria são SOD, CAT e

GPx. Essas enzimas quebram o radical superóxido, H_2O_2 e hidroperóxidos, respectivamente, em moléculas inofensivas (H_2O ou álcool e O_2) (CARRILLO-GONZÁLEZ *et al*., 2021). Proteínas de ligação de íons metálicos, como transferrina e keruloplasmina, também representam essa classe de proteínas que se ligam ao ferro e ao cobre, respectivamente, e assim, impedem a participação do ferro ou do cobre nas reações redox (NIMSE; PAL, 2015; SANTOS-SÁNCHEZ *et al*., 2019). Existem também antioxidantes de defesa de segunda linha que atuam ligando-se aos radicais ativos, impedindo o início da reação em cadeia de oxidação e interrompendo as reações de propagação da cadeia. Na verdade, eles doam um elétron aos radicais livres para neutralizá-los e, por sua vez, tornam-se eles próprios radicais livres, apresentando toxicidade reduzida. Os "novos radicais" menos tóxicos, são facilmente neutralizados e tornados inofensivos por outros antioxidantes desta classe. Como exemplo, podemos citar o ácido ascórbico, o ácido úrico e a glutationa, que são hidrofílicos, o α-tocoferol (vitamina E) e o ubiquinol, que são lipofílicos (SANTOS-SÁNCHEZ *et al*., 2019) e os de natureza anfifílica, como a melatonina. Após o dano dos radicais livres, os antioxidantes de defesa de terceira linha tornam-se ativos. Eles são caracterizados por enzimas que reparam os danos causados pelos radicais livres, reparando DNA, proteínas e lipídios danificados. Exemplos típicos incluem enzimas de reparo de DNA (polimerases, glicosilases e nucleases) e enzimas proteolíticas (proteinases, proteases e peptidases), que estão localizadas no citosol e nas mitocôndrias. Finalmente, existem os antioxidantes de defesa de quarta linha, que impedem a formação de radicais livres e suas reações (STECKIEWICZ *et al*., 2019; AMJAD *et al.,* 2020). No ambiente *in vitr*o, acredita-se que o mesmo aparato de defesa ocorra, mas pelas adversidades do próprio sistema *in vitro*, não sejam plenamente efetivos (SOTO-HERAS, PARAMIO *et al.,* 2020).

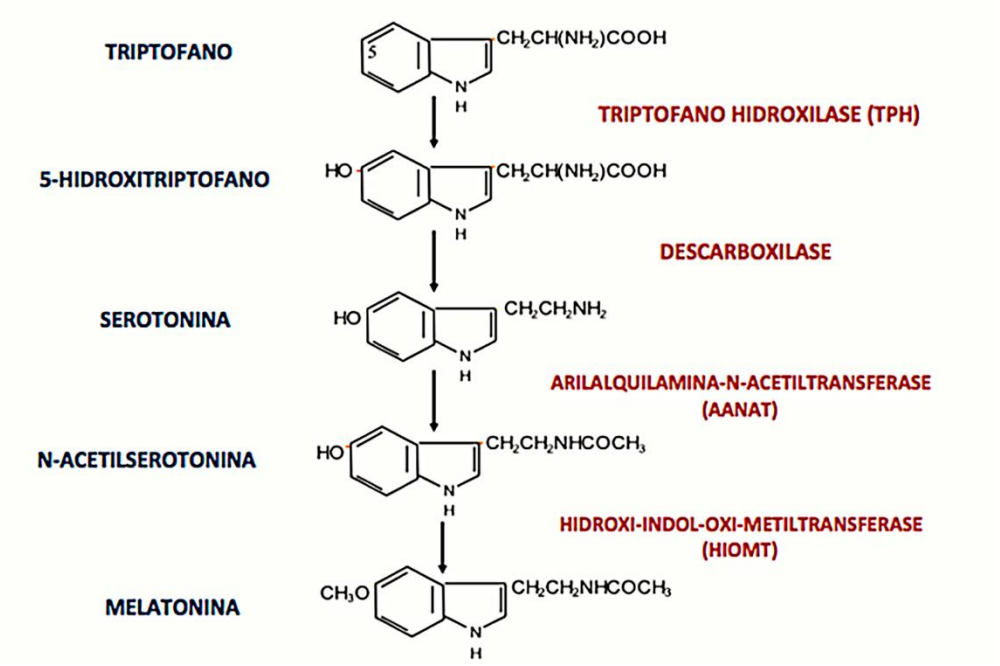
Recentemente, Mengden, Klam e Smitz (2020) demonstraram *in vitro* os mecanismos antioxidantes em CCOs de forma mais específica. Segundo os autores, as enzimas GPx, CAT, SOD e a melatonina participam do processo que se inicia com a quebra da glicose pelas células do cumulus, gerando o piruvato metabolizado pelo oócito por diferentes ações enzimáticas. Além do ATP gerado nesse processo, também são produzidas espécies reativas, aminoácidos e reciclagem de NADPH, o que resulta na estimulação da produção de CAT pelas células do cumulus que posteriormente são enviadas aos oócitos. A CAT também atua contra o peróxido de hidrogênio reativo, enquanto a SOD e suas variantes cobre, zinco e manganês atuam contra o ânion superóxido. Além disso, a melatonina atua na produção de GSH que metaboliza o peróxido em água e O_2 e reduz os hidroperóxidos lipídicos.

2.6 Melatonina e seus mecanismos de ação

Nos mamíferos, a melatonina é o principal hormônio produzido pela glândula pineal e atua no controle de vários processos fisiológicos, como a regulação dos ritmos sono-vigília, temperatura corporal, atividade fisiológica nos ritmos circadianos e propriedades antioxidantes (TALIB *et al*., 2018). Adicionalmente, a melatonina desempenha um papel fundamental na regulação da função ovariana (TAMURA *et al*., 2020).

A melatonina deriva do triptofano, que é convertido em serotonina após reações de hidroxilação e descarboxilação. A síntese de melatonina ocorre em um processo de várias etapas na qual se inicia pela conversão do triptofano em 5-hidroxitriptofano (triptamina) por meio da ação da enzima hidroxilase triptofano (TPH). Em seguida, a triptamina é transformada em 5-hidroxitriptamina (serotonina) via descarboxilase (AAD), com sua subsequente conversão em *N*-acetilserotonina (NAS) via aralquilamina N-acetiltransferase (AANAT). A NAS é a precursora imediata da melatonina (*N*-acetil-5-hidroxitriptmina), e esta etapa é conduzida utilizando hidroxindol-*O*-metiltransferase (HIOMT) (Figura 7) (REITER *et al*., 1991; ROSEBOOM *et al*., 1998). Diversos estudos propuseram que as mitocôndrias são os locais primários de síntese de melatonina devido à presença de enzimas formadoras de melatonina, NAS e AANAT, e da proteína acompanhante 14-3-3, que previne a degradação do NAS e aumenta a afinidade do SNAT pela serotonina (REITER *et al*., 2018). El-raey *et al*. (2011) demonstraram que a AANAT pode estar envolvida na síntese de melatonina em CCOs.

Figura 7. Via de produção da melatonina. A melatonina é sintetizada a partir do aminoácido triptofano que, por ação da enzima triptofano hidroxilase, é convertido em 5-hidroxitriptofano. O 5-hidroxitriptofano é então transformado em serotonina por ação da 5-hidroxitriptofano descarboxilase. Por ação da enzima arilalquilamina-*N*-acetiltransferase, a serotonina é convertida em *N*-acetilserotonina, que após oximetilação pela ação da enzima hidroxindol-*O*-metiltransferase (HIOMT), origina a melatonina.



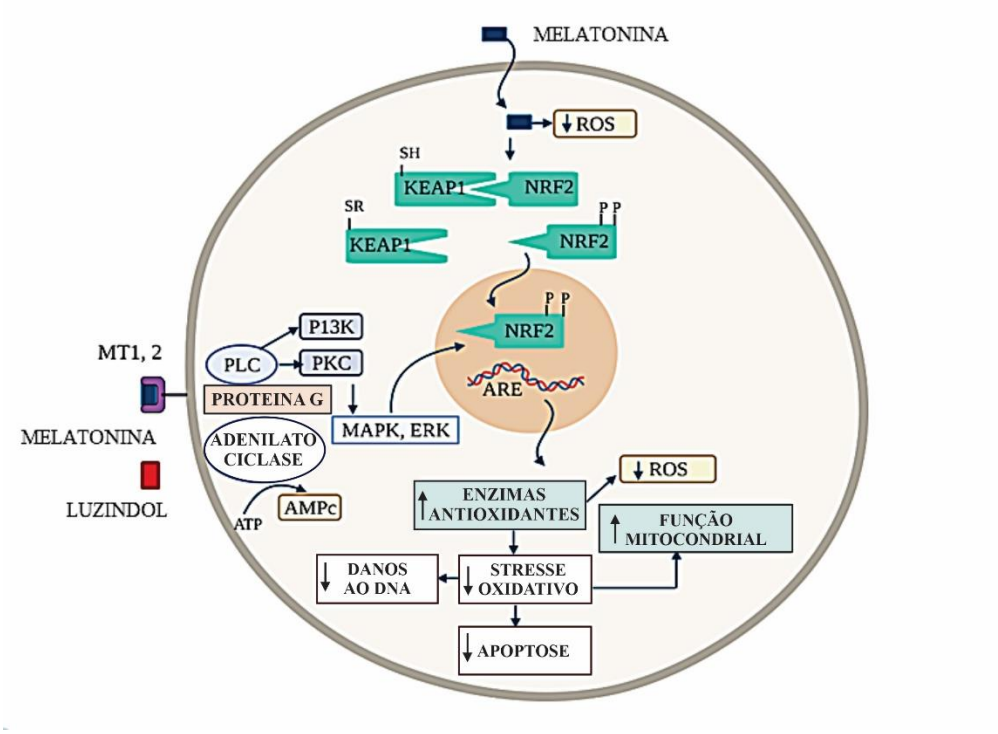
Fonte: Adaptado de CIPOLLA-NETO E AFECHE, 2012.

Numerosos efeitos terapêuticos já foram evidenciados com o uso da melatonina, incluindo sua ação como antioxidante (TAMURA *et al*., 2012; REITER *et al*., 2009) anti-inflamatória (ZHANG *et al*., 2019; anti-apoptótica (FERNÁNDEZ *et al*., 2015; ZHAI *et al*., 2017; ZHI *et al*., 2020) e anti-tumoral (CUCIELO *et al*., 2022), auxiliando no tratamento de distúrbios de fertilidade, osteoporose, doenças cardiovasculares, dentre outras (FERLAZZO *et al*., 2020). Além disso, a melatonina é amplamente usada tanto como medicamento de prescrição, quanto como suplemento sem receita médica em muitos países da Europa e dos Estados Unidos (SAVAGE *et al*., 2020). No Brasil, a Anvisa aprovou o uso da melatonina para a formulação de suplementos alimentares, destinados exclusivamente a pessoas com idade igual ou maior que 19 anos (ANVISA, 2021).

A melatonina exerce suas funções pleiotrópicas por meio de mecanismos dependentes de receptores ou independentes de receptores. Ao se ligar ao seu receptor de melatonina 1 (MT1) ou ao receptor de melatonina 2 (MT2) acoplado à proteína G, várias vias de transdução de sinal são ativadas pela melatonina, incluindo a PI3K/Akt (TAMURA *et al*., 2017; GUO *et al*., 2022). O receptor MT1 é responsável pelos efeitos circadianos e pela modulação da transdução de sinal no sistema reprodutivo (PALA *et al*., 2013; JOCKERS *et al*., 2016). Já o receptor de MT2 modula a apoptose e a proliferação de células da granulosa (HE *et al*., 2016). Portanto, a melatonina e seus receptores, MT1 e MT2, estão envolvidos na regulação de mecanismos reprodutivos importantes. Para a compreensão dos mecanismos de ação da

melatonina, o luzindol (N-acetil-2-benziltriptamina) tem sido amplamente utilizado para antagonizar MT1 e MT2 em pesquisas farmacológicas (DUBOCOVICH *et al*., 1998; BROWNING *et al*., 2000; LIU *et al*., 2016). O luzindol atua bloqueando competitivamente MT1 e MT2 de maneira não seletiva (DUBOCOVICH *et al*., 1997). Na figura 8, é ilustrado uma cascata de sinalização interrompida a partir da ligação de luzindo aos receptores de melatonina.

Figura 8. Mecanismos de ação da melatonina e a ação do bloqueio com luzindol ao ligar-se aos receptores MT1 e MT2 sobre as vias relacionadas a redução do estresse oxidativo.



Fonte: Elaborada pela autora.

A melatonina também pode atuar ligando-se à enzima citosólica quinona redutase 2 (QR2), conhecida como receptor de melatonina 3 (MT3), capaz de neutralizar radicais livres (ČERNYŠIOV *et al*., 2015; BOUTIN *et al*., 2016). A melatonina pode atuar independentemente dos receptores através da eliminação direta de radicais livres (MANCHESTER *et al*., 2015; REITER *et al*., 2016; TAN *et al*., 1993). Os metabólitos formados quando a melatonina doa um elétron têm a capacidade de neutralizar espécies de oxigênio parcialmente reduzidas (HARDELAND *et al*., 2009; GALANO *et al*., 2013). A melatonina também quela metais de transição que participam na geração do radical hidroxila (GALANO *et al*., 2015; ROMERO *et al*., 2014) e reduz indiretamente o estresse oxidativo,

regulando positivamente as enzimas antioxidantes (BICER *et al*., 2022). Adicionalmente, a melatonina atua regulando negativamente enzimas pró-oxidantes (AHMED *et al*., 2022) e estimula a síntese de outros antioxidantes endógenos como CAT e SOD através da via que desencadeia o fator nuclear derivado de eritróide 2 (NRF2) (URATA *et al*., 1999).

Devido ao seu potencial biológico, a melatonina tem despertado a atenção de muitos pesquisadores de diferentes áreas. Estudos revelaram que o pré-tratamento com melatonina melhora o efeito terapêutico de algumas doenças (DE FARIAS *et al*., 2022; ZHANG *et al*., 2021; YANG *et al*., 2020), como a lesão blástica, aliviando a toxicidade de drogas (LIANG *et al*., 2021). Além disso, o pré-tratamento com melatonina melhora a taxa de sobrevivência e a angiogênese das células (MIAS *et al*., 2018). Pan *et al*. (2022) observaram que a melatonina inibiu o aumento do número de linhagem celular de ameloblastos (ALC) de maneira dose e tempo dependentes. Células de diferentes fontes respondem de forma diferente às concentrações de melatonina (TIAN *et al*., 2014). Cucielo *et al*. (2022) revelaram que a melatonina reduz significativamente o metabolismo mitocondrial e o tamanho das células de carcinoma ovariano humano (células SKOV-3).

Na reprodução, diversos estudos demonstraram que a melatonina pode melhorar a capacidade de desenvolvimento dos oócitos tanto *in vitro* quanto *in vivo* (SANANMUANG *et al*., 2020). Em bovinos, recentemente Paulino *et al*. (2022) relataram a presenção dos receptores MT1 e MT2 de melatonina em folículos secundários bovinos. Além disso, a melatonina também já foi encontrada no fluido folicular em altas concentrações, atuando na proliferação de células da granulosa através da ativação de MAPKs (MINGUINI *et al*., 2019). Recentemente, a melatonina foi capaz de aumentar a competência oocitária e o desenvolvimento embrionário em bovinos pré-púberes e adultos, mitigando a produção de ERO (GUTIÉRREZ-AÑEZ *et al*., 2023). A melatonina reduz o conteúdo de ERO de oócitos pós choque térmico, aumentando a taxa de maturação dos oócitos e a proporção de embriões, por meio do aumento da expressão de genes relacionados a função mitocondrial (YAACOBI-ARTZI *et al*., 2020). Além disso, a melatonina também protege os oócitos bovinos de outras substâncias nocivas, por exemplo, prevenindo danos aos oócitos induzidos por paraquat e preservando a capacidade de desenvolvimento embrionário (PANG *et al*., 2019). Há evidências da capacidade da melatonina de melhorar o desenvolvimento de oócitos bovinos (LAN *et al*., 2018). Contudo, apesar dos significativos avanços obtidos com a melatonina, estudos que demonstrem seus efeitos sobre oócitos maturados *in vitro* em sistemas com uma etapa de pré-maturação ou durante o cultivo de folículos antrais iniciais bovinos, bem como seus mecanismos de ação no tecido ovariano bovino cultivado *in vitro* são desconhecidos.

Além da melatonina, estudos têm relatado o potencial de outros antioxidantes não enzimáticos como resveratrol e a N-acetilcisteína, durante o cultivo *in vitro* de folículos ovarianos e oócitos, exercendo diferentes mecanismos de ação, incluindo a estimulação da transcrição de genes relacionados a enzimas antioxidantes (CAT, SOD e GPx) (PIRAS *et al*., 2019; FAN *et al*., 2022). A seguir, iremos apresentar uma revisão de literatura, que abordará aspectos relacionados aos antioxidantes não enzimáticos, incluindo a melatonina.

2.7 CAPÍTULO I

Mechanisms of action of non-enzymatic antioxidants to control oxidative stress during *in vitro* follicle growth, oocyte maturation, and embryo development

[Mecanismos de ação de antioxidantes não enzimáticos no controle do estresse oxidativo durante o crescimento folicular *in vitro*, maturação oocitária e desenvolvimento embrionário]

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Mechanisms of action of non-enzymatic antioxidants to control oxidative stress during in vitro follicle growth, oocyte maturation, and embryo development

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Abstract

In vitro follicle growth and oocyte maturation still has a series of limitations, since not all oocytes matured in vitro have the potential to develop in viable embryos. One of the factors associated with low oocyte quality is the generation of reactive oxygen species (ROS) during in vitro culture. Therefore, this review aims to discuss the role of non-enzymatic antioxidants in the control of oxidative stress during in vitro follicular growth, oocyte maturation and embryonic development. A wide variety of non-enzymatic antioxidants (melatonin, resveratrol, L-ascorbic acid, L-carnitine, N-acetyl-cysteine, cysteamine, quercetin, nobiletin, lycopene, acteoside, mogroside V, phycocyanin and laminarin) have been used to supplement culture media. Some of them, like N-acetyl-cysteine, cysteamine, nobiletin and quercetin act by increasing the levels of glutathione (GSH), while melatonin and resveratrol increase the expression of antioxidant enzymes and minimize oocyte oxidative stress. L-ascorbic acid reduces free radicals and reactive oxygen species. Lycopene positively regulates the expression of many antioxidant genes. Additionally, L-carnitine protects DNA against ROS-induced damage, while acteoside and laminarin reduces the expression of proapoptotic genes. Mogrosides increases mitochondrial function and reduces intracellular ROS levels, phycocyanin reduces lipid peroxidation, and lycopene neutralizes the adverse effects of ROS. Thus, it is very important to know their mechanisms of actions, because the combination of two or more antioxidants with different activities has great potential to improve in vitro culture systems.

Keywords: reactive oxygen species, oxidative stress, antioxidants, assisted reproductive technology

1. Introduction

Ovarian follicle growth and oocyte maturation are complex and regulated processes that involve a precise communication between oocyte and surrounding granulosa cells (Zhang et al., 2022). Increasing evidence shows that in vitro conditions can influence the competence of oocytes and, eventually, the quality of embryos (Zhang et al., 2018). Despite the range of studies carried out in the area, in vitro embryo production (IVP) still has limitations, since not all oocytes have the ability to develop into viable embryos after in vitro maturation (IVM) (Ramos-Ibeas et al., 2019), emphasizing that the culture systems still need to be improved (Rocha et al., 2016). In humans, the majority of oocytes used for in vitro fertilization (IVF) are not developmentally competent to form viable blastocysts (Silber et al., 2017; Chian, 2022). One of the factors associated with low in vitro embryonic quality is the generation of large amount of reactive oxygen species (ROS) during in vitro culture (Lan et al., 2019; Agarwal et al., 2022). Excess ROS can result in oxidative stress that causes oxidation of intracellular molecules, such as amino acids, lipids and nucleic acids and compromises cell viability (Cui et al., 2012; Gualtieri et al., 2021). During oocyte IVM, oxidative stress can induce chromosomal errors, lipid peroxidation and mitochondrial damage, which affect its potential to form a blastocyst (Choi et al., 2008; Mihalas et al., 2017).

The intracellular enzymes, superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), glutathione reductase (GSR) and peroxiredoxins act in cell defense against oxidative stress. They are able to convert oxidized metabolic products in a gradual process into hydrogen peroxide (H_2O_2) and later into water with the help of cofactors (Koruk et al., 2004; Gough et al 2011; Zhan et al 2004). There are also non-enzymatic antioxidants, such as melatonin, resveratrol, ascorbic acid, L-carnitine, N-acetyl-cysteine and cysteamine, quercetin, nobiletin, acteoside, mogroside V, phycocyanin, laminarin and lycopene, that have beneficial effects to control oxidative stress (Truong et al., 2017; Lu et al., 2018). They can interrupt the chain reactions of free radicals through different mechanisms, acting directly and/or indirectly on cellular antioxidant system (Nimse et al. 2015; Moussa et al. 2019). To choose the best combination of antioxidants to supplement in vitro culture media, it is very important to know their mechanisms of actions.

The aims of this review are to discuss the (1) causes of oxidative stress during in vitro follicle growth, oocyte maturation and embryo development, (2) the mechanisms of action of natural antioxidants, and (3) the strategies to use combinations of these antioxidants to improve in vitro culture systems.

2. Oxidative stress during in vitro culture of follicles, oocytes and embryos

Unstable and highly reactive free radical species are naturally produced through cellular metabolism. There are two major types of free radical species: ROS and reactive nitrogen species (NOS) (Agarwal et al., 2005; Pierce et al., 2004). ROS are produced in the healthy follicle during physiological processes and are important for oocyte maturation (Agarwal et al. 2006; Freitas et al., 2017). Despite its essential role, an excessive production of ROS can result in oxidative stress causing the oxidation of cellular molecules such as carbohydrates, amino acids, lipids and nucleic acids and consequently affecting oocyte maturation (Ambrogi et al., 2017). Furthermore, oxidative stress affects mitochondrial activity, meiotic spindle formation, genetic material integrity and chromosomal configuration (He et al., 2016). In this way, these events culminate into DNA malfunction, loss of integrity and membrane selectivity and cellular ATP production, triggering structural and physiological changes in in vitro cultured follicles. Studies show that oxidative stress influences the communication between oocytes and granulosa cells, decreasing oocyte quality and inducing apoptosis in granulosa cells (Chaube et al., 2014; Ghatebi et al., 2019).

In vitro culture conditions, including handling, increase the levels of ROS and oxidative stress, which limits oocyte maturation and embryo development (Soto-Heras and Paramio, 2020). Available culture systems can also impact the transcriptome, and the presence of specific amounts of sugars, lipids or ROS can cause changes in gene expression and methylation of DNA (Cagnone and Sirard, 2016; Tremblay et al., 2017). Considering that follicles, oocytes and embryos are sensitive to these modifications, the in vitro environment is far from that in vivo (Nagano, 2019). New types of culture systems are being developed to allow the growth and competence of oocytes (Zhang et al., 2015). Although modest improvements have occurred in the development and composition of oocyte IVM media, the blastocyst rate rarely exceeds 30-40% in bovine species (Lonergan et al., 2016).

It is important to emphasize that culture of follicles, oocytes and embryos is performed in incubators with 5% CO_2 and approximately 20% O_2, due to the lower cost compared to incubator systems with low atmospheric oxygen tension (~ 5% O_2) (Bontekoe et al., 2012; Christianson et al., 2014). Exposure of cells to high levels of atmospheric O_2 causes an increase in ROS production, which is highly harmful, triggers cellular damage and is associated with poor embryo quality (Ota et al., 2021). Many other factors can cause oxidative stress, such as handling of oocytes, excessive exposure to light, the absence of an efficient combination of antioxidants, temperature, large volume of culture media, and static nature of culture systems

(Castillo-Martín et al., 2014, Li et al., 2016, Ispada et al., 2018). Consequently, production of superoxide ion radical (O_2^-), hydroxyl radical (^-OH), peroxyl radical (HOO^-) and H_2O_2 impairs oocyte quality. These factors affect different cellular components and biological pathways, resulting in negative consequences for oocyte and embryo development (Soto-Heras and Paramio, 2020).

Components of culture media can also cause oxidative stress. Hashimoto et al. (2000) reported that a high concentration of glucose (> 20 mM) in IVM medium increases intra-oocyte ROS levels, reduces glutathione (GSH) and impairs oocyte developmental competence, probably through the promotion of glycolysis and oxidative phosphorylation. In addition, osmolality is another factor that deserves attention. Hyperosmotic medium (310-330 mOsm /Kg) impairs embryo development (Swain et al., 2012) and can induce high ROS production, as reported for other types of cells (Deng et al., 2015). In this way, many factors can cause overproduction of ROS and, consequently, oocyte and follicular cells use their enzymatic antioxidant defense to remove harmful products in order to balance ROS levels within physiological limits (Lushchak, 2014; Lei et al., 2016; Menegon et al., 2016). Increasing production and activity of antioxidant enzymes is, however, not always sufficient to prevent oxidative stress (Ibrahim et al., 2014), requiring the supplementation of culture media with non-enzymatic antioxidants to minimize cellular damages.

3. Mechanisms of action of non-enzymatic antioxidants and their beneficial effects on follicles, oocytes and embryos

3.1. Melatonin

Melatonin is endogenously produced by the pineal gland and is considered a potent and natural antioxidant (Zhang and Zhang, 2014). Acts through both receptor-dependent and receptor-independent mechanisms. The melatonin receptors 1 (MT1) is considered responsible for the circadian effects of melatonin and modulation of signal transduction in the reproductive system (Pala et al., 2013; Jockers et al., 2016). The melatonin receptor 2 (MT2) receptor interacts with melatonin and modulates apoptosis and proliferation of granulosa cells (He et al., 2016). Melatonin can also act by binding the cytosolic enzyme quinone reductase 2 (QR2), known as melatonin receptor 3 (MT3), capable of neutralizing free radicals (Černyšiov et al., 2015; Boutin, 2016). Melatonin can also act independently of receptors (Figure 1). Along with its metabolites, which act as antioxidants, it creates an antioxidant cascade that produces radical

scavenging products in human tissues (Reiter et al., 2016), minimizing oxidative stress through a variety of mechanisms (Galano et al., 2018).

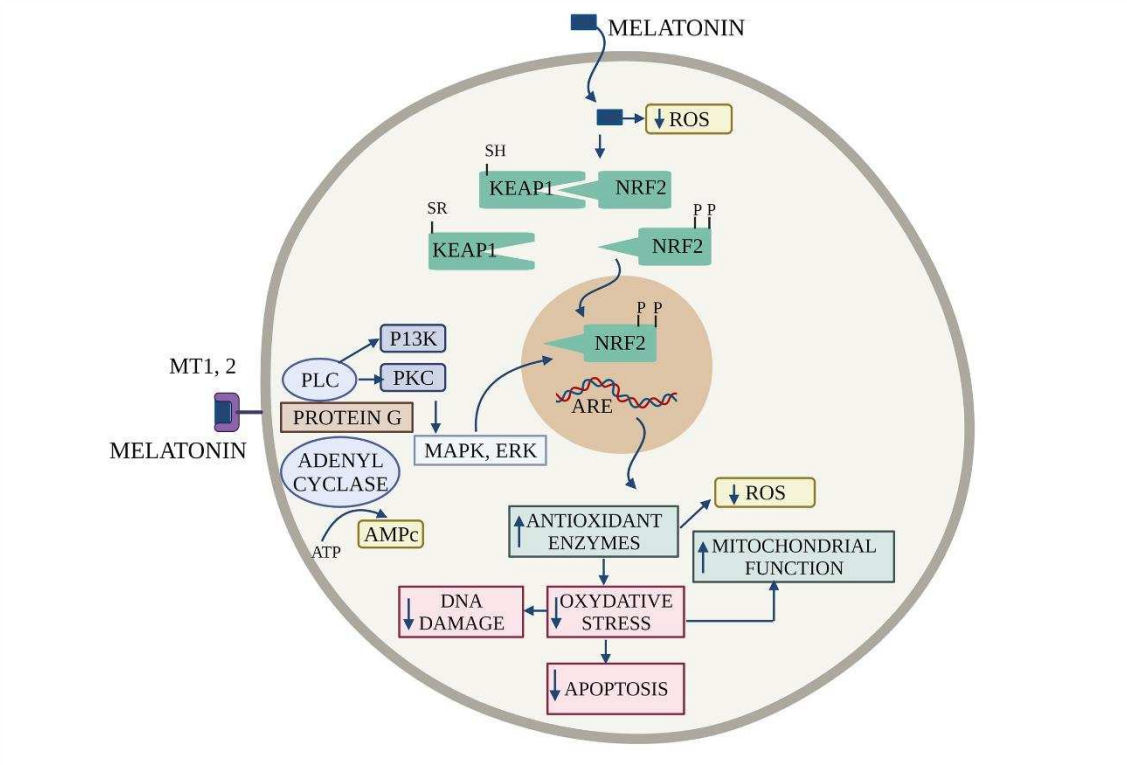
In vitro studies demonstrated that melatonin has a protective effect on murine follicles (Jang et al., 2016, Behram et al., 2017), promotes activation of primordial follicles and maintains stromal cell density during in vitro culture of ovarian bovine cortical tissue (Cavalcante et al., 2019). Cao et al. (2019) reported that melatonin improves the development of porcine cultured secondary follicles and, consequently, the competency of their oocytes. Recently, Paulino et al. (2022) reported that melatonin promotes growth of bovine secondary follicles through its membrane-coupled receptors, because luzindole, a melatonin receptor antagonist, blocks the effects of this hormone on follicle growth and reduces the expression of antioxidant enzymes in cultured follicles. In granulosa cells from bovine antral follicles, melatonin reduces apoptosis and oxidative stress (El-Raey et al., 2011). These data show that reduction of oxidative stress by melatonin is very important to develop an efficient in vitro system to support long-term culture of early follicles from domestic animal species.

Oxidative stress during transportation of ovaries and cumulus-oocyte complexes (COC) is an important factor that limits blastocysts production after oocyte IVM. Indeed, Sánchez-Ajofrín et al. (2020) reported that addition of melatonin in the transport medium of Iberian red deer ovaries increase the rates of cleavage and blastocyst and has a positive effect on expression of essential genes related to embryonic development. Additionally, melatonin improves pig oocyte quality during in vitro COCs maturation (Nakamura et al., 2003) and the proportion of oocytes that extruded the polar body (Kang et al., 2009). In mice oocytes, melatonin protects mitochondria by eliminating ROS, inhibiting mitochondrial permeability transition pore opening, and activating uncoupling proteins (He et al., 2016; Tan et al., 2016). It is known that oxidative stress can increase Ca^{2+} concentration and mitochondrial permeability via opening transition pore, which impairs mitochondrial function and causes apoptosis (Baumgartner et al., 2009). The presence of melatonin in culture medium of aged oocytes increases the speed of development to blastocysts after in vitro fertilization in bovine species (Liang et al., 2017).

During in vitro culture of embryos, melatonin has a positive effect on cleavage rates and increases the total number of blastomeres (Rodriguez-Osorio et al., 2007). Melatonin also has the ability to protect embryos from the harmful effects of heat stress and H_2O_2 (porcine; Rodriguez-Osorio et al., 2007; He et al., 2016, bovine; Cavallari et al., 2019). It has recently been reported that the antioxidant mechanism of melatonin in porcine embryos can be regulated by signaling nuclear factor 2 related to erythroid 2 (Nrf2) (Kim et al., 2019). It is already known that Nrf2 translocate to the nucleus and binds to antioxidant response elements to regulate the

expression of genes related to antioxidant defense (Kansanen et al., 2013; Ma, 2013). Some studies also demonstrated that Nrf2 and nuclear factor kappa B (NF-κB) pathways are involved in the control of inflammatory reaction, which is often related to ROS-mediated oxidative stress (Cuadrado et al., 2014). NF-κB promotes inflammation and subsequently promotes transcription activities related to energetic catabolic support (He et al., 2020; Li et al., 2016; Tominaga et al., 2019; Samed et al., 2022). In addition to the effects related to antioxidant activity, Nrf2 inhibits the actions of NF-κB, favoring cell homeostasis maintenance (Cuadrado et al., 2014). Figure 1 shows the mechanisms of action of melatonin to regulate oxidative stress.

Figure 1. Melatonin exert its effects through its membrane receptors (MT1 and MT2), which results in activation phospholipase C, a cascade involving PI3K, PKC, MAPK and ERK, and finally increasing the expression of antioxidant enzymes. Melatonin can freely pass through the plasma membrane and promote the dissociation of Keap1 and Nrf2 that, after phosphorylation, migrate to the nucleus to activate transcription antioxidant proteins.



3.2. Resveratrol

Resveratrol is a polyphenol phytoalexin that modulates several molecular pathways associated with the cellular redox state (Xia et al., 2017; Pan et al., 2017). In ovarian follicles, Kong et al. (2011) showed that resveratrol decreases apoptosis and atresia, and inhibits the transition from primordial to developing follicle in rats. Furthermore, resveratrol maintained ovarian reserve in rats subjected to induction of toxicity with hexavalent chromium (Banu et al., 2016), and protects against aging-related infertility (Ozcan et al., 2015). In mice, Liu et al. (2017) investigated the toxicity of mancozebe in mice oocytes and showed that resveratrol alleviates the adverse effects of this substance. Resveratrol can alleviate mancozeb-induced infertility by correcting the apoptotic tendency and abnormality of cellular epigenetic modification in mouse oocytes (Liu et al., 2017). Mancozebe is a non-systemic fungicide used to control fungal disease of plants and, consequently, human and animals can ingest this toxic substance through residues present in food and drinking water (Damalas et al., 2011; Canossa et al., 1993). This substance mainly targets mitochondrial enzymes, disturbing mitochondrial function and ATP production, resulting in metabolic disorders and activation of apoptotic pathways (Afsar et al., 1987). Han et al. (2020) showed that resveratrol alleviated the damages caused by doxorubicin in mouse oocytes, by restoring the spindle and chromosome configuration, and reducing ROS levels and inhibiting apoptosis. These data show that resveratrol has great potential to control oxidative stress during chemotherapy, helping to preserve gamete reserve.

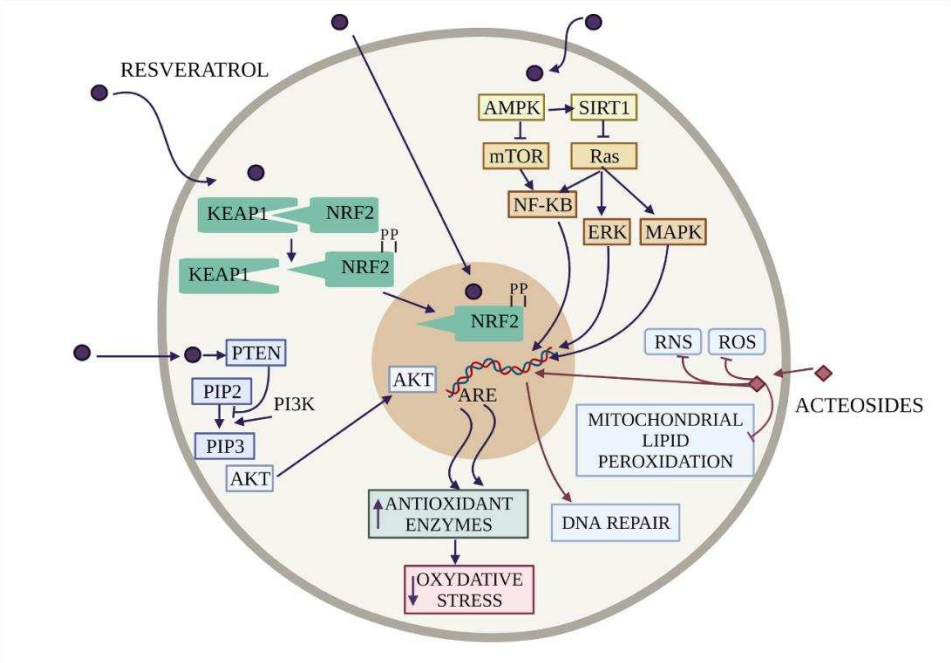
In vitro studies showed that resveratrol supports the growth of human ovarian follicles (Hao et al., 2018). It also delays aging-induced mouse oocytes deterioration, including expression of the anti-aging molecule sirtuin 1 (SIRT1), reducing levels of ROS and improving mitochondrial function (Liang et al., 2018). Kwak et al. (2012) revealed that supplementation of culture medium with resveratrol during oocyte in vitro maturation improves the developmental potential of porcine embryos by increasing the intracellular GSH levels, decreasing ROS levels, and regulating gene expression. Additionally, incubation of oocytes with resveratrol improves embryo development in caprine species (Piras et al., 2019), emphasizing the beneficial effects of resveratrol during these steps of assisted reproduction technologies.

For a better understanding of the mechanisms of action of resveratrol, studies in mice models showed that resveratrol regulates expression of antioxidant genes through AMP-*activated* protein kinase (AMPK), SIRT1, ECH-associated protein 1 similar to Kelch (Keap1) and Nrf2 (Meng et al., 2018). Singh et al. (2017) showed that, under oxidative stress, binding

of SIRT1 to antioxidant response element (ARE) molecules mediates signaling events involved in transcriptional regulation. ARE sequence exclusively detecting changes in cellular redox status and, consequently, triggers transcriptional responses, mediated mainly by Nrf2. Then, the Nrf2 regulates the expression of several antioxidant and detoxification genes involved in cellular antioxidant defense (Ngyuen et al., 2003; Kansanen et al., 2013; Ma, 2013). Resveratrol also attenuates oxidative regulating AMPK-mediated inhibition of targeting of rapamycin in mammals (mTOR) or via activation of transcription factor EB (TFEB) (Kim et al., 2011, Meng et al., 2018). Resveratrol also positively regulates phosphatase and the tensin homologue (PTEN), which decreases a protein kinase B phosphorylation, also known AKT in MCF-7 human breast cancer *cell line*. This action results in positive regulation of mRNA for antioxidant enzymes, like CAT and SOD (Ingles et al., 2014).

In general, resveratrol acts against oxidative damage in five different ways: (i) reducing the generation of ROS; (ii) directing elimination of free radicals; (iii) improving endogenous antioxidant enzymes (SOD, CAT and GSH); (iv) promoting expression of related genes involved in mitochondrial energy biogenesis, and (v) inducing autophagy via mTOR-dependent or TFEB-dependent pathways (Meng et al., 2020). Figure 2 shows the mechanisms of action of resveratrol to regulate oxidative stress.

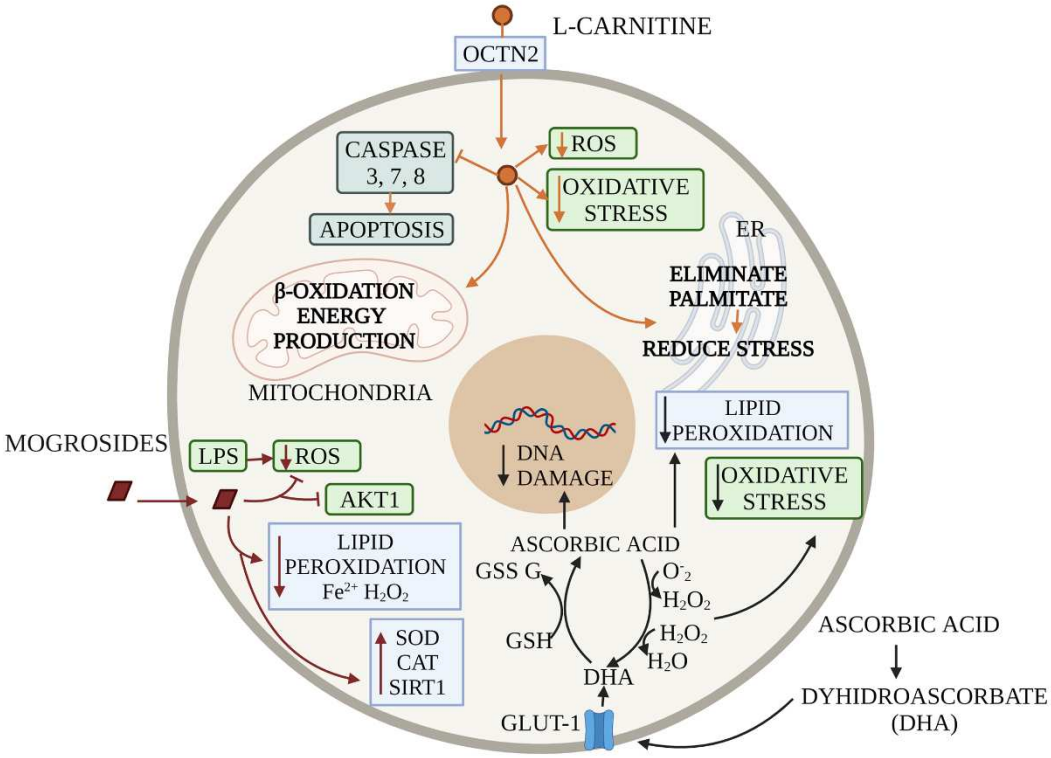
Figure 2. Resveratrol attenuates oxidative damage by promoting the expression of genes related to mitochondrial energetic biogenesis, mainly through the AMPK / SIRT1 / Nrf2, ERK, APK and PTEN / Akt signaling pathways. It also acts through NRF2, modulating a cascade that activates ARE elements, resulting in elevation of antioxidant enzymes (SOD, CAT and GSH). Acteosides act by directly eliminating ROS and RNS. It can also act by quickly repairing oxidative damage to DNA.



3.3. Ascorbic acid

L-ascorbic acid (AA) is an electron donor that reduces free radicals and ROS, having two different biochemical functions, i.e., a powerful antioxidant and an essential cofactor to modulate the ferrous ion and 2-oxoglutarate family (Manning et al., 2013). This substance protects DNA, amino acid residues, and lipids from oxidation induced by free radicals and maintains their integrity (Cimmino et al., 2018). In vitro studies with preantral follicles showed that AA is essential for their development in caprine (Rossetto et al., 2009) and equine (Gomes et al., 2015) species. In porcine COCs, AA increases the nuclear maturation rates of oocytes devoid of cumulus cells (Tao et al., 2010), increases the concentrations of intracellular GSH, reduces the level of ROS, and improves oocyte developmental competence (Tao et al., 2010; Kere et al., 2013). Figure 3 shows the mechanisms of action of AA to regulate oxidative stress. It is important to consider that oxidative stress can alter the epigenetic state and that ascorbic acid can modify the state of epigenome (Niu et al., 2015, Young et al., 2015; Camarena et al., 2016). Yu et al. (2018) showed that the presence of ascorbic acid during IVM of porcine oocytes improve meiotic maturation and developmental competence by reprogramming the global methylation state of DNA, histone and RNA. Ascorbic acid supplementation during in vitro embryo culture also improves the development of blastocysts of porcine and murine species (Boldura et al., 2021; Mallol et al., 2015). Figure 3 shows how AA controls cellular oxidative stress.

Figure 3. Ascorbic acid combat oxidative stress in its reduced form and in its oxidized form (DHA). DHA enters the cells via GLUT1 and it is converted to ascorbic acid and then in DHA. It plays a role in eliminating ROS, reducing lipid peroxidation, and DNA damage. L-carnitine enters the cells through OCTN2 and increases energy production by β-oxidation, scavenges excess ER palmitate to reduce ER stress, scavenges free radicals to reduce oxidative damage and inhibit caspases to prevent apoptosis. Macroside V block ROS produced in response to hypopolysaccharide (LPS) and attenuate phosphorylation AKT1. Its action extends to the reduction of lipid peroxidation and increase of antioxidant enzymes.



3.4. L-carnitine

L-carnitine promotes the transport of fatty acids through the internal mitochondrial membrane for β-oxidation, increasing cellular concentration of ATP in mammalian oocytes and embryos (Dunning et al., 2014; Agarwal et al., 2018). L-carnitine increases the proportion of mature oocytes in mice and pigs with uniform mitochondrial distribution (Zare et al., 2015; Somfai et al., 2011). It has antioxidant activity that can stabilize the mitochondrial membrane and protect DNA against ROS-induced damage (Ismail et al., 2014; Fenkci et al., 2008). Chankitisakul et al. (2013) showed that the addition of L-carnitine during in vitro maturation and subsequent vitrification of oocytes improves the rate of production of bovine embryos. L-

carnitine in mouse acts through the electrogenic force of voltage-gated Na^+ channels and it is transported by Na^+ / organic cationic transporter-2 (OCTN-2) to oocytes (Infante et al., 2002; Dunning et al., 2012). Once in the oocyte, L-carnitine is then converted to acetil-L-cartinine (ALC) by carnitine palmitoyltransferase-I (CPT-I) in the outer mitochondrial membrane, where it will act on the endoplasmic reticulum, mitochondria and even in ooplasm (Mingorance et al., 2011) (Figure 3). In mitochondria, L-carnitine balances the acetyl CoA / CoA ratio to ensure glucose metabolism during energy production (Infante et al., 2002). L-carnitine helps to reduce the concentration of pyruvate that prevents entry into the *tricarboxylic acid cycle* to decrease energy production. Furthermore, L-carnitine eliminates ROS through its antioxidant activity and moves palmitate and other long-chain fatty acids into mitochondria, facilitating their use through β-oxidation (Dunning et al., 2014). In endoplasmic reticulum, it reduces palmitate, transfers it to mitochondria or eliminates it, avoiding lipotoxicity in mouse oocytes (Infante et al., 2002). Figure 3 shows the mechanisms of action of L-carnitine to regulate oxidative stress.

The presence of L-carnitine during in vitro embryo culture improves pig embryo survival (Lowe et al., 2017) and hatching rate of bovine blastocysts (Ghanem et al., 2014). L-carnitine also improved in vitro post-thaw survival rate of bubaline (Verma et al., 2018) and bovine (Held-Hoelker et al., 2017) embryos. It maintains cellular energy in mouse embryos (Abdelrazik et al., 2009) and reduces oxidative stress against linoleic acid peroxidation (Vanella et al., 2000). These functions are essential for the proper development of blastocyst after fertilization of mammalian oocytes (Dunning et al., 2012). It is noteworthy that L-carnitine also promotes cell proliferation and decreases apoptosis by inhibiting TNF-α and other antiproliferative agents in mouse embryos (Abdelrazik et al., 2009).

Unlike other antioxidants, L-carnitine optimizes glucose metabolism and the use of fatty acids for β-oxidation during energy production and thus prevents the excessive production of ROS. The presence of L-carnitine in association with other antioxidants can improve culture systems for follicles, oocytes and embryos.

3.5. Acteoside and Mogroside V

Acteoside is considered a typical phenylethanoid glycoside that eliminates ROS and nitrogen species or acts as a peroxyl radical breaker (Li et al., 2018). In pig oocytes, acteoside increases competence and decreases intracellular ROS levels. Kim et al. (2016) reported that addition of acteoside during IVM of porcine oocytes reduced expression of BAK and BAX in parthenogenetic blastocysts and increased the expression of the anti-apoptotic genes BCL-2 and

BCL-XL. The mechanism of action of acteoside has been described in cells different from oocytes. Pan et al. (1996) demonstrated that this substance and its cis-tanoside analog F inhibit the mitochondrial lipid peroxidation in hepatocellular carcinoma cells (HepG2). With regard to its ability to protect DNA, the acteoside and its derivatives can quickly repair DNA radicals, such as 2'-deoxyadenosine-5'-monophosphate (dAMP) and 2'-deoxyguanosine-5'-monophosphate (dGMP) in *NIH 3T3* mouse embryonic fibroblast cells (Jang et al., 2020; Zheng et al., 2010). These molecules are prolate and can easily reach the DNA loop, and thus are able to quickly repair DNA radical damage (Li et al., 2018; Pan et al., 1996). These beneficial effects on cells and tissues are believed to be associated with the protection of some biomolecules such as lipids and DNA (Li et al., 2018). The mechanisms of action of acteoside are shown in Figure 3.

Mogroside V also has the ability to increase antioxidant enzymes, such as SOD, CAT and SIRT1 as demonstrated in porcine oocytes (Nie et al., 2020). Nie et al. (2019) demonstrated that mogroside V reduces levels of intracellular ROS and increases mitochondrial function during IVM of pig oocytes. In addition, mogroside V can alleviate deterioration in porcine oocyte quality during in vitro oocyte aging, possibly by reversing the abnormalities of cytoskeleton, mitochondrial dysfunction and early apoptosis. It positively regulates the expression of SIRT1 to protect against the deterioration of porcine oocyte induced by in vitro aging (Nie et al., 2020). In a recent study, Yan et al. (2021) reported that administration of mogroside V can reverse adverse effects of exposing pig oocytes to lipopolysaccharides (LPS). This effect is due to its action of preserving mitochondrial, reducing ROS levels and increasing the expression of antioxidant genes (CAT and GPx1). Mogroside V also reduces the occurrence of early apoptosis and improves the development of pig embryos from oocytes exposed to LPS (Yan et al., 2021). Mogroside V is a potent antioxidant that directly eliminates free radicals in other types of cells, like adipocytes (mice: Harada et al., 2016). Mogroside V reduces the increase in lipopolysaccharide (LPS-induced) ROS levels and inhibits activation of the AMPK/AKT-Nrf2 signaling pathway in BV2 lineage microglia cells (Liu et al., 2021). In general, this antioxidant can act directly in the elimination of oxygen free radicals and attenuate the lipid peroxidation induced by Fe^{2+} or H_2O_2 (Li et al., 2014). Considering their mechanism of action (Figure 3), acteoside and mogroside V have great potential to be used alone or in association with other antioxidant substances to control oxidative stress during in vitro culture of oocytes and embryos.

3.6 N-acetyl-cysteine and cysteamine

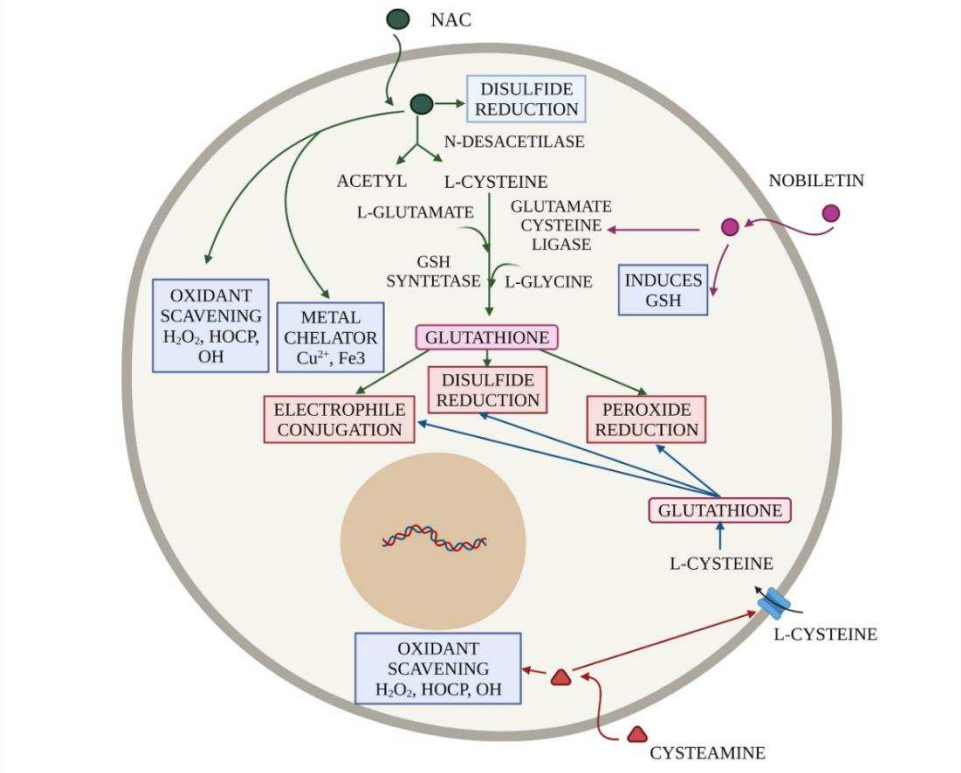
N-acetyl-cysteine (NAC) is a cysteine precursor that suppresses oxidative damage by restoring reduced glutathione levels. Hu et al. (2019) showed that 3.5 mM NAC during in vitro maturation of porcine oocytes protects these cells from heat stress-induced complications and therefore rescues impaired embryonic development. The NAC reversed disorganized spindle assembly and inhibited ERK signaling to protect oocytes from heat stress. Furthermore, NAC corrects erroneous modifications of histone H3 lisyne 27 trimethylation (H3K27me3) and dysregulated expression of imprinted genes, as well as alleviates intra-oocyte ROS accumulation and reduces apoptosis in porcine oocytes (Hu et al., 2019). N-acetylcysteine also minimizes the negative effects of bisphenol during IVM of mouse oocytes and improves rates of fertilization and blastocyst formation (Li and Zhao, 2019).

Regarding to its mechanism of action, NAC interacts with free radicals through its side chains, reacting quickly with the hydroxyl radical (OH^-), nitrogen dioxide (NO_2) and carbon trioxide ion (CO_{3-}), eliminating the ROS produced by cells as demonstrated in vivo in humans (Moraes et al., 2018). N-acetyl-cysteine can also act as a chelator for metallic ions, such as Cu^{2+} and Fe^3, which facilitates the removal of these ions in oocytes Xenopus laevis (Koh et al., 2002). The most reported antioxidant potential of NAC is due to its role as a precursor of GSH (Pei et al., 2018). Glutathione is a tripeptide, formed by the amino acids cysteine, glycine and glutamine, which plays an important role in combating oxidative stress (Meister and Anderson, 1983). The sulfhydryl (SH) group of glutathione confers its action against oxidative damage. There are two forms of glutathione, the reduced form (GSH) and the oxidized form (GSSG) (Lu, 2013). The protective action of glutathione against ROS is facilitated by interactions with its associated enzymes, such as glutathione peroxidase and glutathione reductase. Glutathione peroxidase catalyzes the reduction of hydrogen peroxide, converting it to its oxidized form ($2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$). The oxidized form of glutathione is reduced by the enzyme glutathione reductase in the presence of NADPH (Adeoye et al., 2018).

In the case of cysteamine, it acts by increasing cysteine uptake and substrate supply for GSH synthase, which translates into an increase in GSH synthesis (Meister and Anderson, 1983). Previous studies have shown that addition of cysteamine to the maturation medium improves oocyte maturation and protects oocytes against oxidative stress in bubaline and swine species (Gasparrini et al., 2000; Kobayashi et al., 2006). Some studies showed that cysteamine acts as a scavenger of free radicals in culture media, improving the organization of microtubules in metaphasic II (MII) oocytes and increasing the rate of MII during the mouse oocyte IVM

(Roushandeh et al., 2012; Roushandeh and Roudkena, 2009). Zhenwei and Xianhua (2019) reported that both cysteamine and C-type natriuretic peptide (CNP) increase intra-oocyte GSH levels during oocyte prematuration. These authors suggested that cysteamine can act individually in the synthesis of GSH in bovine cumulus cells, after which it is transferred to the oocyte via gap junctions with communication functionality extended by CNP, which contributes mainly to the accumulation of intra-oocyte GSH. Elamaran et al. (2012) reported that cysteamine up-regulated the expression of anti-apoptotic genes and down-regulated the expression of pro-apoptotic genes during in vitro buffalo embryo development. In bovine embryos, cysteamine enhances cysteine-mediated GSH synthesis (Gasparrini et al., 2006; Sadeesh et al., 2014). Considering that NAC and cysteamine control oxidative stress via GSH (Figure 4), which is different from various other antioxidants, we can highlight the importance of associating them to improve in vitro culture system for follicles, oocytes and embryos.

Figure 4. N-acetyl-cysteine is a precursor of cysteine and restore glutathione stores. It interacts with free radicals through its side chains, quickly reacts with hydroxyl radical (OH-), nitrogen dioxide (NO^2) and carbon trioxide ion (CO^{3-}), resulting in elimination of ROS. NAC also works as a chelator for metallic ions, such as Cu^{2+} and Fe^3, which facilitates their removal. Cysteamine acts by increasing cysteine uptake, a substrate supply for GSH synthetase, which translates into an increase in GSH synthesis. Nobiletin acts by overloading glutamate-cysteine ligase inducing ntracellular GSH production.



3.7 Nobiletin, quercetin and phycocyanin

Nobiletin is a polymethoxylated flavonoid that has attracted great attention because of its antioxidant activities (Cirillo et al., 2017; Goh et al., 2019; Guney et al., 2019; Lin et al., 2019; Cajas et al., 2020). In bovine COCs, Cajas et al. (2020) reported that nobiletin increases steroidogenesis in cumulus cells, improves nuclear and cytoplasmic maturation of the oocytes and decreases the levels of intracellular ROS. Nobiletin induces intracellular GSH through the overloading of glutamate-cysteine ligase, an enzyme that limits the rate of GSH synthesis, and protects against serum withdrawal and H_2O_2-induced cytotoxicity in PC12 cells of mouse (Su et al., 2012). Figure 4 shows the mechanisms of action of nobiletin to regulate oxidative stress.

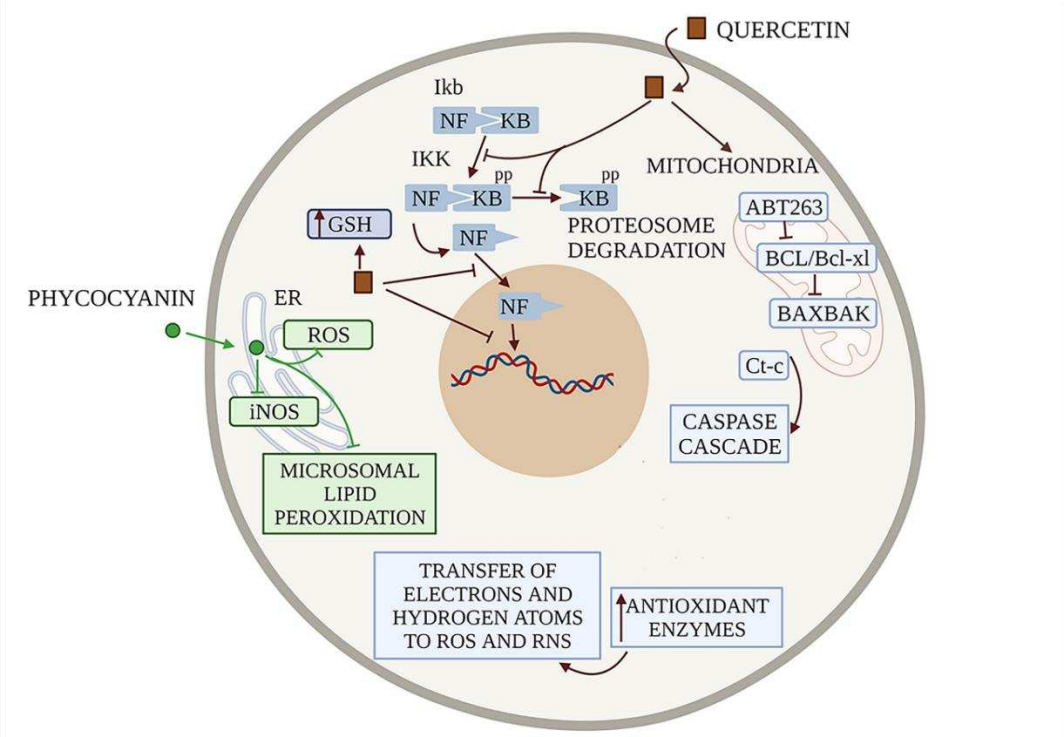
Quercetin is a polyphenolic flavonoid that has antioxidant effects through its action on GSH, signal transduction pathways and ROS (Xu et al., 2019). In the ovaries, quercetin increases the number of growing follicles, prevent follicular degeneration, and decrease apoptosis (Gencer et al., 2014; Nna et al., 2017; Bolouki et al., 2019; Elkady et al., 2019). In COCs, quercetin reduces ROS levels and has beneficial effects on nuclear maturation during IVM of porcine oocytes and subsequent embryo development (Kang et al., 2016). Quercetin also increases intracellular GSH levels, increases porcine oocyte cytoplasmic maturation, and improves blastocyst development (Kang et al., 2013). Concerning to mechanism of action,

quercetin regulates both the enzyme-mediated antioxidant defense system and the non-enzyme-dependent antioxidant defense system. Quercetin increases the expression of endogenous antioxidant enzymes such as Cu/Zn SOD, Mn SOD, catalase (CAT) and GSH peroxidase in CA1 pyramidal neurons (Chen et al., 2017). Regarding the mechanisms of action, its signaling pathways regulate NF-kB and MAPK. Quercetin activates Nrf2 via p38 activation and triggers gene transcription and expression of antioxidant enzymes in human aortic endothelial cells (Chuan et al., 2016). Nrf2 activation can trigger MAPK. This happens due to the fact that the increase in ROS causes an increase in the production of apurinic/apyrimidinic endonuclease 1 / redox effector factor-1 (APE1 / Ref1) and the activation of several signaling events, including apoptotic events mediated by p53, MAPK pathways (Kawamura et al., 2018; Zhao et al., 2017; Vurusaner et al., 2012). Figure 5 shows the mechanisms of action of quercetin to regulate oxidative stress.

Phycocyanin antioxidant activity is about 20 times more efficient than vitamin C (Chopra and Bishnoi, 2008). Li et al. (2016) reported that continuous intragastric administration of phycocyanin significantly reduces the accumulation of ROS in oocytes of mice with D-galactose-induced aging. These authors showed that impaired female reproductive capacity induced by D-galactose can be partially rescued by phycocyanin. Niu et al. (2017) reported that phycocyanin promotes porcine embryonic development by attenuating oxidative stress and apoptosis and protecting mitochondria. Recently, Wen et al. (2020) demonstrated that phycocyanin improve the fertility of obese mice through its potential to reverse DNA damage, which resulted in increased ovarian and oocyte quality. Studies in other types of cells have reported that phycocyanin has the ability to scavenge free radicals, including alkoxy, hydroxyl and peroxyl radicals in human erythrocytes (Pleonsil et al., 2013). In addition, it can act to decrease nitrite production, suppress the inducible expression of nitric oxide synthase (iNOS) and also inhibit microsomal lipid peroxidation in hamsters (Riss et al., 2007; Manconia et al., 2009). Phycocyanin has been reported to inhibit cell aging and protect mitochondrial function in canine kidney cells (Farooq et al., 2014). Figure 5 shows the mechanisms of action of phycocyanin to regulate oxidative stress.

Figure 5. Quercetin signaling pathways can regulate nuclear factor kappa B (NF-KB). All NF-KB proteins contain a Ref-1 homology domain (RHD), which is responsible for DNA dimerization, recognition and binding, as well as interaction with kB receptor (IkB) proteins. The IkB-kinase complex (IKK complex) catalyzes the phosphorylation of IkBs with the result that IkBs are targeted for disruption by the 26S proteasome, thereby releasing NF-KB.

Quercetin may act by blocking this pathway related to an inflammatory response. In mitochondria, quercetin increases the expression of anti-apoptotic genes BCL and blocks the action of pro-apoptotic genes BAX and BAD. Phycocyanin scavenges free radicals, including alkoxy, hydroxyl and peroxyl radicals, and decreases nitrite production, suppresses the inducible expression of nitric oxide synthase (iNOS) and inhibits microsomal lipid peroxidation.



4.8 Laminarin and lycopene

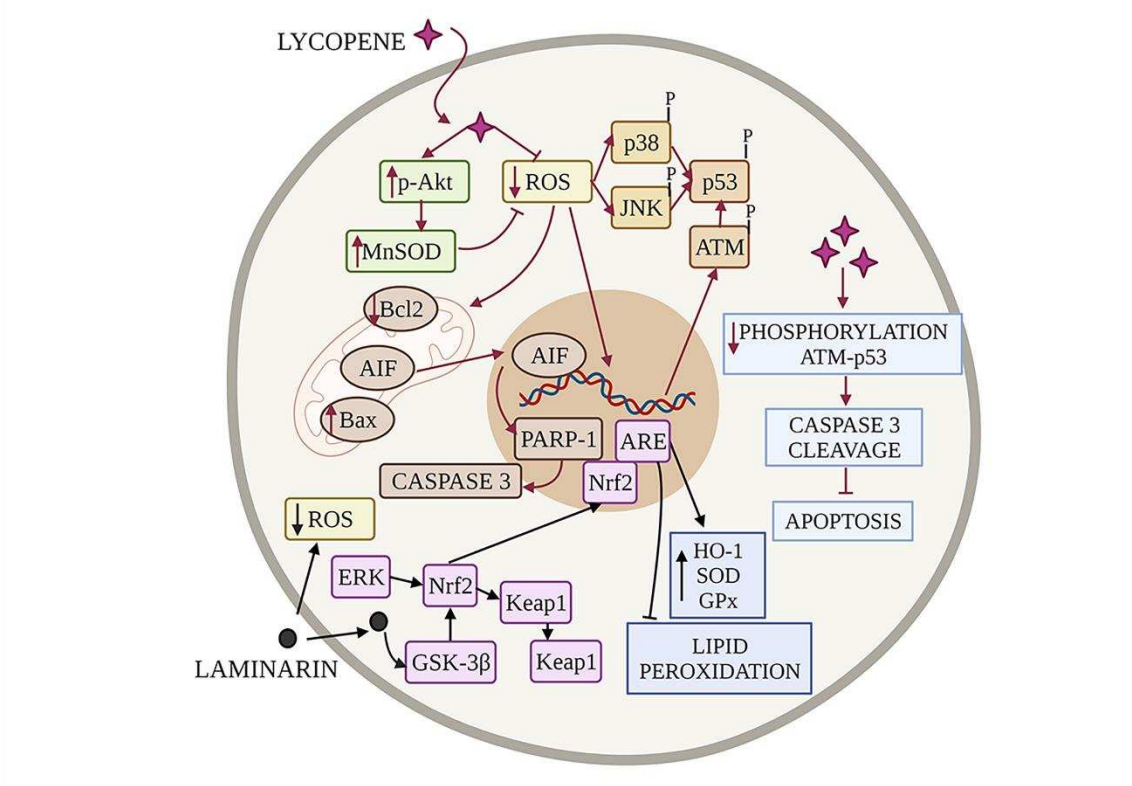
Laminarin is a compound extracted from the brown algae *Laminaria digitate* (Choi et al., 2011) that decreases the levels of mediators of early apoptosis, maintain the potential of mitochondrial membranes and improve the rate of blastocyst formation in porcine oocytes subjected to aging after IVM (Yao et al., 2018). In other types of cells, laminarin inhibits lipid peroxidation in dendritic cells (Song et al., 2017) and reduce oxidative stress in human cancer cells (Ji et al., 2013; Park et al., 2012). It has also been shown that laminarin increases SOD, GSH and CAT levels and alleviates oxidative stress by inhibiting ROS production in fibroblasts (Cheng et al., 2011; Liu et al., 2017).

Lycopene, a natural carotenoid found mainly in tomatoes is considered a potent antioxidant (Kelkel et al., 2011). In chicken ovaries, lycopene attenuates oxidative stress by activating the Nrf2 / hemeoxygenase-1 (HO-1) pathway (Liu et al., 2018). It is known that this pathway positively regulates the expression of many antioxidant genes and relieves oxidative

stress (Sahin et al., 2014). Chowdhury et al. (2017) reported that lycopene neutralizes the adverse effects of ROS and increases the percentage of oocytes at MII stage during IVM of bovine oocytes.

To understand the mechanism of action of lycopene, in vitro studies with humans and mouse cardiomyocytes showed that this substance modulates several redox-sensitive signaling pathways, such as antioxidant response element, small GTPases, MAPK, nuclear factor κB activator protein 1 (AP-1), as well as redox-sensitive proteins involved in cell cycle modulation and apoptosis (p53, Bcl-2 family proteins and Ku protein) (Palozza et al., 2012; Xu et al., 2015). Lycopene prevents oxidative stress by suppressing apoptosis induced by oxidative stress through the Akt-MnSOD axis in human mesenchymal stem cells (Kim et al., 2015). In addition, lycopene decreases MAPK phosphorylation associated with apoptosis (p38 and JNK). These effects of lycopene can result in decreased phosphorylation of the ATM-p53 signaling pathway and the protection of poly (ADP-ribose) polymerase 1 (PARP-1) and caspase-3 cleavage, thus preventing apoptosis and leading to increased cell survival in human mesenchymal stem cells (Kim et al., 2015). Other studies in mouse have reported that the protective effect of lycopene against oxidative stress is through the activation of the Nrf2 / HO-1 pathway (Yue et al., 2015; Sahin et al., 2016). Figure 6 shows the mechanisms of action of laminarin and lycopene to regulate oxidative stress.

Figure 6. Laminarin reduces oxidative stress induced by lipid peroxidation. This is due to its ability to regulate NRF2 and its pathways such as the KEAP1-NRF2 and NRF2/ARE signaling pathway. As a result, there is a reduction in ROS, an increase in antioxidant enzymes and a reduction in lipid peroxidation. Lycopene modulates redox-sensitive proteins involved in cell cycle modulation and apoptosis (p53, Bcl-2 family proteins and Ku protein). Lycopene prevents oxidative stress by suppressing apoptosis induced by Akt-MnSOD. It also acts in a signaling cascade of ATM-p53, protecting cells by reducing ROS and acting directly to prevent apoptosis.



5 Final considerations

In order to reduce damages caused by oxidative stress during in vitro culture of follicles, oocytes and embryos, supplementation of culture media with different antioxidants is crucial for the success of these techniques. Understanding the mechanisms of action of non-enzymatic antioxidants is a critical factor to preserve cellular viability in vitro. Therefore, two or more non-enzymatic antioxidants acting through different mechanisms of action can potentiate the control of oxidative stress. Considering the importance of GSH production against oxidative stress, NAC or cysteamine supplementation can supply the amount of GSH needed for cellular demand. Combined with this, the ability of antioxidants to promote the expression of genes involved in mitochondrial energy biogenesis and pathways related to ROS depletion further strengths the defense against ROS. Melatonin, resveratrol and quercetin can be used for this purpose. Furthermore, it is of great relevance to supplement the medium with substances capable of minimizing lipid peroxidation and DNA damages. Ascorbic acid, macroside V and phycocyanin act against lipid peroxidation while acteoside repair damaged DNA. A combination of antioxidants can increase the efficiency of oocyte maturation and, consequently, in vitro fertilization and embryo development. Previous studies have already shown that combination acetyl-L-carnitine and N-acetyl-L-cysteine improves the development of mice embryos and increase the number of blastocyst cells (Truong et al., 2016, 2017). Further

studies are still needed to enable the use of combined antioxidants in different stages of follicle growth, oocyte maturation and embryo development in different species.

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3 JUSTIFICATIVA

O desenvolvimento de biotécnicas reprodutivas permanece sendo necessário para a maximização e disponibilidade de gametas femininos, visando fornecer um maior subsídio para a produção *in vitro* de embriões de animais de alto valor zootécnico. Dessa forma, é imprescindível a elaboração de pesquisas que busquem o aprimoramento de biotécnicas como o cultivo *in vitro* de folículos ovarianos e a maturação *in vitro* de oócitos.

Nesse cenário, a escolha do modelo experimental bovino se dá pelo fato do Brasil possuir o segundo maior rebanho bovino do mundo destacando-se pela produção de carne que tem contribuído com o produto interno bruto (PIB) brasileiro (IBGE, 2020). Além disso, o Brasil é o terceiro maior produtor de leite do mundo, atrás apenas da Índia e dos Estados Unidos (FAOSTAT, 2020). Vale ressaltar que a espécie bovina possui várias similaridades quanto a foliculogênese da espécie humana e de outros animais de produção, o que pode contribuir para o desenvolvimento de estudos aplicados a reprodução de humanos e animais (modelo de "duplo propósito e duplo benefício"). Embora avanços já tenham sido evidenciados com a utilização de biotécnicas na espécie bovina, em se tratando do cultivo *in vitro,* os resultados limitam-se a formação da cavidade antral a partir de folículos pré-antrais. E ainda, as taxas de embriões obtidos a partir da MIV ainda são consideradas baixas quando comparadas as taxas alcançadas *in vivo*. Isto ocorre por dois principais motivos: primeiro, a assincronia que ocorre entre a maturação nuclear e citoplasmática durante a MIV e segundo, a ocorrência do estresse oxidativo inerente do ambiente *in vitro*. De fato, o estresse oxidativo é capaz de afetar não somente os oócitos maturados *in vitro*, mas também os folículos ovarianos cultivados *in vitro*. Diante do exposto, o presente estudo busca estratégias para mitigar os danos causados pelo estresse oxidativo e melhorar as taxas de oócitos aptos a serem fertilizados *in vitro* por meio da suplementação com melatonina. Além disso, esse estudo fornece bases para novas pesquisas que visem a compreensão dos mecanismos de ação da melatonina durante o cultivo *in vitro*, aumentando assim, seu potencial e utilização nas biotécnicas aplicadas a reprodução.

O ineditismo deste trabalho destaca-se pela avaliação dos efeitos da melatonina e seus mecanismos de ação na ativação folicular e vias a jusante como mTOR durante o cultivo *in vitro* do tecido ovariano bovino. Além disso, os efeitos da adição da melatonina durante o cultivo de folículos antrais iniciais. E finalmente, a avaliação da melatonina durante a maturação *in vitro* de CCOs bovinos em um sistema bifásico com moduladores do AMPc.

4 HIPÓTESES CIENTÍFICAS

Fase 1:

1) A adição de melatonina (1000 pM) durante o cultivo *in vitro* de tecido ovariano bovino proporciona aumento da ativação e desenvolvimento folicular com manutenção da morfologia folicular.
2) A adição de melatonina (1000 pM) durante o cultivo *in vitro* de tecido ovariano bovino proporciona aumento das células do estroma, distribuição de colágeno, teor de tiol e nos níveis de proteínas totais e expressão gênica para SOD, CAT, GPX1 e PRDX6.
3) Os efeitos da melatonina durante o cultivo *in vitro* de tecido ovariano bovino são bloqueados pelo antagonista de receptor luzindol ou rapamicina inibidor de mTOR.

Fase 2:

1) A adição de melatonina durante o cultivo *in vitro* de folículos antrais iniciais bovinos reduz os danos causados pelo estresse oxidativo, favorece o crescimento, a sobrevivência, a manutenção da morfologia e a integridade ultraestrutural de folículos antrais iniciais bovinos.
2) A melatonina aumenta os níveis de expressão de RNAm para SOD, CAT, GPX1 e PRDX6 em complexos granulosa-oócitos oriundos de folículos antrais iniciais cultivados *in vitro*.

Fase 3:

1) A adição de melatonina durante a maturação *in vitro* proporciona melhores taxas de retomada meiótica, projeções tranzonais, atividade mitocondrial, reduz os níveis de EROs e mantém a ultraestrutura de oócitos bovinos.
2) A melatonina aumenta os níveis de expressão de RNAm de SOD, CAT, GPX1 e PRDX6 em complexos cumulus oócitos após a maturação *in vitro.*

5 OBJETIVOS

5.1 Objetivo geral

Analisar a influência da melatonina sobre o desenvolvimento, ultraestrutura e os níveis de expressão de enzimas antioxidantes durante o cultivo *in vitro* de folículos pré-antrais e antrais iniciais e na maturação *in vitro* de complexos cumulus-oócito bovinos.

5.2 Objetivos específicos

1) Investigar os efeitos de melatonina (1000 pM) e do bloqueio de seus receptores MT1 e MT2 ou do bloqueio de mTOR durante o cultivo *in vitro* de tecido ovariano bovino sobre a ativação, desenvolvimento e integridade morfológica.
2) Verificar a influência da melatonina (1000 pM) e do bloqueio de seus receptores MT1 e MT2 ou do bloqueio de mTOR, na densidade de células estromais ovarianas, distribuição de colágeno, ultraestrutura e nos níveis de tiol.
3) Verificar a influência da melatonina (1000 pM) e do bloqueio de seus receptores MT1 e MT2 ou do bloqueio de mTOR nos níveis de RNAm e atividade das enzimas antioxidantes de SOD, CAT, GPX e PRDX6 em tecidos ovarianos bovinos cultivados *in vitro*.
4) Investigar o efeito de diferentes concentrações de melatonina (10^{-6}, 10^{-7} e 10^{-8} M) no crescimento e viabilidade de folículos antrais iniciais bovinos cultivados *in vitro* por 8 dias.
5) Avaliar a influência de diferentes concentrações de melatonina (10^{-6}, 10^{-7} e 10^{-8} M) sobre os níveis de ERO, atividade mitocondrial, ultraestrutura e nos níveis de expressão de RNAm para SOD, CAT, GPX1 e PRDX6 de complexos granulosa-oócito bovinos após o cultivo *in vitro* de folículos antrais iniciais.
6) Investigar os efeitos de diferentes combinações dos bloqueadores meióticos 3-isobutil-1-metilxantina, forscolina e peptídeo natriurético tipo C, durante a pré-maturação *in vitro* de complexos cumulus-oócitos bovinos.
7) Investigar o efeito de diferentes concentrações de melatonina (10^{-6}, 10^{-7}e 10^{-8} M) sobre as taxas de retomada meiótica, níveis de ERO, atividade mitocondrial, ultraestrutura, projeções tranzonais e nos níveis de expressão de RNAm para SOD, CAT, GPX1 e PRDX6 de complexos cumulus-oócito bovinos após a maturação *in vitro.*

6 CAPÍTULO II

Melatonin acts through different mechanisms to control oxidative stress and primordial follicle activation and survival during *in vitro* culture of bovine ovarian tissue

[A melatonina atua através de diferentes mecanismos para controlar o estresse oxidativo e a ativação e sobrevivência dos folículos primordiais durante o cultivo in vitro de tecido ovariano bovino]

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Melatonin acts through different mechanisms to control oxidative stress and primordial follicle activation and survival during *in vitro* culture of bovine ovarian tissue

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Abstract

This study aims to evaluate the effects of melatonin and its mechanisms of action on preantral follicle activation and survival, stromal cell density and collagen distribution in extracellular matrix (ECM). The involvement of melatonin receptors and mTORC1 pathway in these procedures were also investigated. To this end, ovarian fragments were cultured for six days in α-MEM$^+$ alone or supplemented with 1000 pM melatonin, 1000 pM melatonin with 1000 pM luzindole (inhibitor of melatonin receptors), or 1000 pM melatonin with 0.16 µg/ml rapamycin (mTORC1 inhibitor). At the end of culture period, tissues were processed for classical histology, and the follicles were classified as normal or degenerated, as well as in primordial or growing follicles. The ovarian stromal cell density and ECM collagen distribution were also evaluated. Samples of ovarian tissues were also destined to measure the levels of thiol and mRNA for CAT, SOD, GPX1 and PRDX1, as well as the activity of antioxidant enzymes CAT, SOD, and GPX1. The results demonstrated that ovarian tissues cultured with melatonin, melatonin with luzindole or melatonin with rapamycin had significantly higher percentage of morphologically normal follicles than those cultured in control medium (α-MEM$^+$). However, the presence of either luzindole or rapamycin, did not block the positive effects of melatonin

on follicle survival (P>0.05). Although the presence of melatonin in culture medium reduced the percentage of primordial follicles and increased the percentage of development follicles, these positive effects of melatonin were blocked by either luzindole or rapamycin (P<0.05). Melatonin, melatonin with luzindole or melatonin with rapamycin did not influence the number of ovarian stromal cells. In contrast, melatonin significantly increased the percentages of collagen in ovarian tissues, but the positive effects of melatonin were blocked by either luzindole or rapamycin. Tissues cultured with melatonin and rapamycin had higher levels of mRNA for CAT and lower GPx activity when compared to those cultured in control medium. In conclusion, melatonin promotes primordial follicle activation, increases collagen fiber in ECM of *in vitro* cultured bovine ovarian tissue through its membrane-coupled receptors and mTORC1. Oppositely, melatonin increase follicles survival by acting through other pathways, since it can pass through cell membranes and directly regulate oxidative stress.

Keywords: antioxidant, follicular development, luzindole, rapamycin

1. Introduction

The *in vitro* culture of preantral follicles enclosed in ovarian tissue is a valuable tool to study early follicular development and to open new perspectives for using the pool of primordial follicles in assisted reproduction technologies [1-2]. Understanding how primordial follicles remains or not in quiescence or how they become atretic is a subject of high scientific interest and is a critical step for optimization of in vitro culture systems [3]. Therefore, some pivotal features of ovarian tissue, such as *in vitro* maintenance of intracellular interactions between stromal cells and ovarian follicles as well as interactions with extracellular matrix (ECM) components are necessary to regulate follicular activation and growth [4]. Concomitantly, there is a need to control reactive oxygen species (ROS) production, since oxidative stress can lead to damage in cellular membranes, proteins and DNA [5-6]. It is important to highlight that the levels of thiol (-SH) is an important indicator of in vitro oxidative environment [7]. Hence, among the cellular defense mechanisms against oxidative stress, antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPX) act by eliminating free radicals and balancing the cellular redox system *in vivo* or *in vitro* [8-9]. In this sense, various studies have been carried to evaluate the effects of antioxidant substances during in vitro culture of bovine ovarian tissue aiming to optimize survival and activation of primordial follicles [10-11].

Melatonin (N-acetyl-5-methoxytryptamine) is a natural hormone with potent antioxidant activity [12]. It is produced not only in the pineal gland, but also in oocytes [13]. It has been shown that melatonin positively correlates with follicular and oocyte quality and development [14-15]. Melatonin can bind to membrane coupled G protein receptor type 1 (MT1) and type 2 (MT2) that mediate their biological effects via various signal transduction pathways, including phosphatidyl via inositol-3-kinase (PI3K)/Akt [16-17] and trigger positive responses in different types of cells, including ovarian stromal cells [18]. The MT1 receptor controls meiosis during oocyte maturation [19] and modulates embryo development [20], while MT2 receptor regulates apoptosis and proliferation of granulosa cells in cattle [21]. In addition, luzindole, a non-selective MT1 and MT2 melatonin receptor antagonist, has been used to investigate melatonin pathways [22]. Melatonin can also act by binding to the cytosolic enzyme quinone reductase 2 (QR2), known as melatonin receptor 3 (MT3), capable of neutralizing elimination of several ROS and reactive nitrogen species (RNS) [23-24]. Furthermore, as an amphiphilic molecule, melatonin also works through mechanisms not mediated by these receptors, acting in the direct elimination of several reactive species (ROS and RNS) to counteract oxidative stress [25-26]. Besides reducing oxidative stress, melatonin can also regulate primordial follicle activation and survival [27-28].

The main pathways that control primordial follicle activation and development are the fosfatidilinositol-3-quinase/protein kinase B/forkhead box O3a (PI3K-AKT-FOXO3a) and mTORC1[29-30]. In general, FOXO3 localizes in the oocyte nucleus in the dormant follicle, but it is exported to the cytoplasm to activate the primordial follicle when phosphorylated by the PI3K-AKT pathway, which can be activated by the mTORC1 pathway [31-30]. The mTORC1 pathway signaling has been investigated by using molecular inhibitors, such as rapamycin [32]. Previous reports in sheep demonstrated that melatonin-maintained survival and stimulated activation of primordial follicles through the PI3K/Akt/FOXO3a signaling pathway after *in vitro* culture of ovarian tissues [27]. In bovine species, although the effects of different concentrations of melatonin have been investigated on primordial follicle activation and survival [18], the mechanisms of action of this hormone were not reported. Thus, we hypothesized that melatonin regulates primordial follicles activation and survival, as well as oxidative stress in bovine ovarian tissue by binding to its membrane receptors type 1 or type 2 and activating mTORC1 pathway.

The present study aims to investigate the influence of melatonin alone or in association with its receptor antagonist luzindole or with mTORC1 inhibitor rapamycin on primordial follicle activation and survival, ovarian stromal cell density and collagen distribution in ECM.

In addition, their effects on levels of thiol and mRNA for CAT, SOD, GPX1 or PRDX1, as well as the activity of antioxidant enzymes CAT, SOD, GPX1 in *in vitro* cultured bovine ovarian tissue were investigated.

2. Materials 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), unless otherwise indicated in the text.

2.2. Source of ovaries and in vitro culture of ovarian tissue

The ovaries (n = 20) from ten mixed-bred cows were collected in a local slaughterhouse in accordance with procedures approved by the Ethics and Animal Welfare Committee of the Federal University of Ceará (UFC-CE, 04/22). After harvesting, the ovaries were washed once in alcohol 70% for 30 s and twice in alpha-MEM buffered with 20 mM HEPES and supplemented with antibiotics (100 µg/ml penicillin and 100 µg/ml streptomycin) for 30 s. After washing, the ovaries were transported within 1 h to the laboratory in alpha-MEM HEPES solution at 4°C. In the laboratory, large antral follicles were aspirated. Then, in each of five repetitions, four ovaries from two cows were cut in seventy-eight fragments (3 × 3 × 1 mm) under sterile conditions. Immediately, four cortical slices were fixed for histological analysis and extracellular matrix, eight fragments were used to assess of thiol levels and activity of antioxidant enzymes (CAT, SOD and GPX1), and two fragments for quantification of mRNAs for CAT, SOD, PRDX6 and GPX1 (uncultured controls, n= 14). The remaining fragments (n = 64) were randomly distributed among treatments. Culture was performed in 24-well culture dishes for 6 days, at 38.5°C in 5% CO_2 in a humidified incubator. The basic culture medium consisted of α-MEM (pH 7.2–7.4) supplemented with ITS (10 µg/mL insulin, 5.5 µg/mL transferrin, and 5 ng/mL selenium), 2 mM glutamine, 2 mM hypoxanthine, antibiotics (100 IU/mL penicillin and 100 µg/mL streptomycin) and 1.25 mg/mL of bovine serum albumin (α-MEM+). The fragments were cultured in 1 mL of control medium (α-MEM+) alone or supplemented with 1000 pM melatonin [18], melatonin and luzindole (1000 pM) [22] or melatonin and rapamycin (0.16 µg/mL) [32]. Cavalcante et al. [18] reported that 1000 pM melatonin improves primordial follicle activation in the bovine ovarian tissue, while 1000 pM

luzindole [22] blocked melatonin receptors in bovine granulosa cells. In addition, 0.16 µg/mL rapamycin [32] was previously used to block mTOR pathway during primordial follicle activation in mice. Every 2 days, half (500 µL) of the culture medium was replaced with fresh medium.

After the end of the culture period, for each treatment, 32 fragments were stored at -80 ºC for subsequent evaluation of thiol levels and activity of antioxidant enzymes (CAT, SOD and GPX1) (n=8 fragments per treatment) while 8 fragments were stored for quantification of mRNAs for CAT, SOD, PRDX6 and GPX1 (n=2 fragments per treatment) For morphological and extacellular matrix analysis, 24 fragments were fixed and processed (n=6 fragments per treatment). This experiment was repeated five times.

2.3. Assessment of *in vitro* follicular activation and survival

After 6 days of *in vitro* culture, ovarian fragments 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 a graded series of ethanol, clarified with xylene and embedded in paraffin wax. For each piece of ovarian cortex, 7 µm sections were mounted on slides and stained with eosin and hematoxylin. Coded anonymized slides were examined under a microscope (Nikon) at ×100 and ×400 magnification. The developmental stages of follicles were classified as primordial (one layer of flattened granulosa cells around the oocyte) or growing follicles (primary: one layer of cuboidal granulosa cells around the oocyte, and secondary: two or more layers of cuboidal granulosa cells around the oocyte). These 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 pyknotic nucleus. Degenerated follicles were defined as those with a retracted oocyte, which has a pyknotic nucleus and/or is surrounded by disorganized granulosa cells. Overall, from 130 to 240 follicles were evaluated in each treatment. The percentages of healthy primordial and developing follicles were calculated before (uncultured control) and after culture in a particular treatment.

2.4. Ovarian stromal cell density

The evaluation of stromal cell density was performed in uncultured tissues and in tissues cultured for 6 days in the different treatments. For each treatment, ten random fields from different sections from five animals were evaluated with the aid of a camera attached to a microscope (Nikon, Eclipse, TS 100, Japan), and the images were analyzed by Image J Software (Version 1.51p, 2017). The number of stromal cells was manually counted in an area of 100 μm^2 as described previously (18). All evaluations and measurements were performed by a single operator.

2.5. Analysis of extracellular matrix

Collagen fibers in extracellular matrix of the ovarian cortex were stained with Picrosirius Red (Abcam Kit), following the methodology described by Rittié [33] with modifications. Ovarian sections of 7 µm were dewaxed in xylene and incubated in Sirius Red solution (0.1%) for 1 h at room temperature. Next, the excess dye was removed with acetic acid solution (0.5%) and the sections were dehydrated and subjected to slide assembly with subsequent observation under an optical microscope (Nikon, Eclipse, TS 100, Tokyo, Japan) under magnification 400×. For each treatment, the percentage of the area occupied by collagen fibers in ten different fields was measured with the aid of a DS Cooled DS DS-Ri1 camera attached to a microscope (Nikon, Eclipse, TS 100, Tokyo, Japan), the microscope and the images were analyzed by Image J Software (Version 1.51p, 2017) with 400× magnification. Only the collagen fibers were marked in red with the picrosirius color, while the follicles remained colorless (white). The analyzer software automatically excludes the circumference of unstained follicles from the total area marked in red. The Image J Software was used to quantify the percentage of collagen fiber in uncultured and cultured tissues. The staining intensity of collagen fibers was determined by measuring the average pixel intensity of the total area imaged after background subtraction.

2.6. Redox status

Ovarian tissue fragments (100 mg mL^{-1}) 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. The ovarian homogenates were centrifuged at 1500g for 10 min at 4ºC and the supernatant collected for use in the spectrophotometric (Genesis 10s UV-vis; Thermo Scientific) assays described below, using quartz cuvettes [34] and the obtained samples were destined to evaluate of total thiol content and determination of SOD, CAT and GPx activity. Data are expressed as the mean ± S.E.M. enzyme unit per milligram of protein (U/mg protein).

2.7. Total proteins (Bradford method)

The protein concentration was determined using the Bradford method [30]. This method uses Coomassie blue (Quick start/Bradford; Catalogue No. 500–0205; Bio-Rad) to determine the total concentration of proteins in each extract sample. When it comes in contact with proteins, the Coomassie blue stain forms a complex and emits a blue luminescence. The absorbance is directly related to the protein concentration of the sample and was evaluated spectrophotometrically at a wavelength of 595 nm. The total protein concentration in samples 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^{-1}). To develop the standard curve, the calibration factor was obtained by equation $y = ax + b$, where a is the angular coefficient or inclination and b is the linear coefficient. The standard curve was used to standardize the levels of pro-oxidants (thiol) and antioxidants (SOD, CAT and GPx), as described below.

2.8. Determination of pro-oxidant activity based on thiol content

Total thiol content was determined using 5,50-dithiobis 2-nitrobenzoic acid (DTNB; Dinâmica, São Paulo, Brazil D8130) as an index of reduced thiol. Thiol residues react with DTNB (10 mM), cleaving the disulfide bond to form 2-nitro-5-thiobenzoate anion (NTB^2) at a neutral pH. NTB^2 is quantified in a spectrophotometer by measuring absorbance at 412 nm, with results expressed as nMol of reduced DTNB per milligram of protein [36].

2.9. Determination of SOD, CAT and GPx activities

The SOD activity was measured as the inhibition of adrenaline auto-oxidation [37]. Adrenaline oxidation, in the presence of CAT in basic medium, leads to the formation of the O_2^- radical, which SOD reacts with, thus slowing ‘inhibiting’ the oxidation of adrenaline. The

CAT solution (0.048 mg mL^{-1}; c9322; Sigma-Aldrich) was performed adding (7:3) to glycine buffer, pH 10.2 (Dinâmica, São Paulo, Brazil). Three different volumes (10, 20 or 40 mL) of ovary homogenate were then added to the solution and the adrenaline (0.218 mg mL^{-1}; E4260; Sigma-Aldrich) was added to start oxidation. Oxidation was measured at 480 nm every 10 s for 180 s.

The CAT activity was measured as the consumption of H_2O_2 as a substrate at 240nm [38]. A solution of H_2O_2 (PH09717RA; Exôdo 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 ovary homogenate were added. Every 30 s, the consumption of H_2O_2 was measured twice.

The GPx activity was measured by NADPH oxidation. NADPH is consumed by glutathione redutase (GR; G3664; Sigma-Aldrich) 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 [39]. The reaction was prepared by mixing 500 mL potassium phosphate buffer (100 mM), which is composed by 13.6 g/L potassium phosphate monobasic (P0662; Sigma-Aldrich) plus 1.86 g/L EDTA (5mM; E4884; Sigma-Aldrich; ph 7,4) and 38 mg/mL GR, 3 mg/mL GSH and 100 mg ovary homogenate for 10 min at room temperature. Thus, the GPx cysteine could come into contact with GR and GSH. Finally, 100 mL NADPH were 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 evaluated every 10 s for 300 s.

2.10. Quantification of messenger RNAs for SOD, CAT, GPx and PRDX-6

For the total RNA isolation, Trizol reagent was used (Invitrogen, São Paulo, Brazil) according to the manufacturer's instructions. Initially, 1mL of Trizol was added to each sample, and the lysate was then aspirated through 20G needles before being centrifuged at 10,000 g for 3 minutes at 4°C. Then, the lysates were diluted 1:1 in 70% ethanol and placed in a mini-column (spin cartridge by PureLink® RNA Mini Kit, Invitrogen) at 4°C. After binding the RNA to the column, DNA digestion was performed using RNAse-free DNAse (340 units/ml) for 15 minutes at room temperature. After washing the columns three times, the RNA was collected with 30 µl of ultra-pure water. The total RNA concentration was assessed by a nanodrop (Biodrop, Cambridge, England) and 50 ng/µg of total RNA was used for reverse transcription. Before the reverse transcription reaction, the RNA samples were incubated for 5 minutes at

70°C, and then cooled in ice. The reverse transcription was performed in a total volume of 20 µl, composed of 10 µl of sample RNA, 4 µl of reverse transcriptase buffer (Invitrogen, São Paulo, Brazil), 8 RNAsin units, 150 units of Superscript reverse transcriptase, 0.036 U random primers, 10 mM DTT and 0.5 m of each dNTP (Invitrogen, São Paulo, Brazil). The mixture was incubated at 42°C for 1 hour, followed by incubation at 80°C for 5 minutes, and then stored at -20°C. The negative control was prepared under the same conditions, but without the addition of reverse transcriptase. The quantification of messenger RNAs was performed using SYBR Green. Each real-time reaction (15 µl) will contain 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. The primers (Table 1) were designed to amplify specifically messenger RNAs for SOD, CAT, GPx and PRDX-6. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used to normalize mRNA levels. The specificity of each primer pair was confirmed by analyzing the melting curve of PCR products. The thermal cycling profile for the first round of PCR was initially denaturation and activation of the polymerase for 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C, 30 sec at 58°C, and 30 sec at 72°C. The final extension for 10 min at 72°C. All reactions were performed in a Step One Plus instrument (Applied Biosystems, Foster City, CA, USA). The 2^-ΔΔCt method was used to transform the Ct values into normalized relative expression levels [40].

2.11. Statistical analysis

The percentages of primordial and developing follicles, as well as of those of morphologically normal after 6 days of culture in the different treatments were compared by chi-square (GraphPad Prism). Data on collagen fiber distribution, stromal cell density, thiol levels, activity of SOD, CAT and GPx enzymes, as well as the levels of mRNA for SOD, CAT, PRDX6 and GPX1 were analyzed by ANOVA and compared by Krukal-Wallis test. Results were expressed as mean ± S.E.M and the differences were considered significant when $p < 0.05$.

3. Results

3.1. Effect of melatonin, luzindole and rapamycin on follicle survival

The ovarian tissues cultured with melatonin, both melatonin and luzindole or both melatonin and rapamycin had significantly higher percentage of morphologically normal

follicles than those cultured in control medium (α-MEM^{+}) (**Figure 1**). The presence of either luzindole or rapamycin did not block the positive effects of melatonin on follicle survival ($P>0.05$). On the other hand, when compared to the uncultured tissues, irrespectively of treatment, *in vitro* cultured tissues had significantly lower percentage of morphologically normal follicles.

3.2. Effect of melatonin, luzindole and rapamycin on primordial follicle activation

After culture of ovarian tissue in all treatments, a reduction in the percentage of primordial follicles and an increase in the percentage of development follicles were observed when compared to uncultured tissues **(Figure 2).** The presence of melatonin in culture medium reduced the percentage of primordial follicles and increased the percentage of developing follicles when compared to tissues cultured in control medium. However, these positive effects of melatonin were, blocked by either luzindole or rapamycin ($P<0.05$).

3.3. Effect of melatonin, luzindole and rapamycin on ovarian stromal cell density

Melatonin, both melatonin and luzindole or both melatonin and rapamycin did not influence the number of ovarian stromal cells when compared to uncultured tissues or tissues cultured in control medium ($P>0.05$). Representative histological sections illustrating the stromal cell density are shown in **Figure 3A-F**.

3.4. Effect of melatonin, luzindole and rapamycin on extracellular matrix

Compared with non-cultured tissues, tissues cultured in control medium alone or supplemented with both melatonin and luzindole or both melatonin and rapamycin had lower percentages of collagen fibers ($P<0.05$), which was not observed for those tissues cultured with melatonin **(Figure 4).** On the other hand, the melatonin significantly increased the percentages of collagen in ovarian tissues, but the positive effects of melatonin were blocked by either luzindole or rapamycin ($P<0.05$).

3.5. Effect of melatonin, luzindole and rapamycin on the levels of thiol

Ovarian tissues cultured in control medium alone or supplemented both melatonin and luzindole or both melatonin and rapamycin had lower levels of thiol when compared with the non-cultured tissues (P<0.05), which was not observed for those tissues cultured with melatonin **(Figure 5).** On the other hand, the thiol content was significantly (P<0.05) higher in tissues cultured in presence of melatonin when compared with those cultured in control medium.; however, these positive effects of melatonin were, blocked by either luzindole or rapamycin (P<0.05).

3.6. Activity of antioxidant enzymes (CAT, SOD, and GPx)

No significant differences were observed in the activity of CAT (**Figure 6A**) or SOD (**Figure 6B**) among uncultured and cultured tissues, irrespectively of treatment. However, GPx (**Figure 6C**) activity was significantly lower in tissues cultured with both melatonin and rapamycin, when compared to those cultured in control medium (P <0.05).

3.7. Quantification of messenger RNAs

Figura 7 shows that in vitro culture of ovarian tissues in control medium significantly reduced the levels of mRNA for SOD, CAT, GPX1 and PRDX6. Tissues cultured in control medium alone or with only melatonin had significantly reduced levels of CAT mRNA (P<0.05) when compared to tissues cultured with both melatonin and rapamycin **(Figure 8A)**. Concerning the mRNA levels for SOD, a significantly higher expression was observed in tissues cultured with only melatonin and both melatonin and rapamycin when compared to tissues cultured with both melatonin and luzindole **(Figure 8B)**. The levels of mRNA for PRDX6 **(Figure 8C)** and GPX1 **(Figure 8D)** did not differ among treatments.

4. Discussion

There are strong evidences that melatonin acts through pathways associated with its receptors during follicular growth, activation, and oocyte maturation [41,27, 42-44]. Previous studies showed that melatonin increases the rates of bovine primordial follicle activation and survival [18]. The present study demonstrates that when either melatonin receptors or mTORC1 pathway are blocked, the positive effects of melatonin on bovine primordial follicle activation, collagen distribution in ECM and antioxidant activity are inhibited. Previous studies suggest

that MT1 and MT2 blockage by luzindole, and mTORC1 blockage by rapamycin inhibited PI3K/AKT downstream pathway, controls primordial follicle activation and survival [27,28]. In bovine species, *in vitro* primordial follicle activation is associated with primordial follicle loss of phosphatase and tensin homologue (PTEN) and cytoplasmic translocation of FOXO3 [45-46]. Regarding the mTOR pathway, as far as we know, this is the first study to demonstrate a relationship between melatonin and the mTORC1 pathway during primordial follicle activation in bovine species. Previous report suggested a relationship between melatonin and the mTORC pathway during follicular activation in rat ovary [47]. Moreover, a recent study by Liu et al. [48] demonstrated that melatonin can activate the PI3K/Akt/mTOR pathway in rat ovarian granulosa cells. Thus, melatonin promotes the activation of bovine primordial follicles cultured *in vitro* through its receptors, modulating the PI3K/FOXO3 or PI3K/Akt/mTOR pathways.

In our study, melatonin increased the percentage of collagen fibers and activating mTORC1 pathway. Collagen is the main constituent of the ECM [49-50] and recently, Zhao et al. [51] showed that melatonin promotes the synthesis and secretion of type II collagen and reduces ECM degradation. On the contrary, melatonin increased follicles survival, but its effects were not blocked by either luzindole or rapamycin. These findings demonstrate that although a large number of studies observed the action of melatonin on follicle viability and development occurs through receptors [27, 42-45], the melatonin can also pass through cell membranes, and act through other pathways to reduce oxidative stress. Previously, García et al. [52] reported that melatonin protects cell membranes from free radical attack by reducing oxidative stress and by optimizing electron transfer through the electron transport chain. Luchetti et al. [53] reported that melatonin increases cell viability via improving phosphorylation and the activation of extracellular signal-regulated kinase 1/2 (ERK 1/2), and inhibits the activation of the stress kinases p38, MAPK and JNK which are responsible for cell apoptosis.

The current research has shown strong evidences of the relationship between the beneficial effects of melatonin and its antioxidant activity. To clarify this aspect, the thiol level was evaluated in our study. The thiol group (-SH) present in reduced glutathione (GSH) has been used as an index of the oxidated environment of a medium [7]. Considering that the oxidation of GSH by GPx only occurs in the presence of H_2O_2, a low thiol content in a sample indicates a higher rate of GSH oxidation, while higher thiol content represents a lower oxidation rate [54]. Thus, melatonin contributed for a less oxidizing environment, but this activity was inhibited in the presence of luzindole or rapamycin. Melatonin has beneficial effects on

controlling oxidative stress, and this action occurs after receptor activation [27,55-57]. Additionally, it is known that the antioxidant activity of melatonin relays on its indirect action mediated by receptors, probably stimulating other antioxidant enzymes, such as sirtuin-3 (SIRT3) and others [58].

In contrast, previous reports showed that the antioxidant action of melatonin is related to the increase in SOD, CAT or GPX1 proteins or in the gene expression of these enzymes [42,56]. The current study did not find effects of melatonin on activity of SOD and CAT enzymes. Interestingly, for total protein levels of GSH-PX, we observed that bovine ovarian tissue cultured with rapamycin showed a significant reduction when compared to tissue cultured with melatonin alone. The GPx oxidizes reduced glutathione (GSH) to form oxidated glutathione (GSSG) and water, reducing the levels of oxidative stress [59-60]. This study shows that the presence of rapamycin increased the expression of CAT mRNA. Recently, Yang et al. [61] demonstrated that rapamycin can regulate the expression of nuclear factor 2-related transcription factor E2 (Nrf2), a master component of the transcriptional pathway of many antioxidant genes, detoxifying enzymes including CAT [62]. Based on this, we hypothesized that rapamycin can increase CAT levels by activating Nrf2 during *in vitro* culture of ovarian tissue. The complex interactions between the mTOR pathway and increased CAT levels require further studies. Additionally, our results show that in vitro culture of bovine ovarian tissue reduces the expression of mRNA for antioxidant enzymes. Soto-Heras and Paramio [63] also reported that in vitro cultured oocytes and embryos have an imbalance in the cellular defense system, leading to an increase in ROS. We also showed that luzindole reduced SOD activity in cultured tissues. These findings are in agreement with a recent study in which luzindole alone or associated with melatonin decreased the expression of SOD, and did not influence the expression of GPX1 or PRDX-6 in bovine ovarian follicles cultured for 18 days [56].

In conclusion, melatonin promotes primordial follicle activation, increases collagen fiber in ECM of *in vitro* cultured bovine ovarian tissue through its membrane-coupled receptors and mTORC1. Interestingly, melatonin increase follicles survival by acting through other pathways, since it can pass through cell membranes and directly regulate oxidative stress.

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List of tables and figures

Table 1. *Primers* used for amplification of messenger RNAs.

Target gene	Primer sequence (5' → 3')	Sense (S), anti-sense (As)	GenBank accession no.	Amplicon length (bp)
GAPDH	TGTTTGTGATGGGCGTGAACCA ATGGCGCGTGGACAGTGGTCA TAA	S As	GI: 402744670	183
PRDX6	GCACCTCCTCTTACTTCCCG GATGCGGCCGATGGTAGTAT	S As	GI: 59858298	105
GPx1	AACGTAGCATCGCTCTGAGG GATGCCCAAACTGGTTGCAG	S As	GI:15660264 5	121
SOD	GTGAACAACCTCAACGTCGC GGGTTCTCCACCACCGTTAG	S As	GI: 31341527	165
CAT	AAGTTCTGCATCGCCACTCA GGGGCCCTACTGTCAGACTA	S As	GI:40269337 5	165

Figure 1. Percentages of morphologically normal follicles in uncultured tissues, and in tissued cultured in α-MEM^{+} alone or supplemented with melatonin, both melatonin and luzindole or both melatonin and rapamycin. * Asterisk represents significant differences between the uncultured and cultured tissues ($P < 0.05$). [a,b] Different lowercase letters represent significant differences among cultured treatments ($P < 0.05$). MT: melatonin, LUZ: luzindole, RAP: rapamycin.

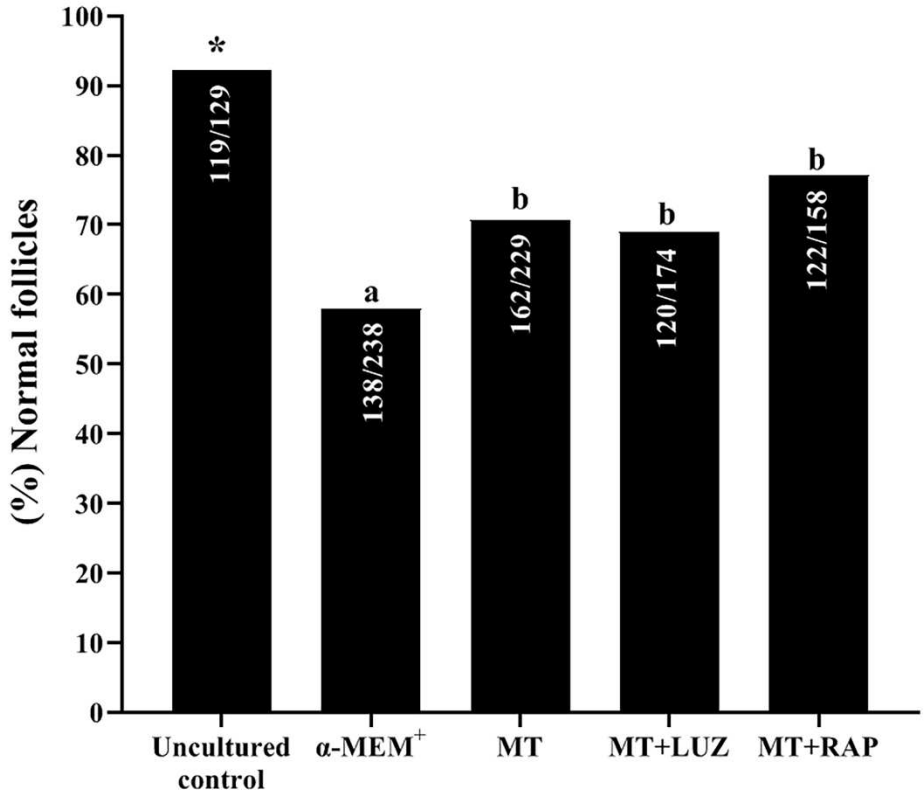


Figure 2. Percentages of primordial and developing follicles in uncultured tissues, and tissues cultured in α-MEM^{+} alone or supplemented with melatonin, both melatonin and luzindole or both melatonin and rapamycin. * Asterisk represents significant differences among the uncultured and cultured tissues ($P < 0.05$). A, B,C: uppercase letters indicates differences in the percentages of primordial follicles among cultured tissues in different treatments; a,b,c Lowercase letters indicates differences in the percentages of developing follicles among tissues cultured in different treatments ($P < 0.05$). MT: melatonin, LUZ: luzindole, RAP: rapamycin.

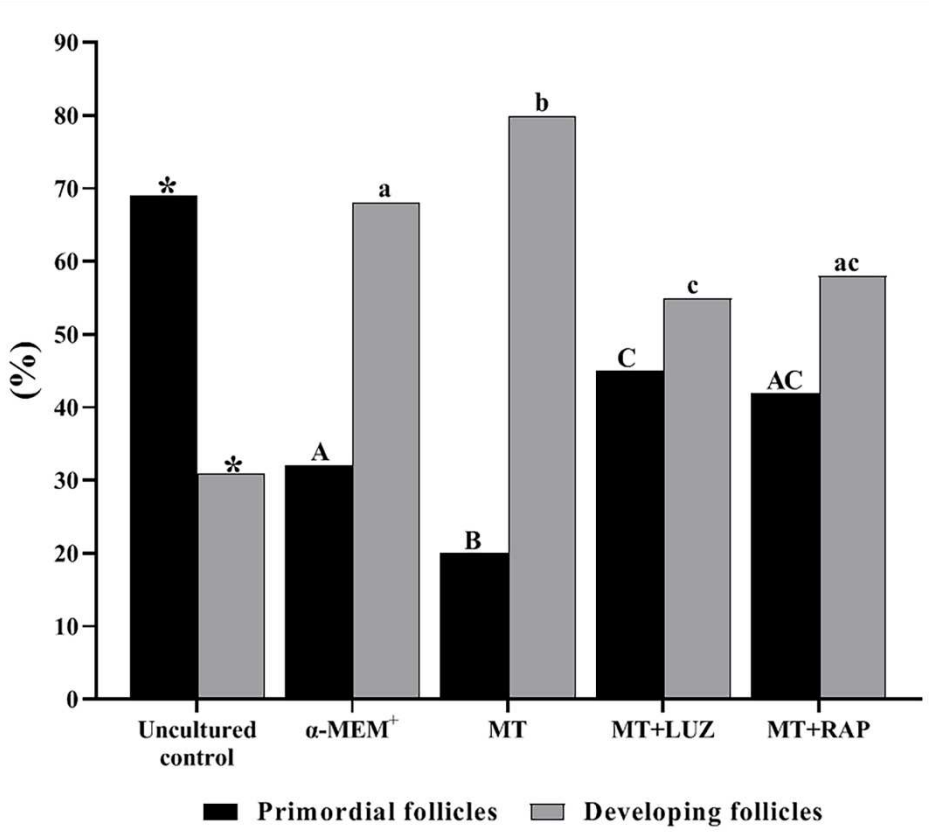


Figure 3. Ovarian stromal cell density in uncultured tissues (A), and tissues cultured in α-MEM$^+$ alone (B) or supplemented with melatonin (C), both melatonin and luzindole (D) or both melatonin and rapamycin (E) Magnification = 400x. Scale bars = 100µm. The F image shows no differences among treatments ($P > 0.05$). ns: no significant difference. MT: melatonin, LUZ: luzindole, RAP: rapamycin.

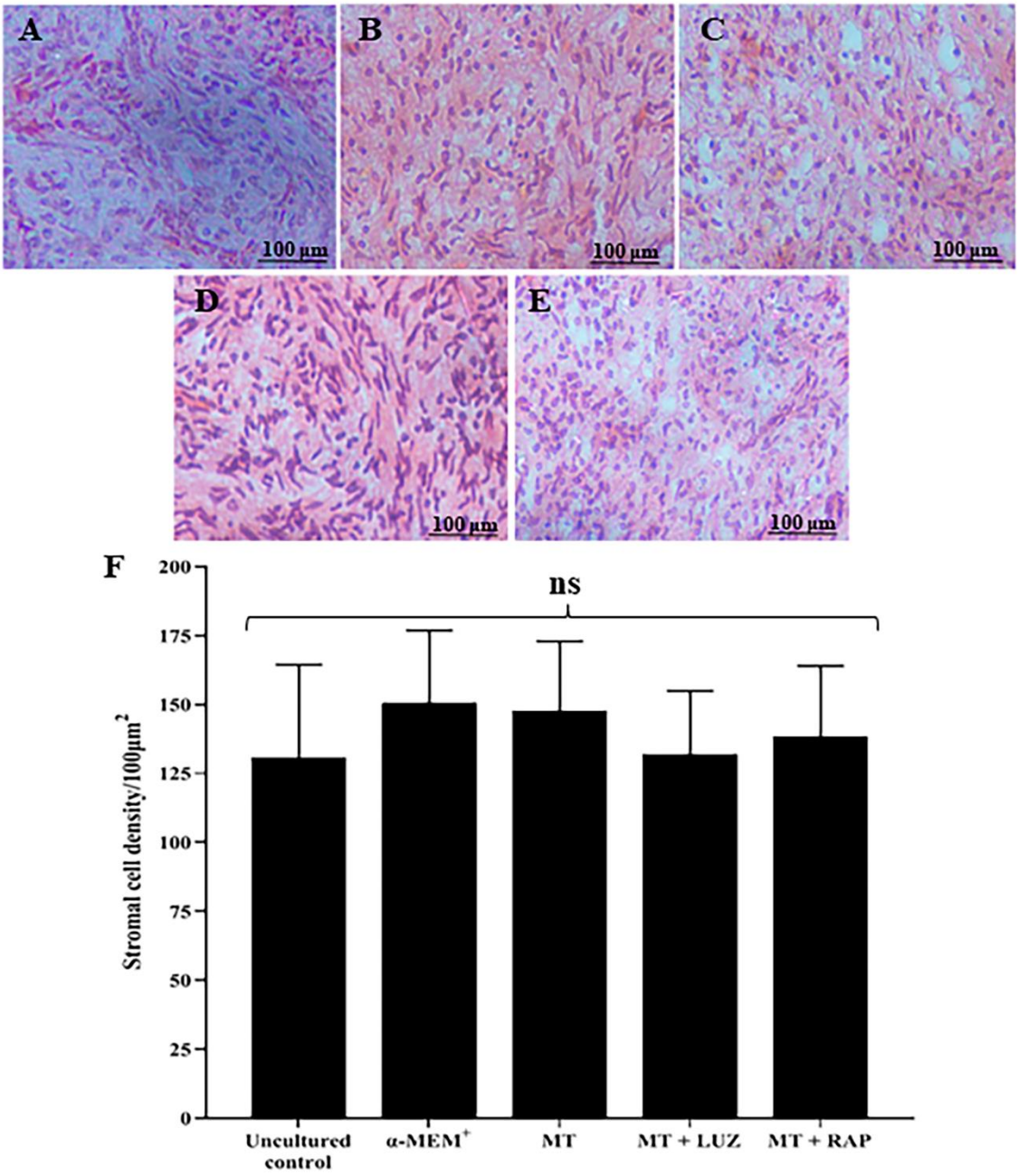


Figure 4. Collagen fibers in uncultured tissues (A) and tissues cultured in α-MEM^{+} alone (B) or supplemented with melatonin (C), both melatonin and luzindole (D) or both melatonin and rapamycin (E). Magnification = 400x. Scale bars = 100µm. F: area of collagen fibers between treatments. *Asterisks represents significant differences between the uncultured and cultured tissues ($P < 0.05$). [a,b] Different lowercase letters represent significant differences among tissues cultured in the different treatments ($P < 0.05$). MT: melatonin, LUZ: luzindole, RAP: rapamycin.

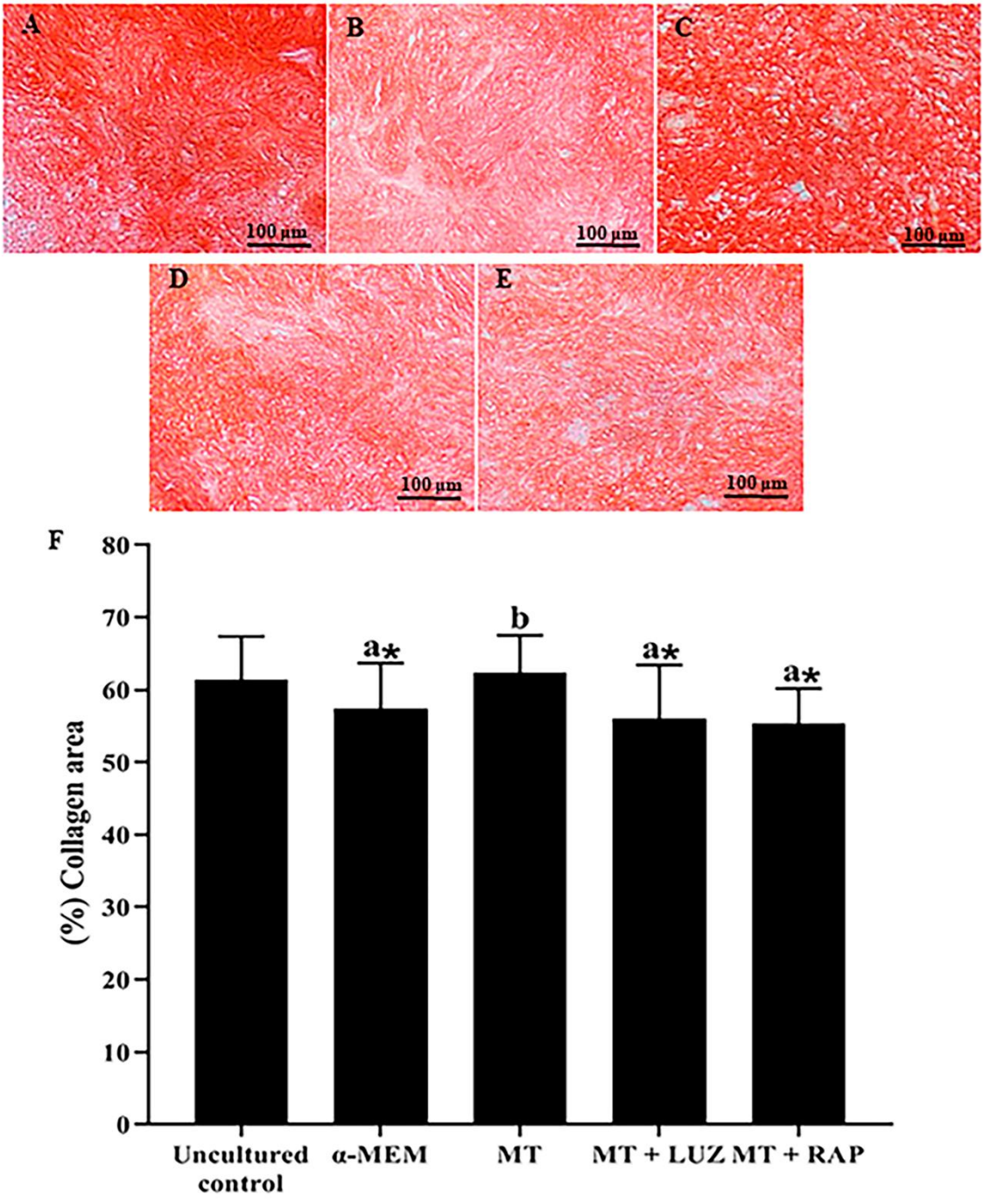


Figure 5. Levels of thiol in uncultured ovarian tissues, and in tissues cultured in α-MEM^{+} alone or supplemented with melatonin, both melatonin and luzindole or both melatonin and rapamycin *Asterisks represents significant differences between the uncultured and cultured tissues ($P < 0.05$). [a,b] Different lowercase letters represent significant differences among cultured tissues ($P < 0.05$). MT: melatonin, LUZ: luzindole, RAP: rapamycin.

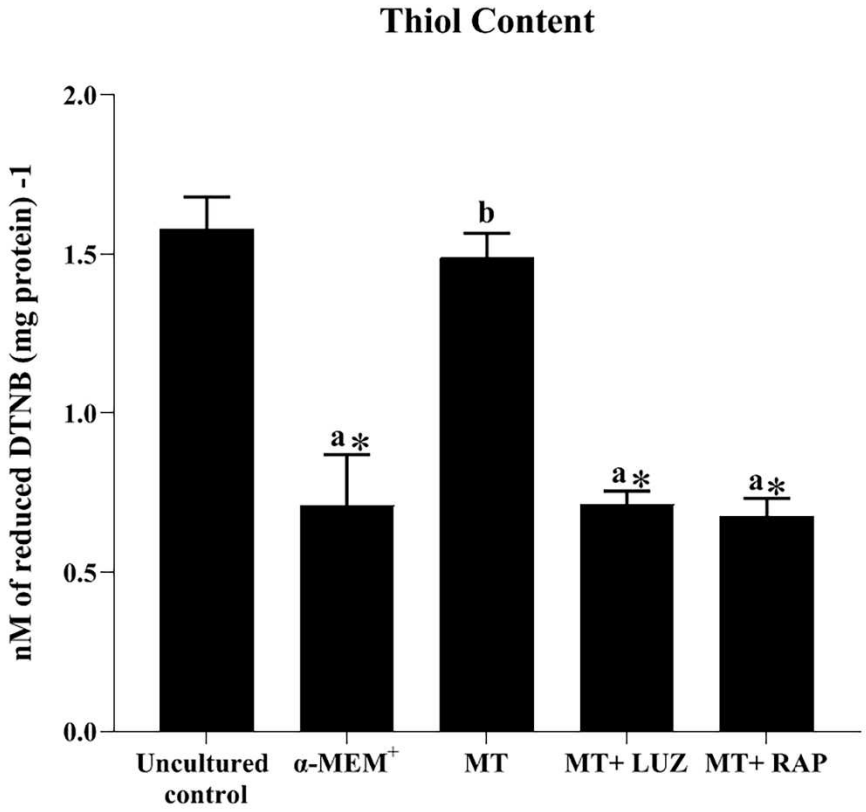


Figure 6. Redox status measured by activity of CAT (A), SOD (B), and GPx (C) in uncultured ovarian tissues, and in tissues cultured in α-MEM^{+} alone or supplemented with melatonin, both melatonin and luzindole or both melatonin and rapamycin. *Asterisks represents significant differences between the uncultured and cultured tissues ($P < 0.05$). [a,b] Different lowercase letters represent significant differences among tissues cultured treatments ($P < 0.05$). ns: no significant difference. MT: melatonin, LUZ: luzindole, RAP: rapamycin.

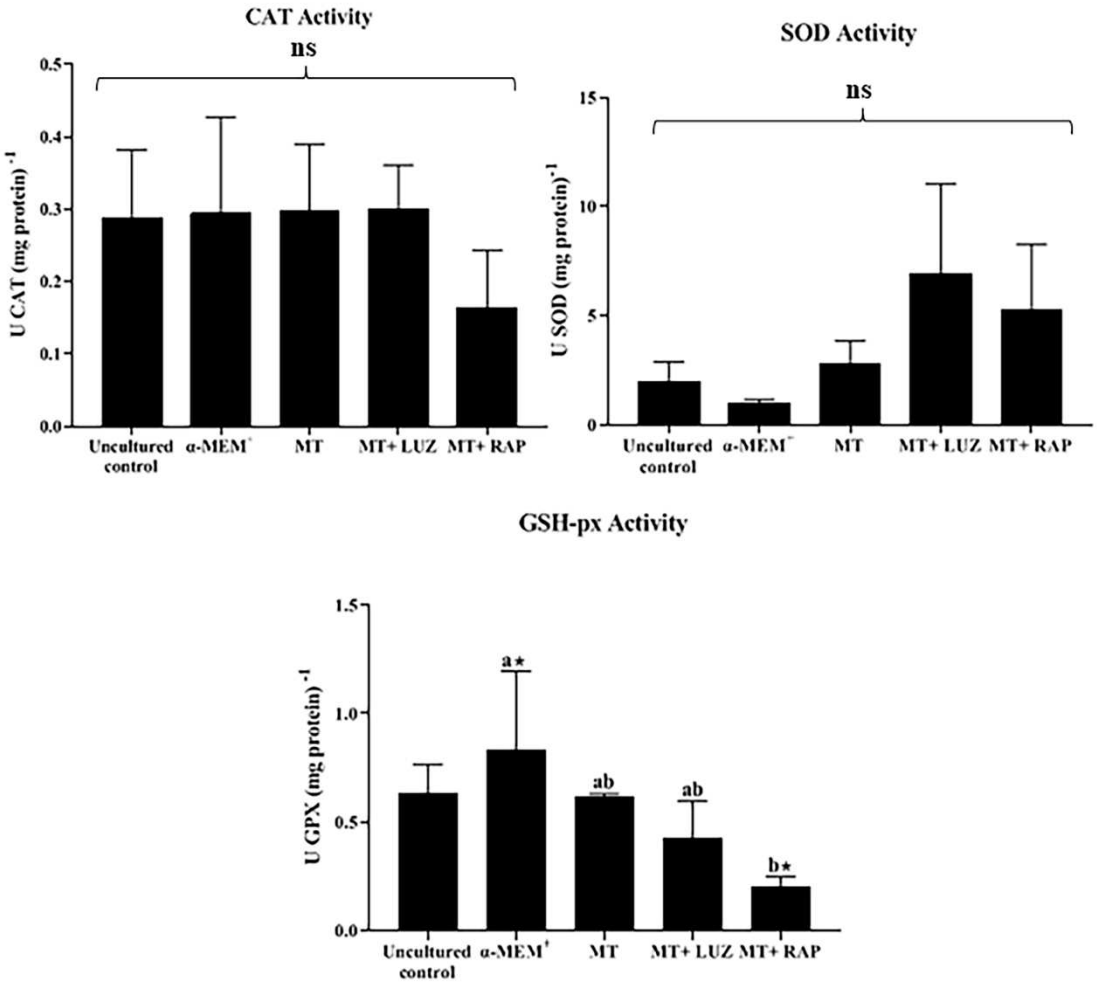


Figure 7. Relative mRNA levels for CAT(A), SOD(B), GPX1 (C) and PRDX6(D) in uncultured ovarian tissues, and in tissues cultured in α-MEM+ alone. *Asterisks represents significant differences between the uncultured and cultured tissues ($P < 0.05$).

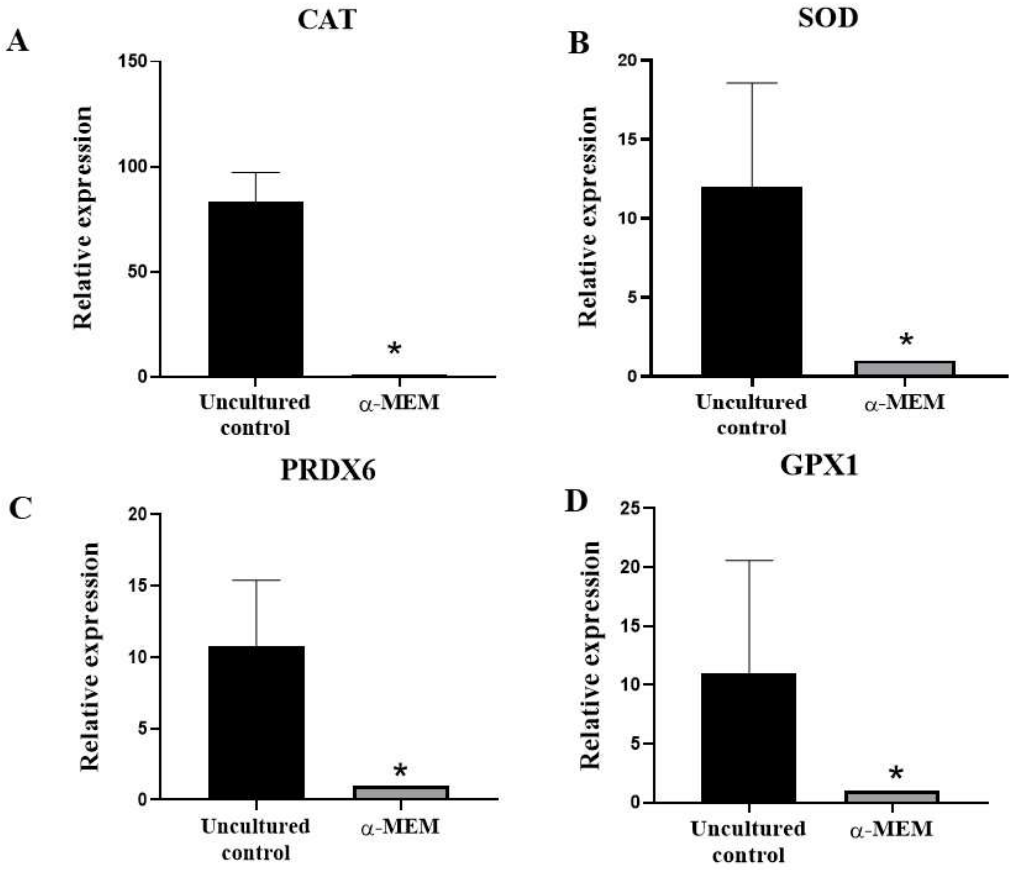
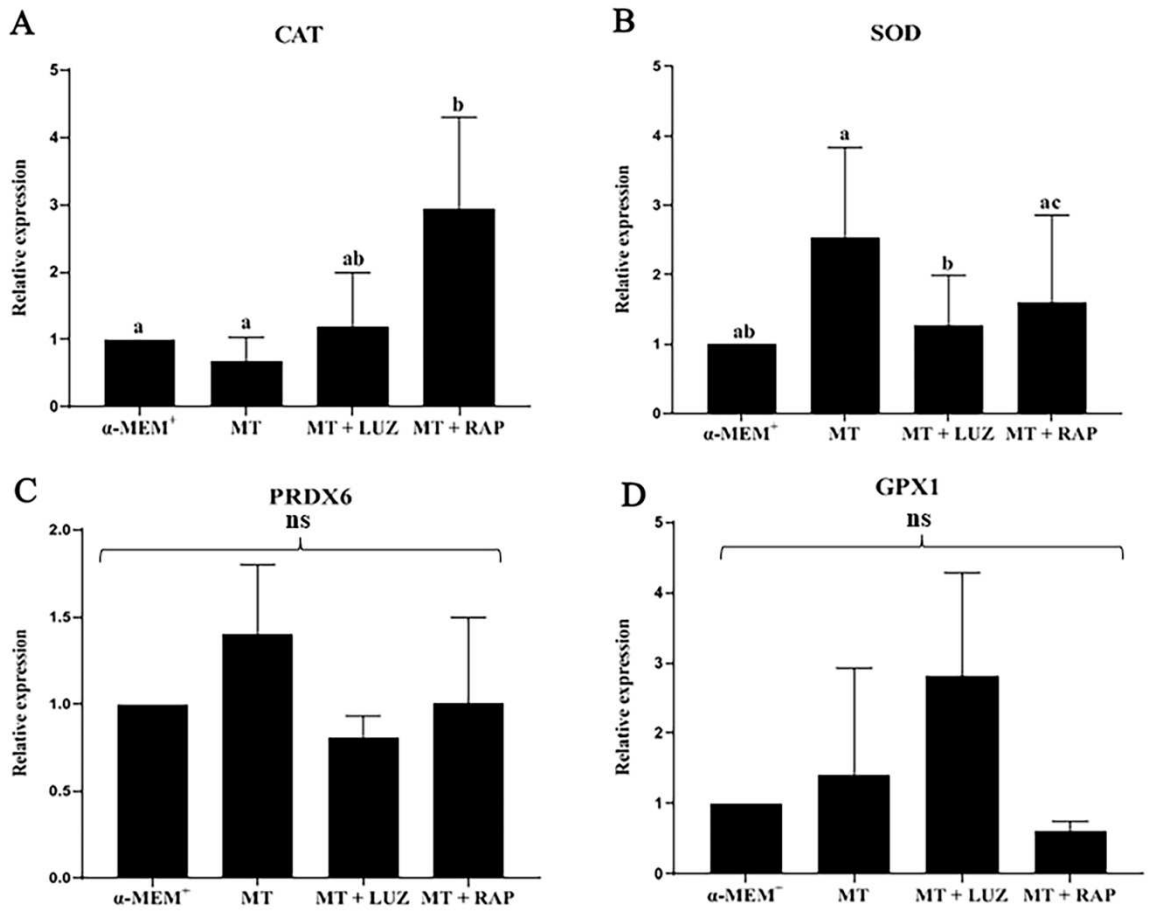


Figure 8. Relative mRNA levels for CAT(A), SOD(B), GPX1 (C) and PRDX6(D) in uncultured ovarian tissues, and in tissues cultured in α-MEM^{+} alone or supplemented with melatonin, both melatonin and luzindole or both melatonin and rapamycin. [a,b,c] Different lowercase letters indicate significant differences among treatments. ns: no significant difference. MT: melatonin, LUZ: luzindole, RAP: rapamycin.



7 CAPTÚLO III

Melatonin improves the viability and ultrastructure of oocyte-granulosa complexes from bovine initial antral follicles cultured *in vitro*

[A melatonina melhora a viabilidade e a ultraestrutura dos complexos oócitos-granulosa de folículos antrais precoces bovinos cultivados *in vitro*]

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Melatonin improves viability and ultrastructure of bovine oocyte-granulosa complexes of *in vitro* cultured early antral follicles

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Abstract

This study aims to investigate the effects of melatonin on follicular growth, viability and ultrastructure, as well as on the levels of mRNA for antioxidant enzymes, reactive oxygen species (ROS) and meiotic progression in oocytes from in vitro cultured bovine early antral follicles. To this end, isolated early antral follicles (500-600 μm) were cultured in TCM-199^{+} alone or supplemented with 10^{-6}, 10^{-7} or 10^{-8} M melatonin at 38.5 °C with 5% CO_2 for 8 days. Follicle diameters were evaluated at days 0, 4 and 8 of culture. At the end of culture, ultrastructure, chromatin configuration, viability (calcein-AM and ethidium homodimer-1 staining), and the levels of ROS and mRNA for catalase (CAT), superoxide dismutase (SOD) and peroxiredoxin 6 (PRDX6) and glutathione peroxidase (GPx) were investigated in oocyte-granulosa cell complexes (OGCs). The results showed that early antral follicles cultured with 10^{-6} M and 10^{-8} M melatonin had a progressive and significant increase in their diameters throughout the culture period ($P < 0.05$). Additionally, oocytes from follicles cultured with 10^{-7} or 10^{-8} M melatonin had increased fluorescence for calcein-AM, while those cultured with 10^{-6} or 10^{-7} M had reduced fluorescence for ethidium homodimer-1. Different from follicles cultured in other treatments, those cultured with 10^{-8} M melatonin had well-preserved ultrastructure of oocyte and granulosa cells. Melatonin, however, did not influence the levels of ROS, the mitochondrial activity, oocyte meiotic resumption and expression mRNA for *SOD,*

CAT, GPX1 and *PRDX6*. In conclusion, the presence of 10^{-8} M melatonin in culture medium improves viability and preserves the ultrastructure of oocyte and granulosa cells of early antral follicles cultured in vitro.

Key-words: oocyte-granulosa cell complexes, ultrastructure, viability, melatonin

1. Introduction

In the last decades, *in vitro* culture of preantral follicles has raised great scientific interest as an alternative to have competent oocytes for *in vitro* embryo production (Chaves et al., 2011). However, in bovine species, the most promising results achieved are limited to follicular growth up to antrum formation (Paulino et al., 2022; Nascimento et al., 2022). In primates, however, *in vitro* culture of early antral follicles resulted in yield of competent oocytes (rhesus monkeys: Peluffo et al., 2010, baboons: Xu et al., 2011). Although these promising results have been achieved, the development of an efficient culture system for bovine early antral follicles remains an obstacle to be overcome (Ferreira et al., 2022). According to Marino et al. (2019), the maintenance of follicular viability, growth and ultrastructural integrity of oocyte and granulosa is a prerequisite to have effective early antral follicle growth *in vitro*. Another critical limitation is the increase in production of ROS during in vitro culture, which can influence mitochondrial activity, meiotic spindle formation and chromosomal configuration (He et al., 2016). The antioxidant enzymes involved in cellular defense, such as CAT, SOD, PRDX and GPx, are responsible for converting superoxide radicals into water (Sovernigo et al., 2017). The SOD converts the superoxide radical into hydrogen peroxide and oxygen, while CAT, GPx and PRDX convert these radicals into water (Baszyński et al., 2022; Jia et al., 2022).

As an alternative to improve follicular ultrastructure and to control oxidative stress during in vitro culture of bovine early antral follicles, melatonin has been highlighted due to the its protective effect on murine preantral follicles (Jang et al. 2016, Kandemir et al., 2017), and the ability to improve the development of secondary follicles, and oocyte competence in pigs (Cao et al., 2019). In addition, melatonin promotes activation of primordial follicles during *in vitro* culture of bovine ovarian cortical tissue (Cavalcante et al., 2019), and growth of in vitro cultured secondary follicles (Paulino et al., 2022). Therefore, we formulate the hypothesis that melatonin is able to improve viability, ultrastructural integrity and mitochondrial activity, as well as to preserve chromatin and to regulate the levels ROS and expression of mRNA for antioxidant enzymes of oocyte-granulosa complexes (OGC) of *in vitro* cultured early antral follicles.

The present study aims to investigate the effects of different concentrations of melatonin on follicular development and viability, levels of ROS, expression of mRNA for CAT, SOD, PRDX6 and GPX1, mitochondrial activity, ultrastructure, and chromatin configuration of oocytes from bovine early antral follicles cultured *in vitro*.

2 Material and methods

2.1 Chemicals

Unless otherwise indicated, all chemicals used in the present study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2 Ovaries

Ovaries (n = 200) from adult cows were collected at a local abattoir. Each ovary was washed once in 70% ethanol, followed by two washes in TCM-199 supplemented with HEPES, penicillin (100IU), and streptomycin (0.1 µg/mL). Soon after, the ovaries were placed into tubes containing 15 mL of this medium then transported to the laboratory at 4°C within 1 h. This study was approved and carried out in accordance with the rules and guidelines of the Ethics and Animal Welfare Committee of the Federal University of Ceará (number 04/22).

2.3 Follicle isolation and *in vitro* culture

Ovarian cortical slices (1–3 mm) were obtained from the ovaries and placed TCM-199 medium supplemented with HEPES, penicillin (100IU), and streptomycin (0.1mg/mL). Then, early antral follicles (500–600 µm in diameter) were visualized under a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan) and manually dissected from the ovarian cortex using 26-gauge needles. After isolation, only follicles with a visible oocyte surrounded by granulosa cells, intact basement membrane, and the presence of an antral cavity were classified as morphologically normal follicles and selected for *in vitro* culture as previously described (Cordeiro et al., 2023). The isolated follicles were individually cultured in droplets of 150 µL of culture medium under mineral oil in Petri dishes (60 × 15mm, Corning, USA). The control medium was TCM-199 (pH 7.2–7.4) supplemented with FSH (50 µg/mL), insulin (10 µg/mL), transferrin (5.5 µg/mL) and selenium (5 ng/mL) (ITS), 0.015 mg/mL bovine serum albumin (BSA), penicillin (100IU),

streptomycin (0.1mg/mL), glutamine (50 µg/mL), and hypoxanthine (50 µg/mL) (TCM-199^{+}) (HE et al. 2020; ALM, et al. 2006). The follicles were distributed randomly and cultured in TCM-199^{+} alone or supplemented with 10^{-6}, 10^{-7} or 10^{-8} M melatonin. Melatonin (23.2mg) was dissolved in 5 mL of ethanol and then diluted in TCM-199 medium to obtain a 20,000pM solution. In each treatment, approximately 115 follicles were cultured. Follicular culture was performed at 38.5°C with 5% CO_2 in air for 8 days. Every 4 days of culture, half (75 µl) of the medium was replaced with fresh medium. This experiment was repeated 10 times.

2.4 Follicular morphology, growth and viability

Throughout the culture period, follicles that had spherical oocyte surrounded by homogeneous granulosa cells, intact basement membrane and external stromal-thecal layer were considered morphologically normal. Degenerate follicles contained a dark oocyte with disorganized granulosa cells or extruded oocyte (Rosseto *et al*., 2013). For follicular diameter, two perpendicular measurements from the basement membrane were performed in normal follicles using an inverted microscope coupled with a NIS-Elements 2.4 software (Nikon, Nikon Instruments Inc., Japan). Follicular morphology and growth were evaluated at days 0, 4 and 8 of culture. The individual growth was defined as the difference between follicular diameters between days 0 and 8 of culture.

After 8 days of culture, OGCs were extruded from morphologically normal follicles with the aid of 25 G needles and incubated with 100 µL of TCM-199 containing 4 mM calcein-AM and 2 mM ethidium-1 homodimer (Molecular Probes, Invitrogen, Karlsruhe, Germany) at 37°C for 15 min. Whereas the first probe detected the intracellular esterase activity in the cytoplasm of viable cells, the later labeled the nucleic acids of non-viable cells after plasmatic membrane disruption. The OGCs were examined under fluorescence microscope (Nikon, Eclipse, TS 100, Japan) at 100x magnification, and considered viable if the cytoplasm was stained positively in green with calcein-AM and the chromatin was not labeled in red with ethidium homodimer-1 (Van den Hurk et al., 1998). Fluorescence intensity either for calcein or ethidium homodimer-1 was analyzed by ImageJ software (version 1.46; National Institutes of Health, Bethesda, MD, USA). To evaluate morphology and growth, a number of 74 to 87 follicles were examined per treatment. To analyze viability, a number of 60 oocyte granulosa complexes were examined, 15 per treatment.

2.5 Ultrastructural analysis

Ultrastructural evaluation of OGCs was performed as previously described (Soares et al., 2018). Briefly, OGCs were fixed in Karnovsky solution (2% paraformaldehyde and 2% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.2), and post fixed in 1% osmium tetroxide, 0.8% potassium ferricyanide and 5mM calcium chloride in 0.1M sodium cacodylate buffer for 1 h. After washing in sodium cacodylate buffer, the OGCs were counterstained with 5% uranyl acetate. Then, the samples were dehydrated in acetone and embedded in epoxy resin (Epoxy-Embedding Kit, Fluka Chemika- BioChemika). Semi-thin sections (2 μm) were cut, stained with toluidine blue and analyzed by light microscopy at 400× magnification. Subsequently, ultra-thin sections (70 nm) were obtained and counterstained with uranyl acetate and lead citrate, and examined under a transmission electron microscope (Fei Tecnai Spirit, North American Sales, USA). A number of five OGCs were analyzed per treatment.

2.6 Analysis of mitochondrial activity and ROS levels in oocytes from cultured follicles

To evaluate mitochondrial activity, denuded oocytes (n = 15 per treatment) recovered from cultured follicles were incubated in phosphate buffer saline (PBS) with 100 nM Mitotracker Red (Mitotracker® Red, CMXRos, Molecular Probes, Melbourne, VIC, Australia) at 37°C for 20 minutes. Subsequently, the oocytes were washed in PBS and evaluated with the aid of an epifluorescence microscope (TS100; Nikon Corp.). Considering that the fluorescent dye accumulates only in active mitochondria, mitochondrial distribution was assessed based on the cytoplasmic location of red fluorescence. Mitochondrial distribution patterns were classified as homogeneous, with staining spread throughout the cytoplasm, or heterogeneous, with peripherical and central aggregations of mitochondria. The corrected total fluorescence was determined by subtracting out background signal. A total of 48 oocytes were evaluated, i.e., 12 oocytes per treatment.

To investigate the levels of ROS, denuded oocytes (n = 15 per treatment) were washed in 0.1% polyvinyl alcohol in PBS (PBS-PVA) and incubated with 10 mM 6-carboxy-2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA, Molecular Probes®, Eugene, OR, USA) at 38.5°C in the dark for 30 min. Then, the oocytes were washed with PBS-PVA and placed on glass slides with ProLong® Gold (Molecular Probes, Eugene, OR, USA). The background signal intensity was subtracted from the measured values. Oocytes cultured in control medium without melatonin were used as control. The fluorescence intensity was considered directly proportional to ROS concentration. A total of 48 oocytes were evaluated.

The slides were evaluated using an epifluorescence microscope (Nikon, TS100). Fluorescence intensity either for mitochondrial activity or ROS levels was analyzed by ImageJ software.

2.7 Evaluation of chromatin configuration in oocytes from cultured follicles

The OGCs were isolated from morphologically normal follicles (n = ~ 30 per treatment) and then, the granulosa cells were removed by pipetting or vortexing. The recovered denuded oocytes were fixed in 4% paraformaldehyde for 15 minutes and transferred to 0.1% Triton X-100 for up to 72 hours. Finally, the oocyte chromatin was stained adding 10 µg/mL of Hoechst 33342 for 10 min, and classified as germinal vesicle (GV), germinal vesicle breakdown (GVBD) or degenerated (pycnotic chromatin) in a fluorescence microscopy (Nikon, TS100; 400x magnification). The fluorescence intensity was measured by using the ImageJ software (version 1.46; National Institutes of Health, Bethesda, MD, USA).

2.8 Quantification of messenger RNAs for antioxidant enzymes

Total RNA isolation was performed by using Trizol® reagent (Invitrogen, São Paulo, Brazil), according to the manufacturer's instructions. Initially, for each treatment, 1mL of Trizol was added to each sample, containing a pool of 10 oocyte-granulosa cell complexes recovered from cultured bovine antral follicles. The lysates were aspirated through 20G needles before being centrifuged at 10,000 g for 3 min. Then, the lysates were diluted (1:1) in 70% ethanol and placed in a mini-column. After binding the RNA to the column, DNA digestion was performed using RNAse-free DNAse (340 units/ml) for 15 min. After washing the columns three times, the RNA was collected with 30 µl of ultra-pure water. The total RNA concentration was assessed by a spectrophotometer (Amersham, Biosciences Cambridge, England) and 1 µg of total RNA was used for reverse transcription. Before the reverse transcription reaction, the RNA samples were incubated for 5 min at 70°C, and then cooled in ice. For reverse transcription reaction, all products were purchased from Invitrogen, São Paulo, Brazil. The reverse transcription was performed in a total volume of 20 µl, composed of 10 µl of sample RNA, 4 µl of reverse transcriptase buffer, 8 RNAsin units, 150 units of Superscript reverse transcriptase, 0.036 U of random primers, 10 mM DTT, and 0.5 m of each dNTP. The mixture was incubated at 42°C for 1 h, 80°C for 5 min, and then stored in a freezer at -20°C. The negative control was prepared under the same conditions, but without the addition of reverse

transcriptase. This PCR analysis was repeated three times. The quantification of messenger RNAs was performed using SYBR Green. Each real-time reaction (15 µl) will contain 7.5 µl of SYBR Green Master Mix (PE Applied Biosystems, Foster City, CA, USA), 5.5 µl of ultra-pure water, 1 µl of cDNA and 0.5 µM of each primer. The primers (**Table 1**) were designed to amplify specifically messenger RNAs for *SOD, CAT, GPX,* and *PRDX-6*. The *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* gene was used to normalize mRNA levels. The specificity of each primer pair was confirmed by analyzing the melting curve of PCR products. The thermal cycling profile for PCR was performed for 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C, 30 sec at 58°C, and 30 sec at 72°C. The final extension was performed for 10 min at 72°C. All reactions were performed in a Step One Plus instrument (Applied Biosystems, Foster City, CA, USA). The 2^ΔΔCt method was used to transform the Ct values into normalized relative expression levels (Livak and Schmittgen, 2001).

2.9 Statistical analysis

Statistical analyses were performed using GraphPad Prism software, version 9.0. Data of percentages of morphologically normal follicles, growth rates and chromatin configuration were compared using Fisher's exact test. Data of follicular diameter, levels of active mitochondria and ROS, as well as the levels of calcein and ethidium homodimer-1 were initially subjected to normal distribution analysis using D'Agostinho & Pearson Test. Comparisons between treatments were performed by ANOVA and Tukey test. The levels of RNAs in the different treatments were evaluated by ANOVA and Kruskal-Wallis test, followed by Dunn's multiple comparison test. Results are expressed as the mean ± SEM, and differences were considered significant when $P < 0.05$.

3. Results

3.1 Effects of melatonin on follicular growth and viability

Early antral follicles cultured with 10^{-6} M and 10^{-8} M melatonin had a progressive and significant increase in their diameters throughout the culture period ($P < 0.05$). On the other hand, follicles cultured in TCM-199^{+} alone or with 10^{-7} M melatonin increased their diameters only up to day 4 of culture (**Table 2**). After 8 days of culture, no significant differences among follicular diameters in the different treatments were observed (**Table 2**). At light microscopy,

the percentage of normal follicles ranged from 98.5 to 100.0% and did not differ among treatments.

When follicular growth in different culture periods (days 0 to 4 or days 4 to 8) were compared, follicles cultured with TCM-199^{+} alone or supplemented with 10^{-7} M melatonin had reduced growth in the second half of culture period. On the other hand, those cultured in medium supplemented with 10^{-6} or 10^{-8} M melatonin had similar growth in both periods (Table 3).

Oocytes from follicles cultured with 10^{-6} or 10^{-7} M melatonin, but not 10^{-8} M melatonin, showed a significant reduction in fluorescence intensity for ethidium homodimer-1 when compared to those cultured in control medium. In addition, the presence of 10^{7} or 10^{-8} M melatonin increased oocyte fluorescence intensity for calcein-AM, when compared to those cultured in the control medium **(Figure 1E)**.

3.2. Ultrastructural analysis of OGCs from cultured early antral follicles

According to ultrastructural features, the oocytes and granulosa cells were classified as normal or as degenerated (early or advanced stage). Oocytes from follicles cultured in control medium showed early signs of degeneration, i.e., large number of vacuoles, presence of mitochondria with absence of cristae, autophagosomes and lipid droplets **(Figure 2A-B).** It was not possible to observe microvilli and perivitelline space. Furthermore, transzonal projections (TZPs) were rarely observed in the zona pellucida. The granulosa cells were morphologically normal and had irregularly shaped nuclei with no signs of nuclear membrane disruption. Mitochondria with visible and prominent cristae with a preserved Golgi complex were also observed in these cells **(Figure 2C-D)**. Additionally, the smooth endoplasmic reticulum was well developed and had concentric cisterns **(Figure 2-D).**

Oocytes from follicles cultured with the 10^{-6} M melatonin had early signs of degeneration, like low organelle density, mitochondria with absence of cristae, large number of vacuoles and swollen endoplasmic reticulum. Furthermore, a low number of TZPs was observed in the zona pellucida **(Figure 3A-B)**. The cytoplasm of granulosa cells was morphologically normal and had mitochondria with prominent cristae, as well as dilated and well-developed smooth endoplasmic reticulum **(Figure 3C-D)**. Additionally, vacuoles, and suggestive clusters of glycogen storage were found in granulosa cells. Moreover, the nucleus of these cells had few indentations and no signs of membrane rupture **(Figure 3C-D)**.

Regarding follicles cultured with 10^{-7} M melatonin, oocytes showed advanced signs of degeneration and had the presence of cellular debris, low density of organelles, large amount of vacuoles, as well as a more pronounced perivitelline space. Additionally, the TZPs were not seen in zona pellucida (**Figure 4A-B**). In granulosa cells, the nucleus showed less indentations and heterochromatin. The mitochondria had altered cristae and a rounded shape. Additionally, the endoplasmic reticulum was well developed, with the presence of concentric cisterns **(Figure 4A-D)**.

Oocytes from follicles cultured with 10^{-8} M melatonin were morphologically normal and had low number of vacuoles, mitochondria with relatively intact cristae and well-preserved Golgi complex. The endoplasmic reticulum was well developed. Furthermore, a layer of well-preserved microvilli projected in the oocyte surface was observed, as well as intact zona pellucida in close contact with oocyte membrane. A large number of TZPs was observed in zona pellucida **(Figure 5A-B).** Concerning to granulosa cells nuclei, little heterochromatin was observed without indentations. Mitochondria with visible and well-demarcated cristae were found, as well as well-developed endoplasmic reticulum and suggestive intracytoplasmic glycogen storages were seen **(Figure 5C-D).**

3.3 Mitochondrial activity and ROS levels in oocytes from cultured follicles

The fluorescence labeling intensity for mitochondrial activity **(Figure 6A-E)** and ROS levels (**Figure 7A-E**) were similar in oocytes cultured in the different treatments. Oocytes from follicles cultured in control medium alone or supplemented with melatonin had mitochondria distributed mainly in the periphery of the ooplasm.

3.4 Chromatin configuration of oocytes from cultured early antral follicles

Oocytes from early antral follicles cultured in the different treatments did not have significant differences in the percentages of oocytes at GV (TCM199^{+}: 74.2% (23/31), MEL 10^{-6} M: 64.5% (20/31), MEL 10^{-7} M: 70% (21/30), MEL 10^{-8} M: 53.3% (16/30) or GVDB (TCM199^{+}: 22.6% (7/31), MEL 10^{-6} M: 32.3% (10/31), MEL 10^{-7} M: 30% (9/30), MEL 10^{-8} M: 46.7% (14/30) stages among the treatments. Similarly, the percentages of oocytes with degenerated chromatin (TCM199^{+}: 3.2% (1/31), MEL 10^{-6} M: 3.2% (1/31), MEL 10^{-7} M: 0% (0/30), MEL 10^{-8} M: 0% (0/30) did not differ (Table 4).

3.5 Expression of mRNA for *SOD, CAT, PRDX6* and *GPX1* in OGCs from early antral follicles cultured in vitro

Regarding antioxidant enzymes relative mRNA expression, no differences were observed among treatment ($P < 0.05$) when comparing the mRNA levels for *SOD* (**Figure 8A**), *CAT* (**Figure 8B**), *PRDX6* (**Figure 8C**) and *GPX1* (**Figure 8D**) in OGCs from follicles cultured in the different treatments.

Discussion

This is the first report to demonstrate that melatonin improves viability and the ultrastructural characteristics of oocyte and granulosa cells of early antral follicles cultured in vitro. The presence of 10^{-7} or 10^{-8} M melatonin in culture medium increases calcein staining in oocytes of cultured early antral follicles. These findings suggest that the enzymatic machinery of intracellular esterases is active, and therefore, performing key catalytic functions in the reactions of ester hydrolysis, in particular, the acetylesterases that hydrolyze acetyl esters (Oesch-Bartlomowicz and Oesch, 2007). Pathak and Bansal (2019) demonstrated the relationship between non-specific esterases and viability and cell growth in preantral and antral ovarian follicles of buffaloes. Additionally, in our study, 10^{-7} M melatonin decrease the ethidium homodimer labeling in oocytes, indicating a protective effect of melatonin on cell membranes (Galano et al., 2018). When cytoplasmic membrane integrity is lost, ethidium homodimer-1 can easily enter the cell and, due to its high affinity with nucleic acids, binds to DNA, emitting red fluorescence (Martinez-Madrid et al., 2004; Maltaris et al., 2006).

In our study, 10^{-8} M melatonin helped to preserve ultrastructure of oocyte and granulosa cells organelles, as well as zona pellucida and TZPs. Mehrzadi et al. (2020) reported that melatonin modulates autophagy, endoplasmic reticulum stress and inflammation and, consequently, influences viability and growth in various cell types. In previous study, melatonin inhibited autophagy in mouse granulosa cells cultured in vitro (Shen et al., 2018). In rats, this hormone inhibits autophagy in rat ovarian granulosa cells through activation of the PI3K/Akt/mTOR pathway (Liu et al., 2022). In growing follicles, the TZPs between granulosa cells and oocytes allow the transfer of small metabolites, RNA, and signaling factors, ensuring proper oocyte development (Anderson and Albertini, 1976; Kidder et al., 2010). On the other hand, high concentrations (10^{-6} M and 10^{-7} M) of melatonin cause ultrastructural alterations (e.g., mitochondria with absence of cristae or irregular shape) indicating clear signs of

autophagy. The indicative of cellular death by autophagy is also supported by the presence of autophagosomes and overdeveloped endoplasmic reticulum. Previous report indicated an increase in the expression of endoplasmic reticulum stress-related genes after melatonin treatment, associating it with autophagy (Tasdemir et al., 2012). Various studies showed that melatonin potentially modulate autophagy through inactivation of mTOR/PI3K/AKT signaling pathway in cancer cells (Kim et al., 2014; Chen et al., 2021). Thus, our findings indicate that melatonin (10^{-8} M) attenuates autophagy and helps to maintain cellular homeostasis. Furthermore, it is worth mentioning that from day 0 to day 4 a marked growth was observed in the 10^{-7} M melatonin treatment, while in the final cultured period (4 to 8) a drastic reduction in growth was evident. It is known that this fast growing may promote an early oocyte exhaustion, known as oocyte aging, which may occur in the vitro culture environment (Liang et al., 2017), and ultimately, jeopardize the ultrastructural organelles integrity.

Despite the fact that previous reports demonstrated the potential of melatonin to reduce ROS levels (Tamura et al., 2008), in this study, this hormone did not influence the levels of ROS in cultured early antral follicles. Additionally, melatonin did not impact the mitochondrial activity in oocytes from cultured early antral follicles, with all treatments showing a heterogeneous mitochondrial distribution. Previous studies have already demonstrated that the modulation of the antioxidant response by melatonin through ROS levels, as well as mitochondrial activity, depends on its direct action, that is, by penetration into cells, which may trigger pathways directly related to oxidative stress, such as the Nrf2 pathway. /HO-1 (Kang et al., 2022) or increasing and activating sirtuin 3 (SIRT3), the enzyme responsible for modulating ROS production (Zhou et al., 2019). The absence of direct effects of melatonin on ROS levels indicated that this hormone is acting through different mechanism, via binding to melatonin membrane receptor type 1 (MT1) and type 2 (MT2). Recently, Paulino et al. (2022) showed that these receptors are expressed in bovine ovarian follicles and that melatonin binds to these receptors to promote the growth of secondary follicles. By binding to MT1 or MT2 receptors, melatonin can trigger several cellular responses, including activation of the PI3K/AKT pathway, which is related to maintenance viability and ultrastructure (Li et al., 2022). However, future studies are still need to evaluate the specific putative effect of melatonin in the activation of PI3K/AKT pathway in bovine early antral follicles.

The presence of melatonin in culture medium did not influence expression of SOD, CAT, PRDX-6 or GPXI mRNA. However, it was observed by Paulino et al. (2022) that melatonin at a concentration of 10^{-7} M increased the mRNA expression of the CAT and SOD enzymes when compared to treatment using luzindole (blocker of its MT1 and MT2 receptors)

in bovine secondary follicles cultured *in vitro*. In this case, we must consider that although we used larger follicles in our study, the evaluation was carried out using the granulosa-oocyte complex, and not the entire follicular structure. Another important fact is the culture period, in our study cultured was carried out for just eight days, while in the study in question it was carried out for 18 days. It is well known that superoxide dismutase is responsible for the dismutation of superoxide into H_2O_2, and its activity has been directly related to oocyte quality (Matos et al., 2009). Intriguingly, the improvement in the SOD activity should produce greater levels of H_2O_2, acting as a substratum to increase the relative mRNA expression for PRDX6 and GPX1, which was not observed in this study. This may be due to the fact that melatonin did not act directly by modulating oxidative stress, since did not influence the levels of ROS and mitochondrial activity.

In conclusion, the presence of 10^{-8} M melatonin in culture medium of early antral follicles improves oocyte viability and preserves the ultrastructure of organelles, zona pellucida and membranes of oocyte and granulosa cells. Future studies with in vitro culture of early antral follicles aiming to follow the follicular development during a prolonged period are necessary to allow the complete oocyte maturation in vitro.

CRediT authorship contribution statement

MSc Bianca Silva, Dr. José Silva and Dr. Francisco Aguiar conceptualized, designed and draftedthe manuscript. MSc Bianca Silva, MS Francisco Costa, MSc Venância Azevedo, MSc Laís Paulino and MSc Danisvânia Nascimento. Dr. José Silva, Dr. Mariana Donato in editing the manuscript. Dr. Ana Batista contributed in analyzing the data, editing the manuscript and providing facilities. All authors approved the final draft of the manuscript.

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List of figures

Table 1. *Primers* used for amplification of messenger RNAs.

Target gene	Primer sequence (5' → 3')	Sense (S), anti-sense (As)	GenBank accession no.	Amplicon length (bp)
GAPDH	TGTTTGTGATGGGCGTGAACCA ATGGCGCGTGGACAGTGGTCA TAA	S As	GI: 402744670	183
PRDX6	GCACCTCCTCTTACTTCCCG GATGCGGCCGATGGTAGTAT	S As	GI: 59858298	105
GPx1	AACGTAGCATCGCTCTGAGG GATGCCCAAACTGGTTGCAG	S As	GI:15660264 5	121
SOD	GTGAACAACCTCAACGTCGC GGGTTCTCCACCACCGTTAG	S As	GI: 31341527	165
CAT	AAGTTCTGCATCGCCACTCA GGGGCCCTACTGTCAGACTA	S As	GI:40269337 5	165

Table 2. Early antral follicle diameters (mean ± SEM) before and after 4 and 8 of *in vitro* culture in TCM-199^{+} alone or supplemented with melatonin (MEL).

Treatments	Day 0	Day 4	Day 8
TCM-199^{+}	532.7±6.5^{A}	605.8±8.8^{B}	629.5±8^{B}
MEL 10^{-6} M	544.4±5.2^{A}	593.5±7.4^{B}	634.7±8.4^{C}
MEL 10^{-7} M	546.8±5.2^{A}	598.6±8.0^{B}	619.4±6.9^{B}
MEL 10^{-8} M	534.6±5.1^{A}	577.0±7.5^{B}	608.1±7.9^{C}

A, B, C Differences between days of culture (columns, $P < 0.05$).

Table 3. Follicular growth (mean ± (SEM) in the different periods (days 0 to 4 or days 4 to 8) after culture in vitro TCM-199+ alone or supplemented with different concentrations of melatonin.

Tratamentos	Dia 0-4	Dia 4-8
TCM	73.1 ± 8.2^{aA}	23.7 ± 9.4^{aB}
10.6	49.1 ± 5.5^{bA}	41.1 ± 6.5^{aA}
10.7	51.8 ± 5.4^{abA}	20.7 ± 6.2^{aB}
10.8	42.4 ± 5.3^{bA}	31.1 ± 4.6^{aA}

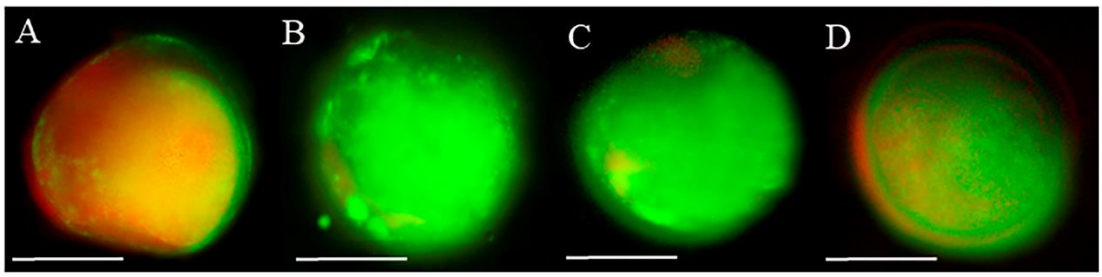
[a, b] Diferenças entre linhas,
[A, B, C] Diferenças entre colunas.

Table 4. Percentages of GV, GVBD and degenerated chromatin in oocytes from follicles cultured for 8 days in control medium alone or supplemented with different concentrations of melatonin (MEL).

Treatments	Total	GV (%)	GVBD (%)	Degenerated chromatin (%)
TCM-199+	45	71.1% (32/45)	26.7% (12/45)	2.2% (1/45)
MEL 10^{-6} M	43	65.1% (28/43)	32.3% (14/43)	2.3% (1/43)
MEL 10^{-7} M	42	71.4% (30/42)	28.6% (12/42)	0% (0/42)
MEL 10^{-8} M	43	53.5% (23/43)	46.5% (20/43)	0% (0/43)

No differences ($P < 0.05$) among treatments were observed.

Figure 1. Calcein-AM (green) and ethidium homodimer-1 (red) staining in oocytes from bovine antral follicles cultured TCM-199^{+} alone (A) or supplemented with 10^{-6} (B), 10^{-7} (C) or (D) 10^{-8} M melatonin (MEL) for 8 days. The fluorescence intensity is shown in in figure E. a,b Differences between treatments for calcein-AM. A, B differences between treatments for ethidium homodimer-1. Scale bars = 50 μm.



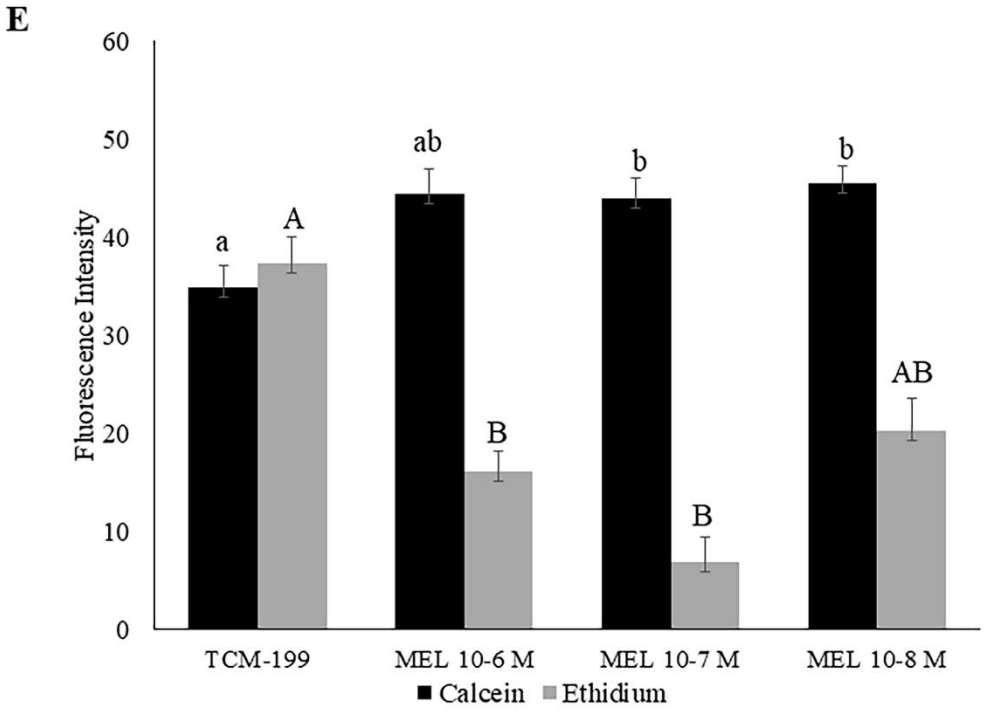


Figure 2. Electron micrograph of oocyte (A-B) and granulosa cells (C-D) from early antral follicles cultured in TCM-199^{+}. Symbols: ZP: zona pellucida; PVS: perivitelline space; Va: vacuoles; V: vesicles; M: mitochondria; GC: Golgi complex; N: Nucleus; ER: endoplasmic reticulum; L: lipid droplets. Blackarrow: autophagosomes; White arrow: degenerated mitochondria. Bars: 1 µm (A); 500 µm (B); 2µm (C); 1 µm (D).

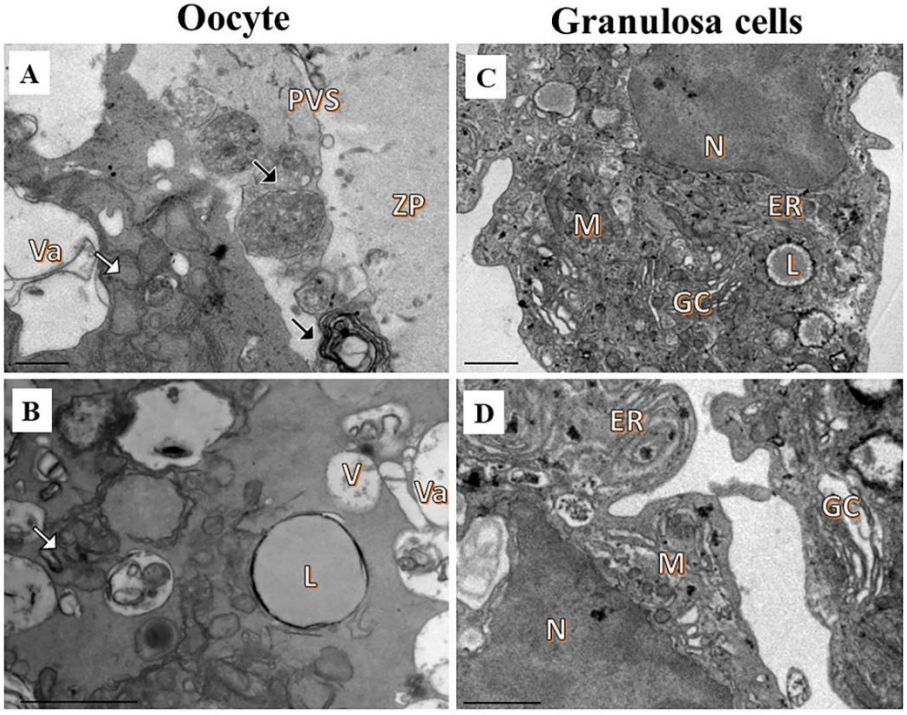


Figure 3. Electron micrograph of oocyte (A-B) and granulosa cells (C-D) from early antral follicles cultured with 10^{-6} M melatonin. Symbols: ZP: zona pellucida; Va: vacuoles; V: vesicles; ER: endoplasmic reticulum; M: mitochondria; mv: microvilli; L: lipid droplets; GC: Golgi complex; N: Nucleus. Black arrow: autophagosomes; White arrow: degenerated mitochondria; Bluearrow: glycogen storage; White circle: nuclear envelope ruptured. Bars: 1 µm (A); 500 µm (B); 1 µm (C); 500 µm (D).

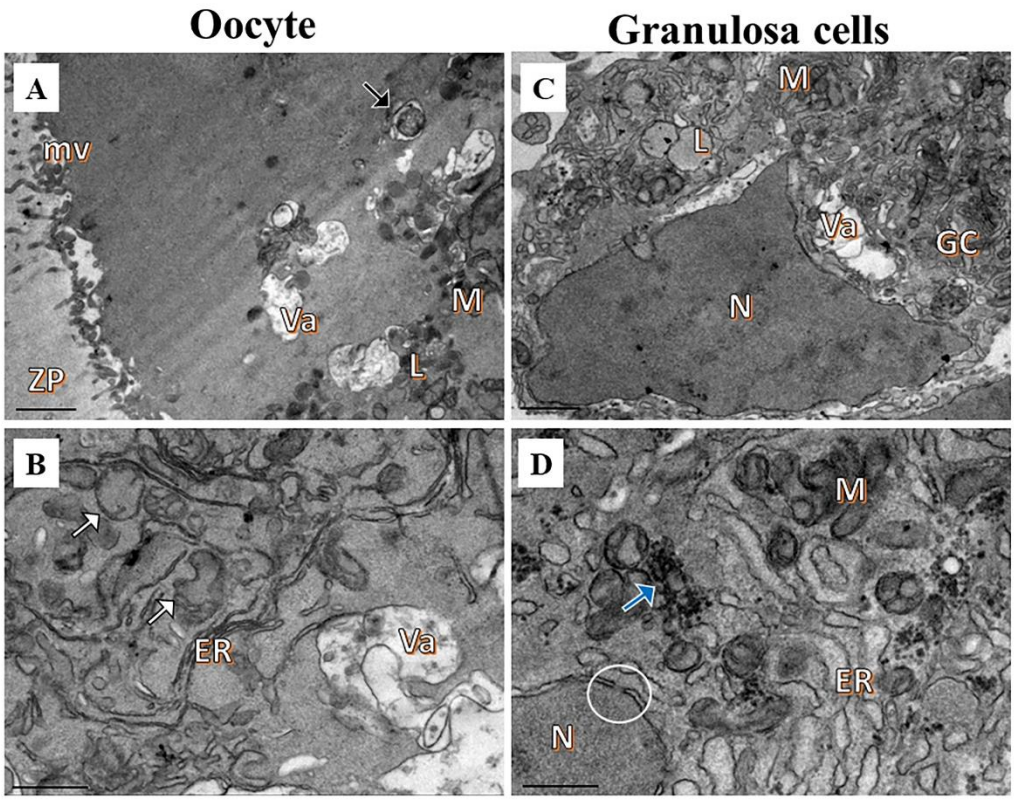


Figure 4. Electron micrograph of oocyte (A-B) and granulosa cells (C-D) from early antral follicles cultured with 10^{-7} M melatonin. Symbols: ZP: zona pellucida; PVS: perivitelline space; Va: vacuoles; V: vesicles; ER: endoplasmic reticulum; M: mitochondria; mv: microvilli; L: lipid droplets; N: Nucleus. Black arrow: autophagosomes; White arrow: degenerated mitochondria. Bars: 1 μm (A); 500 μm (B); 1 μm (C); 500 μm (D).

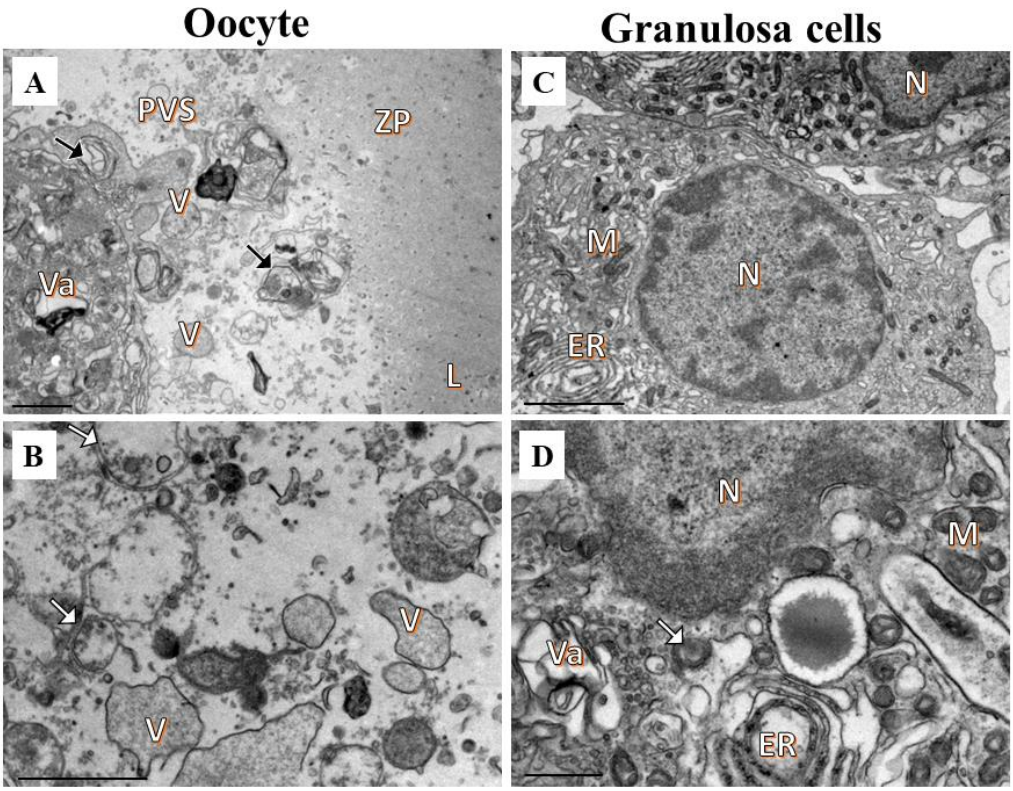


Figure 5. Electron micrograph of oocyte (A-B) and granulosa cells (C-D) from early antral follicles cultured with 10^{-8} M melatonin. Symbols: ZP: zona pellucida; Pv: perivitelline space; Va: vacuoles; R: endoplasmic reticulum; M: mitochondria; mv: microvilli; L: lipid droplets; N: Nucleus. Blue arrow: glycogen storage; Black arrowhead: transzonal projections. Bars: 1 µm (A); 500 µm (B); 1 µm (C); 500 µm (D).

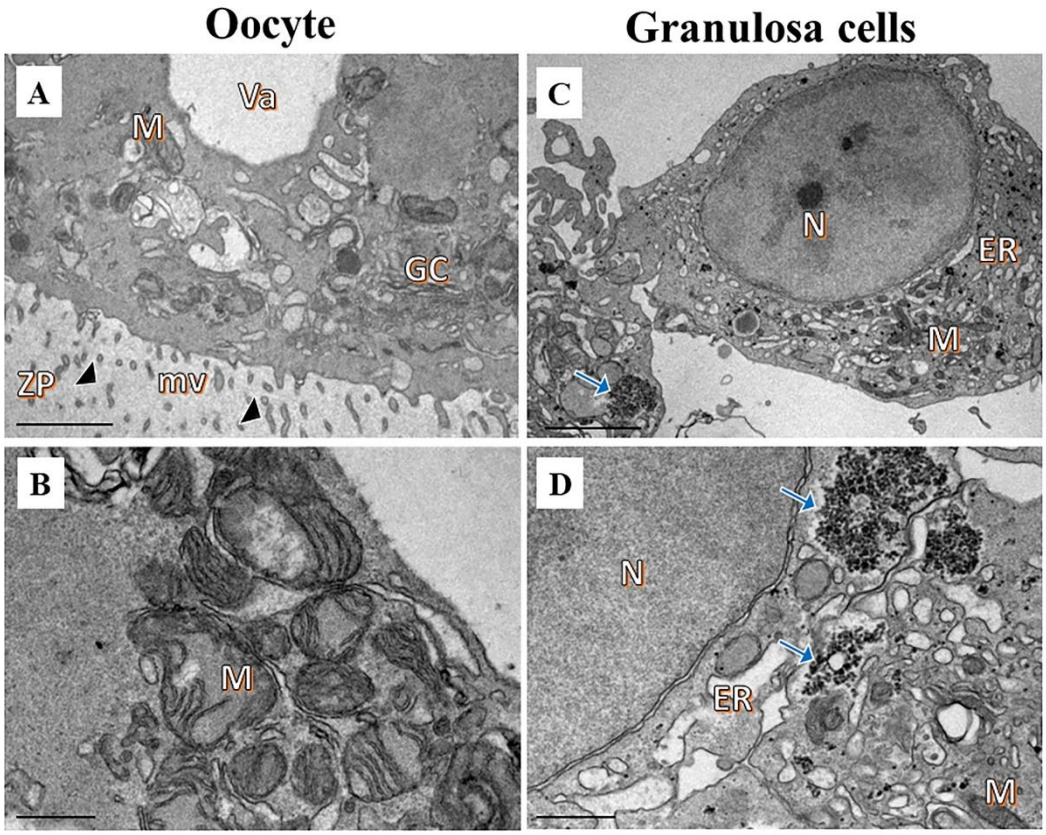


Figure 6. MitoTracker Red staining in oocytes from follicles cultured *in vitro* for 8 days in (A) TCM-199+ control medium or supplemented with(B) 10^{-6} M, (C) 10^{-7} M or (D)10^{-8} M of melatonin (MEL). (E) Oocyte fluorescence intensities.No differences (P > 0.05) where found among treatments. Scale bars = 50 µm.

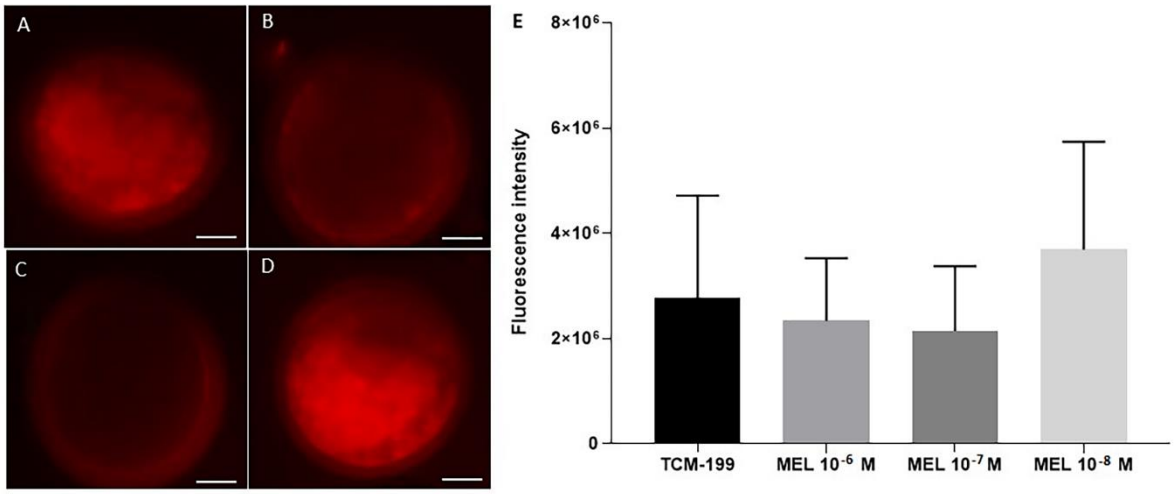


Figure 7. Mean fluorescence staining intensity for ROS after in vitro follicular culture for 8 daysin (A) TCM-199+ control medium or supplemented with (B) 10^{-6} M, (C) 10^{-7} M or (D)10^{-8} M of melatonin (MEL). (E) Oocyte fluorescence intensities. No differences (P > 0.05) where found among treatments. Scale bars = 50 µm.

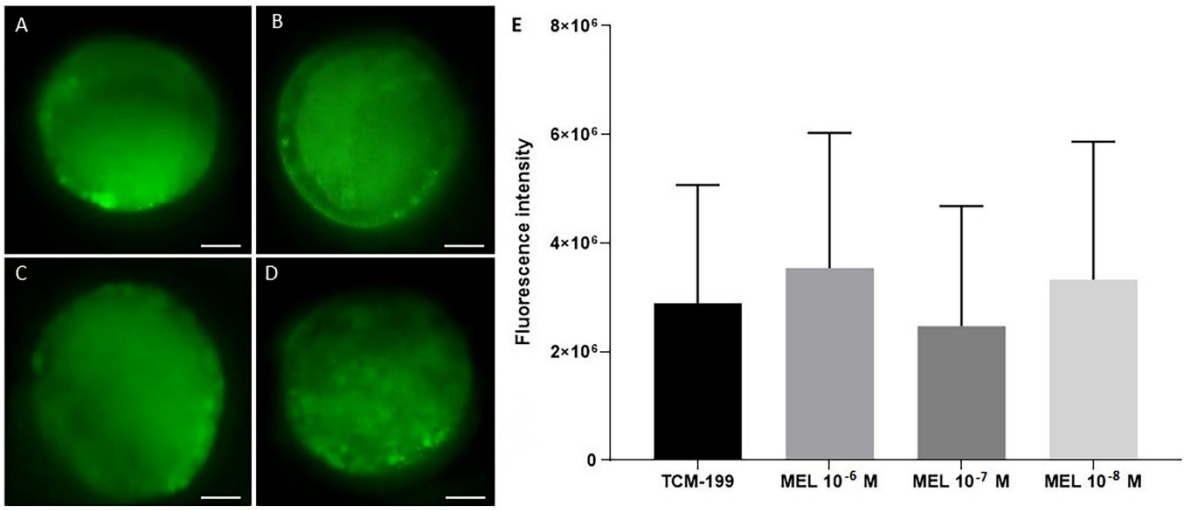
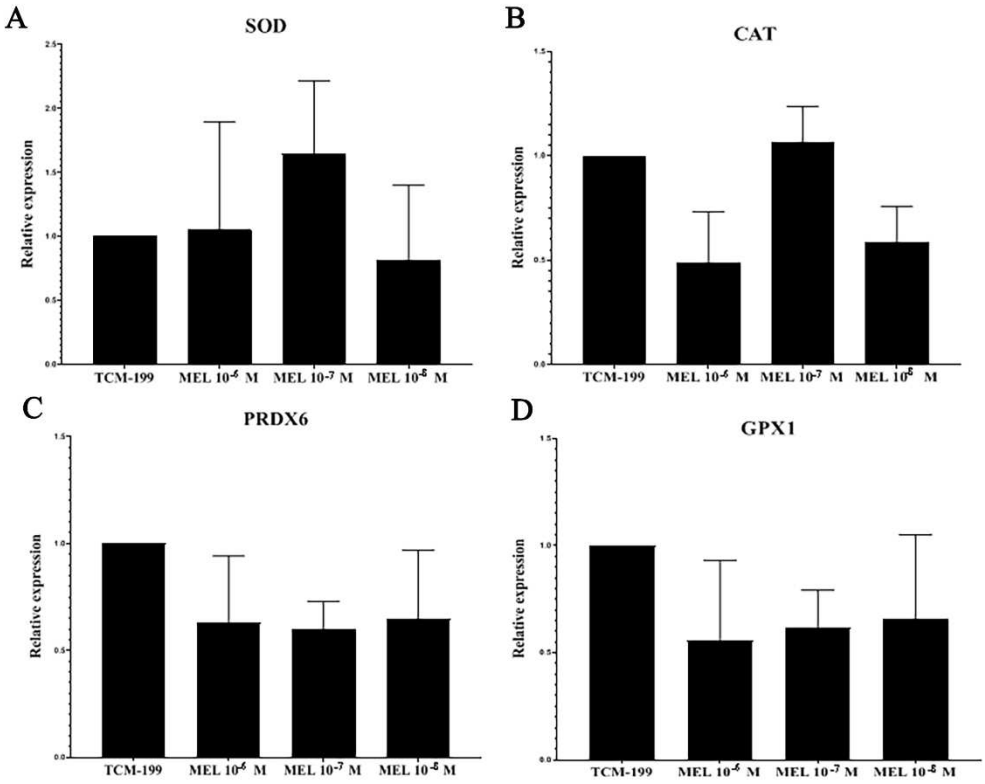


Figure 8. Relative mRNA levels for (A) SOD, (B) CAT, (C) PRDX6 and (D) GPX1 in oocytes from follicles cultured for 8 days in TCM199^{+} alone or supplemented with different concentrations (10^{-6} M, 10^{-7} M, and 10^{-8} M) of melatonin (MEL).



8 CAPÍTULO IV

Effects of cyclic adenosine monophosphate modulating agents during oocyte pre-maturation and the role of melatonin on in vitro maturation of bovine cumulus-oocyte complexes

[Efeitos de agentes moduladores de adenosina monofosfato cíclico durante a pré-maturação oocitária e o papel da melatonina na maturação *in vitro* de complexos cumulus-oócitos bovinos]

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Effects of cyclic adenosine monophosphate modulating agents during oocyte pre-maturation and the role of melatonin on in vitro maturation of bovine cumulus-oocyte complexes

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Abstract

This study investigated the effects of cyclic adenosine monophosphate modulating during cumulus-oocyte complexes (COCs) pre-maturation and the role of melatonin on in vitro maturation (IVM) of bovine COCs. In experiment one, COCs were pre-matured for 8 h in control medium or with 3-isobutyl-1-methylxanthine (IBMX) and forskolin, IBMX and C-type natriuretic peptide, c-type natriuretic peptide and forskolin or IBMX, forskolin and c-type natriuretic peptide. Then, meiotic progression was evaluated. In experiment two, COCs were pre-matured, followed by IVM in control medium alone or with 10^{-6}, 10^{-7} or 10^{-8} M melatonin. After IVM, chromatin configuration, transzonal projections (TZPs), reactive oxygen species, mitochondrial distribution, ultrastructure and mRNA expression for antioxidant enzymes were evaluated. In experiment 1, COCs pre-matured with both and forskolin or C-type natriuretic peptide, forskolin and IBMX had lower meiotic resumption rate when compared to control. Considering that IBMX had not an additional effect to potentiate inhibition of meiotic resumption, a combination of C-type natriuretic peptide and forskolin was chosen. In

experiment 2, COCs matured with 10^{-8} M melatonin had greater rates of meiotic resumption when compared to the other treatments (P<0.05). The COCs matured with 10^{-7} or 10^{-8} M melatonin had greater mitochondrial activity (P<0.05), while those matured with 10^{-6} or 10^{-8} M of melatonin had greater levels of TZPs. Ultrastructure of oocyte and cumulus cells after IVM with melatonin was relatively well preserved. COCs matured with 10^{-8} M melatonin increased mRNA expression for superoxide dismutase (SOD) and catalase (CAT) (P<0.05), when compared to non-cultured and pre-matured COCs, respectively. In conclusion, bovine COC pre-maturation with C-type natriuretic peptide and forskolin, followed by IVM with 10^{-8} M melatonin improves meiotic resumption rates, TZPs, mitochondrial distribution and mRNA expression for SOD and CAT.

Keywords: meiotic resumption, antioxidant, *in vitro* pre-maturation, melatonin and cAMP

1. Introduction

The asynchrony between nuclear and cytoplasmic maturation during oocyte *in vitro* maturation (IVM) is one factor that limits *in vitro* production of blastocyst in bovine species (Thomas et al., 2004; Botigelli et al., 2017). This asynchrony may be partially explained by triggering meiotic maturation resumption after isolation of oocytes from follicles. Although oocytes from medium antral follicles (3 - 6 mm) undergo meiotic resumption *in vitro* (Lima et al., 2018) a premature extrusion of the first polar body before an appropriate cytoplasmic maturation can occur (Rizos et al., 2002; Sutton et al., 2003). Thus, modulatory pathways investigation aiming to favor a suitable synchronicity of oocyte maturation is desirable. Cyclic adenosine monophosphate(cAMP) is a critical signaling molecule controls resumption of meiosis. The cAMP high concentrations in the ooplasm maintain the oocyte at germinal vesicle stage, allowing cytoplasmic competence acquisition. In this context, pharmacological modulation of cAMP during pre-maturation can inhibit or delay meiotic resumption (Albuz et al., 2010; Oliveira et al., 2010; Liang et al., 2017; Sugimura et al., 2018). The control of cAMP levels may occur through the inhibition of phosphodiesterase 3 enzyme (PDE3) due to the effect of C-type natriuretic peptide, which acts inducing the cyclic guanosine monophosphate (cGMP) production in the granulosa cells (Zhang et al., 2010). Subsequently, cGMP is transferred through transzonal projection (TZPs) to the oocyte, inhibiting PDE 3, sustaining high levels of cAMP, and maintaining oocyte meiotic arresting (Zhang et al., 2010; Thomas et al., 2004; Luciano et al., 2011; Li et al., 2016). The 3-isobutyl-1-methylxanthine (IBMX) is another PDE inhibitor that stopped cAMP degradation, while activation of adenylate cyclase by forskolin increased the levels of cAMP (Albuz et al., 2010; Zhang et al., 2010).

Another limiting factor during oocyte IVM is the increase in production of reactive oxygen species (ROS) (Ambrogi et al., 2017). Under normal conditions, cells produce suitable levels of ROS, but a disbalance of ROS levels can oxidize cellular molecules and produce lipid peroxidation, mitochondrial damage, and apoptosis (Ambrogi et al., 2017). As a consequence, it is very important to evaluate ultrastructure of organelles and membranes after oocyte IVM in presence of antioxidant substances. As an antioxidant, melatonin protected the cells against oxidative stress by modulating mitochondria-related functions, strengthening their antioxidant defense system by scavenging free radicals (Manchester et al., 2015). Additionally, melatonin regulated mRNA levels of critical antioxidant enzymes, such as superoxide dismutase (SOD) and glutathione peroxidase (GPX) (Wang et al., 2014; Li et al., 2015; El Sheikh et al., 2019). Previous reports have demonstrated that melatonin increased the maturation rates in cattle oocytes (El-Raey et al., 2011; Magalhães et al., 2021) and improved the nuclear and cytoplasmic maturation of porcine and ovine oocytes (Yang et al., 2020; Barros et al., 2020). However, to the best of our knowledge, no study investigated the effects of melatonin on oocyte ultrastructure and on expression of antioxidant enzymes in oocytes from medium-sized antral follicle that had been previously pre-matured *in vitro*. We hypothesized that a combination of forskolin, IBMX and C-type natriuretic peptide modulates oocyte meiotic resumption and favors cytoplasmic maturation and that IVM of pre-matured oocytes in presence of melatonin controls oxidative stress and helps to preserve oocyte integrity and TZPs.

This study aimed to investigate the effects of different combinations of cAMP modulating agents during pre-maturation of cumulus-oocyte complexes (COCs) from medium-sized antral follicles, as well as the influence of different concentrations of melatonin on oocyte meiotic resumption, integrity of organelles, membranes and transzonal projections, mitochondrial distribution, expression of antioxidant enzymes, and on ROS levels during bovine oocyte IVM.

2. Material and methods

2.1 Chemicals and collection of bovine COCs

Unless otherwise indicated, the chemicals used in the present study were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The COCs were obtained from 88 ovaries from mixed breed cows (n=44) in a local slaughterhouse in Sobral, Ceará, Brazil (mean temperature: 27.6ºC, average annual pluviosity:796 mm) between the months of January and

March, 2021. After collection, the ovaries were transported to the laboratory in 0.9% NaCl with 100 IU/mL penicillin and 50 mg/mL streptomycin, at 33 °C within 1 h. Then, COCs were aspirated from medium-sized follicles (3–6 mm in diameter) using a 10 mL disposable syringe connected to an 18 Gauge needle. The COCs with compact cumulus cells and homogeneous oocyte cytoplasm were selected for *in vitro* pre-maturation. For the uncultured control, five groups of 20 COCs were collected and stored at -80 °C until total RNA extraction. This study was approved and carried out in accordance with the rules and guidelines of the Ethics and Animal Welfare Committee of the Federal University of Ceará (number 04/22).

2.2 Experiment 1: Effects of cAMP modulating agents on *in vitro* pre-maturation bovine oocytes

To examine the effects of cAMP modulating agents on meiotic arrest, bovine COCs were collected from bovine ovaries (n=36) and pre-matured in TCM-199 containing Earle's salts and L-glutamine (Sigma) supplemented with 0.2 mM pyruvic acid, 5.0 mg/ml LH (Lutropin®-V, Bioniche, Belleville, ON, Canada), 0.5 mg/ml FSH (Folltropin®-V, Bioniche, Belleville, Canada), 0.4% BSA, and 100 IU/ml penicillin and 50 µg/ml streptomycin sulfate. For the treatments, the COCs were cultured in TCM-199^{+} alone or supplemented with both 500 mM IBMX and 100 µM FORSKOLIN, both 500 mM IBMX and 100 nM C-type natriuretic peptide, both 100 nM CNP and 100 µM forskolin or a combination of 500 mM IBMX, 100 µM forskolin and 100 nM C-type natriuretic peptide. The concentrations of these substances were chosen according to previous studies (Soto-Heras et al., 2019; Razza et al., 2019). In each treatment, a number of 105 to 118 COCs were cultured in 4-well plates in an incubator, totaling 543 oocytes analyzed (Forma™ 310, Thermo Scientific - Massachusetts, USA) (Forma™ 310, Thermo Scientific - Massachussetts, USA) for 8 h at 38.5°C with 5% CO_2 in air. After the pre-maturation period, oocyte meiotic progression was evaluated. For that, the COCs were transferred to tubes containing 70 µL of PBS and vortexed for 3 minutes. Subsequently, the denuded oocytes were incubated in 4% paraformaldehyde for 15 minutes and then transferred to 0.1% Triton X-100. The oocytes were then incubated with 10 µg/mL of Hoechst 33342 and analyzed in an inverted epifluorescence microscope (Nikon, TS100). Oocytes were classified according to the nuclear maturation stage as germinal vesicle (GV) when they had uncondensed chromatin. Resumption of meiosis in oocytes was characterized by nuclear chromatin condensed and germinal vesicle breakdown (GVBD) (Bezerra et al., 2016). Oocytes with abnormal chromatin configuration was considered degenerated when nuclear pyknosis or

condensed chromatin were observed (Lin et al., 2016). Only oocytes with normal chromatin were considered to calculate the percentages of oocytes in GV or resumption of meiosis. This experiment was repeated five times and a total of 543 oocytes were analyzed.

2.3 Experiment 2: Effects of melatonin on oocyte chromatin configuration, mitochondrial distribution, and on levels of ROS and antioxidant enzymes

2.3.1 Assessment of meiotic progression in matured oocytes

Before maturation, the COCs obtained from 164 bovine ovaries (n=82) were pre-matured in TCM-199^{+} containing 100 µM FORSKOLIN and 100 nM C-type natriuretic peptide (best combination defined in experiment 1). The COCs were cultured in 4-well plates for 8 hours at 38.5°C, with 5% CO_2 in air. Then, they were randomly matured in TCM 199^{+} alone or supplemented with 10^{-6}, 10^{-7}, or 10^{-8} M melatonin. Melatonin concentrations were chosen according to results of previous studies (Wang et al., 2014; Rodrigues-Cunha et al., 2016). For IVM, in each treatment, a number of 148 to 174 oocytes were cultured in 4-well culture dish (Nunc, Roskilde, Denmark) containing 300 µL of maturation medium for 22 h. The IVM medium was TCM-199 containing Earle's salts and L-glutamine (Sigma) supplemented with 0.2 mM pyruvic acid, 5.0 mg/ml LH (Lutropin®-V, Bioniche, Belleville, ON, Canada), 0.5 mg/ml FSH (Folltropin®-V, Bioniche, Belleville, Canada), 0.4% BSA, and 100 IU/ml penicillin and 50 µg/ml streptomycin sulfate. Oocyte maturation was carried out at 38.5 °C in a humidified atmosphere of 5% CO_2 in air. After maturation of COCs, chromatin configuration was evaluated. Oocytes having nuclear membrane were considered at GV stage, while those without nuclear membrane, i.e., that were at GVBD, metaphase I or metaphase II stages, were considered to have resumed meiosis (Bezerra et al., 2016). In addition, mitochondrial distribution, ROS levels, chromatin configuration, and the levels of mRNA for CAT, SOD, PRDX6 and GPX1 were analyzed. For each treatment, 10 COCs were fixed for ultrastructural analysis of organelles and membranes. This experiment was repeated six times and a total of 660 oocytes were evaluated.

2.3.2 Assessment of mitochondrial distribution in matured oocytes

After maturation of COCs in the different treatments, oocytes were incubated in PBS with 100 nM Mitotracker Red (Mitotracker® Red, CMXRos, Molecular Probes, Melbourne, Victoria,

Australia) at 37°C for 20 minutes. Subsequently, the oocytes were washed in PBS and evaluated with the aid of an epifluorescence microscope (TS100; Nikon Corp.). Considering that the fluorescent dye accumulates only in active mitochondria, mitochondrial distribution was assessed based on the cytoplasmic location of red fluorescence. Mitochondrial distribution patterns were classified as homogeneous, with staining spread throughout the cytoplasm, or heterogeneous, with peripherical and central aggregations of mitochondria. The fluorescence intensity was measured by using the Image J software (version 1.46; National Institutes of Health, Bethesda, MD). The corrected total fluorescence was determined by subtracting out background signal. A total of 48 oocytes were evaluated, i.e., 12 oocytes per treatment.

2.3.3 Levels of ROS in matured oocytes

Denuded oocytes were washed in 0.1% polyvinyl alcohol in phosphate buffered saline (PBS-PVA) and incubated with 10 mM 6-carboxy-2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA, Molecular Probes®, Eugene, OR) at 38.5°C in the dark for 30 min. Then, the oocytes were washed with PBS-PVA and placed on glass slides with ProLong® Gold (Molecular Probes, Eugene, OR). The slides were evaluated using an epifluorescence microscope (Nikon, TS100) under the 460 nm wavelength. The fluorescence intensities were analyzed individually using the Image J software (version 1.46; National Institutes of Health, Bethesda, MD). The background signal intensity was subtracted from the measured values of the treatment micrographs. Oocytes cultured in TCM-199 without melatonin were used as control. The fluorescence intensity was considered directly proportional to ROS concentration. A total of 48 oocytes were evaluated.

2.3.4 Analysis of transzonal projections (TZPs) in matured COCs

After IVM, the oocytes were fixed in 4% paraformaldehyde for up to 1 hour at room temperature. After this period, they were transferred to a fixation solution, composed of PBS plus 0.1% BSA and 0.1% Tween20 for 8-12 hours at 4°C. Then, the COCs were transferred to a blocking solution composed of PBS plus 0.5% BSA, 0.2% sodium azide, 1% milk powder, 10% goat whey, 1% of donkey serum, 0.1 M glycine and 0.1% Triton X for 1h under agitation and protected from light. Subsequently, the oocytes were incubated for 2h at room temperature with Alexa 488 Phalloidin antibody (Invitrogen, cat# T6199 – 1:50 dilution) also under shaking and protected from light. After the incubation period, the oocytes were submitted to three

washings in blocking solution, for 5 minutes each, under agitation and protected from light. Finally, oocytes were placed in 60-well slides (µ-Slide Angiogenesis IbiTreat, Ibidi GmbH – Germany) containing 10 µL of mounting medium (50% glycerol and 50% PBS) with DAPI (ABCAM - 104139) and examined by confocal laser scanning microscopy (Zeiss LSM 700META, Weimer, Germany). Fluorescence intensity was quantified and analyzed by image J software. The background signal intensity was subtracted from the measured values of the treatment micrographs. Oocytes cultured in TCM-199 without melatonin were used as control. The region corresponding to zona pellucida was manually delimited and the mean fluorescence intensity in this area was measured. A total of 20 oocytes were evaluated, i.e., five oocytes per treatment.

2.3.5 Ultrastructural analysis of COCs

The COCs were fixed in Karnovsky solution (2% paraformaldehyde and 2% glutaraldehyde in 0.1M sodium cacodylate, buffer pH 7.2), and postfixed in 1% osmium tetroxide, 0.8% potassium ferricyanide and 5mM calcium chloride in 0.1M sodium cacodylate buffer for 1 h. After washing in sodium cacodylate buffer, the COCs were counterstained with 5% uranyl acetate. The samples were then dehydrated in acetone and embedded in epoxy resin (Epoxy-Embedding Kit, FlukaChemika- BioChemika). Semi-thin sections (2 µm) were cut, stained with toluidine blue and analyzed by light microscopy at a 400× magnification. Subsequently, ultra-thin sections (70 nm) were obtained and counterstained with uranyl acetate and lead citrate, and examined under a transmission electron microscope (Fei Tecnai Spirit, North American Sales, USA). A total of 20 oocytes were evaluated, i.e., five oocytes per treatment.

2.3.6 Quantification of messenger RNAs in COCs

The levels of mRNA for SOD, CAT, GPX and PRDX-6 were evaluated in COCs pre-matured in TCM-199^{+} containing forskolin and C-type natriuretic peptide, as well as in COCs that had been pre-matured followed by maturation in control medium alone or supplemented with 10^{-8} M melatonin. For the total RNA isolation, Trizol reagent was used according to the manufacturer's instructions (Invitrogen, São Paulo, Brazil). Initially, 1 mL of Trizol was added to each sample, and the lysate was then aspirated through 20G needles before being centrifuged at 10,000 g for 3 minutes. Then, the lysates were diluted 1:1 in 70% ethanol and placed in a

mini-column (Invitrogen). After binding the RNA to the column, DNA digestion was performed using RNAse-free DNAse (340k units/mL, Invitrogen) for 15 minutes. After washing the columns three times, the RNA was collected with 30 µl of ultra-pure water. The total RNA concentration was assessed by a spectrophotometer (Amersham, Biosciences Cambridge, England) and 1 µg of total RNA was used for reverse transcription. Before the reverse transcription reaction, the RNA samples were incubated for 5 minutes at 70°C, and then cooled in ice. The reverse transcription was performed in a total volume of 20 µl, composed of 10 µl of sample RNA, 4 µl of reverse transcriptase buffer (Invitrogen), 8 RNAsin units (Invitrogen), 150 units of Superscript reverse transcriptase (Invitrogen), 0.036 U random primers (Invitrogen), 10 mM DTT and 0.5 M of each dNTP (Invitrogen). The mixture was incubated at 42°C for 1 hour, then at 80°C for 5 min, and then stored at -20°C. The negative control was prepared under the same conditions, but without the addition of reverse transcriptase. The messenger RNAs quantification was performed using SYBR Green. 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. Real-time PCR was performed on a Step One Plus instrument (Applied Biosystems, Foster City, CA, USA). The primers (Table 1) were designed to amplify specifically messenger RNAs for SOD, CAT, GPX and PRDX-6. Ubiquitin, phosphoglycerokinase (PGK) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes were used to normalize mRNA levels. According to van Tol et al. (2008), these three housekeeping genes are the most stable in bovine oocytes and cumulus cells matured in vitro. The specificity of each primer pair was confirmed by analyzing the melting curve of PCR products. The thermal cycling profile for the first round of PCR was initially denaturation and activation of the polymerase for 10 min at 95°C, followed by 40 cycles of 15s at 95°C, 30s at 58°C, and 30s at 72°C. The final extension was performed at 72°C for 10 min. All reactions were performed in a Step One Plus instrument (Applied Biosystems, Foster City, CA, USA). The 2^-ΔΔCt method was used to transform the Ct values into normalized relative expression levels. This analysis was repeated four times and 100 COCs were analyzed per treatment.

2.4 Statistical analysis

The statistical analyses were performed in Graph Pad Prism 8.4. Data of mRNA expression, staining intensity of TZPs, active mitochondria and ROS levels were analyzed by Shapiro–Wilk and Kolmogorov Smirnov tests to verify normal distribution and homogeneity

of variance. The data of maturation rates in different treatments were compared using Fisher's exact test. Data of TZP and levels of active mitochondria and ROS staining intensity was evaluated by ANOVA and Tukey test. The levels of RNAs before and after pre-maturation in different treatments were evaluated by ANOVA and Kruskal-Wallis test, followed by Dunn's multiple comparison test. Results are expressed as the mean ± SEM, and differences were considered significant when $P < 0.05$.

3. Results

3.1 Experiment 1: Effects of cAMP modulating agents on *in vitro* pre-maturation bovine oocytes

Greater percentages of oocytes at GV stage were observed in COCs pre-matured with both C-type natriuretic peptide and forskolin or a combination of IBMX, C-type natriuretic peptide and forskolin when compared to those matured in other treatments ($P < 0.05$). The only exception was for COCs cultured with IBMX and forskolin or a combination of IBMX, C-type natriuretic peptide and forskolin that had similar levels of oocytes at GV stage. Therefore, we selected the treatment with both C-type natriuretic peptide and forskolin for experiment 2. Similar results among treatments were observed for GVDB assessment (**Table 2**).

3.2 Experiment 2: Effects of melatonin on oocyte chromatin configuration, mitochondrial distribution, and levels of ROS and antioxidant enzymes

3.2.1 Effects of melatonin on meiotic progression in matured oocytes

The results show that COCs matured with 10^{-8} M melatonin had significantly reduced percentages of GV oocytes ($P < 0.05$) when compared with those cultured in TCM-199^{+}control medium alone or supplemented with other concentrations of melatonin. Furthermore, COCs matured with 10^{-8} M melatonin had greater ($P < 0.05$) percentage of meiotic resumption when compared with the other treatments (**Table 3**). On the other hand, melatonin did not influence the percentages of oocyte with degenerated chromatin.

3.2.2 Mitochondrial distribution and intracellular ROS levels in matured oocytes.

Characteristics of mitochondrial distribution after oocyte IVM are shown in Figure 1. Oocytes matured in control medium alone or with 10^{-6} M of melatonin showed heterogeneous mitochondrial distribution (**Figure 1A-B)**, while those matured with 10^{-7} M or 10^{-8} M melatonin had homogeneous distribution (**Figure 1C-D**). Furthermore, oocytes matured with 10^{-7} or 10^{-8} M melatonin showed greater fluorescence than those matured in control medium (**Figure 1E**).

The intracellular ROS levels in oocytes matured with 10^{-6}, 10^{-7} or 10^{-8}M melatonin did not show a significant difference when compared to those matured in control medium. However, oocytes matured with 10^{-7}M melatonin had greater levels of ROS than those cultured with 10^{-6} M melatonin, while oocytes matured with 10^{-8}M melatonin had similar levels of fluorescence compared with both melatonin treatments (**Figure 2A-E**).

3.2.3 Analysis of transzonal projections (TZPs)

The results show increased levels of fluorescence for TZPs in zona pellucida of oocytes matured with 10^{-6} M or 10^{-8} M melatonin when compared to those cultured in control medium. However, TZPs staining in oocytes matured with 10^{-7} M melatonin did not differ when compared to the other treatments ($P > 0.05$) (**Figure 3A-E**).

3.2.4 Ultrastructural analysis of COCs

Non-cultured oocytes exhibited well-preserved mitochondria, endoplasmic reticulum and Golgi complex, as well as cortical granules, electron-dense lipid droplets and large number of vesicles. A continuous layer of microvilli projected from the oolemma surface into the perivitelline space was observed (**Figure 4A-B**). The cumulus cells were attached to zona pellucida (**Figure 4A**) and had irregularly-shaped nuclei and cytoplasm with mitochondria and well-developed endoplasmic reticulum (**Figure 4-C**). The ultrastructural characteristics of pre-matured COCs (**Figure 5A-C**) and oocytes matured in control medium (**Figure 6A-C**) were similar to those seen in uncultured COCs; however, the oocytes in the matured control medium (**Figure 6-A)** had a small number of vesicles and the presence of clustering of cortical granules at the periphery of the oocyte.

Overall, slightly changes in the ultrastructure of oocyte and cumulus cells were observed after maturation in presence of melatonin. Notably, melatonin at a concentration of 10^{-6} M showed similar oocyte ultrastructural characteristics when compared to the oocytes matured in

control medium without melatonin (**Figure 7A-B**); however, we observed changes in the pattern of mitochondria, with a slight degree of dilatation (**Figure 7-C**), as well the presence of isolated cortical granules (**Figure 7-B)**. It was also verified that the cumulus cells were not well-adhered to the zona pellucida (**Figure 7A)**. In cumulus cells (**Figure 7-C**), slight degree of dilatation in mitochondria, a well-developed and dilated rough endoplasmic reticulum were observed. Concerning the oocytes cultured with 10^{-7} M of melatonin, similar oocyte and cumulus cells ultrastructural characteristics were observed when compared to those of oocytes matured with 10^{-6} melatonin (**Figure 8A-C**), except by the presence of clustering of cortical granules at the periphery of the oocyte. Besides, oocytes matured in the presence of 10^{-8} M of melatonin presented smaller amount of vacuolization and a layer of microvilli projected on the surface of the oolemma. Cortical granules with a solitary distribution were observed in the periphery of the oolemma (**Figure 9A-B**). Granulosa cells had indented nucleus, preserved mitochondria with visible and well-demarcated ridges, and well-developed endoplasmic reticulum (**Figure 9-C**). Finally, a reduction in the perivitelline space was similarly observed between the oocyte and zona pellucida in the *in vitro* matured oocytes (**Figure 9A-B**).

3.2.5 Expression of mRNA for SOD, CAT, PRDX6 and GPX1 in pre-matured COCs and after *in vitro* maturation

The COCs matured with 10^{-8} M melatonin showed a significant increase in the levels of mRNA for SOD after 22 hours of maturation, when compared to non-cultured or pre-matured COCs, as well as with those matured in control medium (**Figure 10A**). The mRNA levels for CAT were significantly greater in COCs matured in the presence of melatonin when compared to pre-matured COCs or those matured in control medium (**Figure 10B**). The levels of transcripts for PRDX6 were significantly greater in pre-matured COCs when compared to non-cultured COCs or COCs matured with melatonin (**Figure 10C**). Furthermore, levels of transcripts for GPX1 were significantly greater in pre-matured COCs when compared to non-cultured COCs (**Figure 10D**).

4. Discussion

This study shows that the presence of C-type natriuretic peptide and forskolin or a combination of C-type natriuretic peptide, forskolin and IBMX in pre-maturation medium of COCs from medium antral follicles increased the percentages of oocytes at GV stage after

culture. Previous studies have demonstrated that FORSKOLIN stimulates activity of adenylate cyclase and increases the levels of cAMP, while IBMX reduces cAMP degradation (Albuz et al., 2010; Li et al., 2016; Van Tol et al., 2008). The C-type natriuretic peptide induces the production of cGMP in the granulosa cells (Zhang et al., 2010; Wang et al., 2014) that, after transportation to the oocyte, inhibits PDE 3 and increases the levels of cAMP, which maintains oocyte meiotic arrest (Thomas et al., 2004; Li et al., 2016). Knowing that oocytes from these medium follicles can have a premature extrusion of the first polar body before an appropriate cytoplasmic maturation (Conti et al., 1998; Rizos et al., 2002), the development of protocols to modulate meiotic resumption followed by IVM in the presence of melatonin can innovatively optimize the current IVM rates of bovine oocytes. As a consequence, follow up studies evaluating blastocyst production from IVM oocytes using melatonin are warranted.

Noteworthy, after 22 h of IVM, 10^{-8} M melatonin increased oocyte meiotic resumption rates. Such results are consistent with a previous report by El-Sheikh et al. (2019) that used similar concentrations (10^{-8} or 10^{-9} M) of melatonin during IVM, and reported greater rates of embryonic development in cattle. Moreover, Tian et al. (2014) demonstrated that 10^{-9} M melatonin improved bovine oocyte maturation rates, whereas 10^{-3} M of melatonin delays oocyte maturation. A potential explanation for a better efficiency of low concentrations of melatonin on the resumption of meiosis is the fact that after prolonged exposure period (about 8 h), melatonin physiological concentrations (30-400 pM) induce activation and desensitization of its receptors (mainly MT1 and MT2) (Nikolaev et al., 2021). In contrast, supraphysiological melatonin concentrations (~1000 nM) overcome the effective ligand capacity for melatonin receptors (Reppert et al., 1996). The improved effects in meiotic resumption rates may be due to the ability of melatonin to inhibit adenylate cyclase activity through binding to its G-protein coupled-receptors (Chang et al., 2016; Suofu et al., 2017). After binding, adenylate cyclase is initially inhibited by inhibitory G protein, leading to a decrease in cAMP (Chan et al., 2002), and ultimately, enhancing meiotic resumption (Li et al., 2016). Another consequence of the signaling cascade activated via melatonin was described by Peetit et al. (1999) and by Tian et al. (2014), which showed that MT2 activation inhibits cGMP production. Previous report had been demonstrated that the reduction in cGMP levels is also critical in meiotic resumption rates by increasing oocyte PDE3A activity and reducing cAMP levels that are responsible for the meiotic resumption (Norris et al., 2009).

In our findings, the presence of 10^{-7} or 10^{-8} M melatonin in culture medium increased the levels of mitochondrial activity, indicating that melatonin was able to support mitochondrial function for oocyte meiotic resumption. Indeed, melatonin improved mitochondrial membrane

function during oocyte maturation in several studies (Lan et al., 2020; Zou et al., 2020; Jin et al., 2020). Moreover, it is reported that mitochondria distribution pattern in the ooplasm is a dynamic process (Yamochi et al., 2016), and it is an important indicator of oocyte quality. Oocytes cultured with 10^{-7} or 10^{-8} M melatonin had a homogeneous mitochondrial distribution profile in our study. The beneficial role of a homogeneous mitochondrial distribution during oocyte maturation has been reported (Nagai et al. 2006; Brevini et al., 2007). Potentially, an increase in mitochondrial activity may be related to an increase in ROS production (Turrens, 1997; Finkel et al., 2000). However, in our study, melatonin did not influence the levels of ROS, but it enhanced the levels of mitochondrial staining. Probably, other enzymes, such as sirtuin-3 and hemi oxygenase 1 are modulating ROS levels (Kang et al., 2022).

Our findings demonstrated that 10^{-6} M and 10^{-8} M melatonin increase TZP staining after maturation, which is similar to previous reports of Zhang et al. (2021). The TZPs are vital for cumulus cells-oocyte communication, which favors exchange of critical molecules, pivotal metabolic interactions, and ultimately, regulation of meiosis (Anderson and Albertini, 1976; Kidder and Vanderhyden., 2010). It is important to highlight that pyruvate produced in granulosa cells flows, via TZPs, into the oocyte, where it is used to generate ATP. TZPs also play a crucial role in regulating the progression of oocyte development by transporting cGMP from granulosa cells, which arrest meiosis in oocytes (Clarke, 2022). During oocyte maturation, LH causes a rapid drop in the cGMP content of these cells (Egbertet al., 2016). Remarkably, the efficient gap junction communication promoted by melatonin may contribute to an adequate flow of metabolites between cumulus cells and oocytes, including critical RNAs which are important for oocyte development.

In our study, 10^{-8} M melatonin increased the relative mRNA levels for SOD and CAT after 22 h of IVM. SOD is an enzyme responsible for metabolizing the reactive superoxide anion (O^{2-}) into H_2O_2 and O_2 (Matos et al., 2009) while CAT, catalyzes the metabolism of H_2O_2 to produce water and molecular oxygen (Wang et al., 2017). Previous studies reported that melatonin increased mRNA levels of Zn-SOD (Li et al., 2016) and Cu-SOD (He et al., 2016) in oocytes, as well as in granulosa cells of pigs (Tanabe et al., 2015), and mice (Antolin et al., 1996), probably contributing with the reduction of oxidative stress in COCs. Regarding PRDX6 e GPX1, these transcripts had their levels increased in pre-matured COCs. PRDX6 is an important antioxidant enzyme present in the intracellular environment responsible to promote cellular protection from the damage caused by exposure to high levels of ROS (Wang et al., 2016). In cumulus-oocyte complexes and early embryos, PRDX6 activity controls hydrogen peroxide levels (Harvey et al., 2002), while GPX1 reduces the levels of hydroperoxide, such as

hydrogen peroxide or lipid hydroperoxide (Yoboue et al., 2017). Additionally, GPX1 helps to repair damages caused by lipid peroxidation and catalyzes the oxidation of glutathione to hydroperoxide (Kurutas, 2016). Hence, it is likely that the high hydrogen peroxide levels, which is one of the derivative products of other antioxidant enzymes activity may have stimulated an increase in PRDX6 expression.

Some changes were observed in mitochondria (e.g., irregular ridges) after maturation with melatonin in the highest concentrations (10^{-6} or 10^{-7} M). The irregularities seen in the mitochondrial cristae could be due to the oocyte meiotic stage once immature oocytes have unstructured cristae with a limited energy production capacity. In fact, it is likely that the energy to support oocyte maturation is mainly provided by the surrounding cumulus and granulosa cells and not directly by the oocyte mitochondria (Kirillova et al., 2021). Additionally, recent studies have reported that melatonin may have a pro-oxidant effect on mitochondria isolated from rat liver cells, human mesangial cell lines, and kidney cells of mice (Zhang et al., 2011; Martinis et al., 2012). Therefore, the vast majority of studies have been reported that melatonin at physiological concentrations (i.e., 10^{-8} M) protects mitochondria from oxidative damage. In this context, we believe that the addition of supraphysiological concentration of melatonin (10^{-6} and 10^{-7} M) produced lower meiotic resumption rates due to its putative pro-oxidant effect on immature mitochondria.

5. Conclusion

The C-type natriuretic peptide and forskolin or a combination of CNP, forskolin and IBMX increases the percentages of oocytes at GV stage during pre-maturation of bovine COCs from medium antral follicles. Additionally, the presence of 10^{-8} M of melatonin in maturation medium improves the meiotic resumption rates, preserves TZPs between oocyte and cumulus cells, increases the mitochondrial activity and relative mRNA expression for SOD and CAT in IVM COCs. This proposed protocol opens new perspectives for using melatonin to supplement maturation medium and to improve oocyte competence in vitro, which consequently can have a positive impact on embryo production in livestock species.

CRediT authorship contribution statement

MSc Bianca Silva, Dr. José Silva and Dr. Francisco Aguiar conceptualized, designed and drafted the manuscript. MSc Bianca Silva, MSc Laryssa Barrozo, MSc Francisco Costa, MSc

Venância Azevedo, MSc Laís Paulino, MSc Everton conducted the experiments. Dr. José Silva, Dr. Christina Peixoto, Dr. Mariana Donato and Dr Ana Rodrigues edited the manuscript. Dr. Ana Batista contributed in analyzing the data and editing the manuscript. All authors approved the final draft of the manuscript.

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List of figures and tables

Table 1. *Primers* used for amplification of messenger RNAs.

Target gene	Primer sequence (5' → 3')	Sense (S), anti-sense (As)	GenBank accession no.	Amplicon length (bp)
GAPH	TGTTTGTGATGGGCGTGAACCA ATGGCGCGTGGACAGTGGTCATAA	S As	GI: 402744670	183
PGK	AGCCTTCCGAGCTTCACTTT AAACCTCCAGCCTTCTTTGGCA	S As	GI:77735550	119
Ubiquitin	GAAGATGGCCGCACTCTTCTGAT ATCCTGGATCTTGGCCTTCACGTT	S As	GI: 57163956	173
PRDX6	GCACCTCCTCTTACTTCCCG GATGCGGCCGATGGTAGTAT	S As	GI: 59858298	105
GPx1	AACGTAGCATCGCTCTGAGG GATGCCCAAACTGGTTGCAG	S As	GI:156602645	121
SOD	GTGAACAACCTCAACGTCGC GGGTTCTCCACCACCGTTAG	S As	GI: 31341527	165
CAT	AAGTTCTGCATCGCCACTCA GGGGCCCTACTGTCAGACTA	S As	GI:402693375	165

Table 2. Percentages (%) of oocytes at germinal vesicle (GV) or germinal vesicle breakdown (GVBD) stages after 8 h of in vitro pre-maturation in the presence of different cAMP modulators [3-isobutyl-1-methylxanthine (IBMX), forskolin (FSK) and C-type natriuretic peptide (CNP).

Treatments	Oocyte chromatin configuration	
	GV (%)	**GVDB (%)**
TCM-199^{+}	45.3^{a} (48/106)	54.7^{a} (58/106)
IBMX+FSK	51.9^{ac} (56/108)	48.1^{ac} (52/108)
IBMX+CNP	41.0^{a} (43/105)	59.0^{a} (62/105)
CNP+FSK	70.7^{b} (75/106)	29.3^{b} (31/106)
IBMX+FSK+CNP	59.3^{bc} (70/118)	40.7^{bc} (48/118)

^{a,b,c} Differences between treatments in each column, $p < 0.05$.

Table 3. Percentages (%) of oocytes at germinal vesicle (GV), with resumption of meiosis or degenerated chromatin after IVM with different concentrations of melatonin (MEL).

Treatments	Total	GV (%)	Resumption of meiosis (%) *	Degenerated chromatin (%)
TCM-199^{+}	154	18.9 ^{a} (28/148)	81.1 ^{a} (120/148)	3.9 (6/154)
MEL 10^{-6} M	168	6.8 ^{b} (11/161)	93.2 ^{b} (150/161)	4.2 (7/168)
MEL 10^{-7} M	182	4.0 ^{b} (7/174)	96.0 ^{b} (167/174)	4.4 (8/182)
MEL 10^{-8} M	156	0.0 ^{c} (0/153)	100.0 ^{c} (153/153)	1.9 (3/156)

^{a,b,c} Differences between treatments in each column $p < 0.05$. * Oocytes that were at GVBD, MI and MII together.

Figure 1. Mitochondrial activity in matured oocytes with different concentrations of melatonin. Oocytes matured in TCM control medium (A) or supplemented with 10^{-6} M (B), 10^{-7} M (C), or 10^{-8} M of melatonin (D). The fluorescence intensity is shown in figure E. [a,b,c] Differences between treatments $p < 0.05$. Scale bars = 50µm.

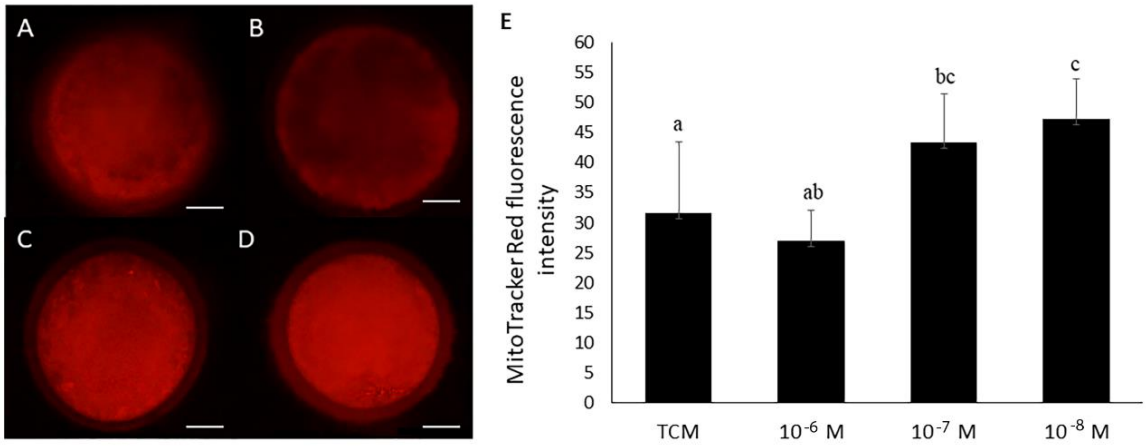


Figure 2. Fluorescence staining intensity for ROS in matured oocytes with different concentrations of melatonin. Oocytes matured in TCM control medium (A) or supplemented with 10^{-6} M (B) 10^{-7} M (C) or 10^{-8} M of melatonin (D). The fluorescence intensity is shown in panel E. [a,b] Differences between treatments $p < 0.05$. Scale bars = 50µm.

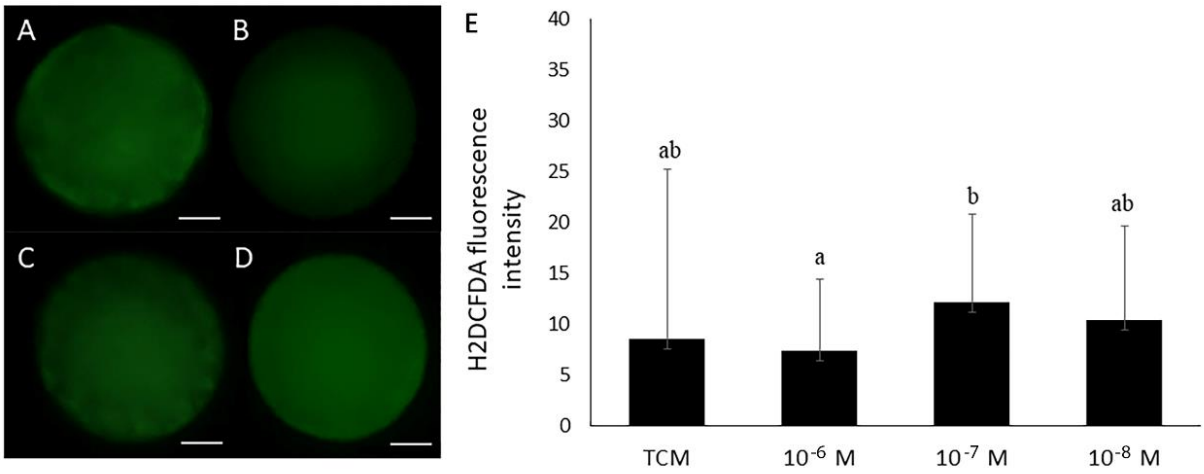


Figure 3. TZPs staining in bovine COCs after IVM in control medium (A) or supplemented with 10^{-6} M (B), 10^{-7} M (C), or 10^{-8} M of melatonin (D). The fluorescence intensity is shown in panel E. [a,b] Differences between treatments $p < 0.05$. Scale bars = 50µm. Arrow: indicates presence of transzonal projections.

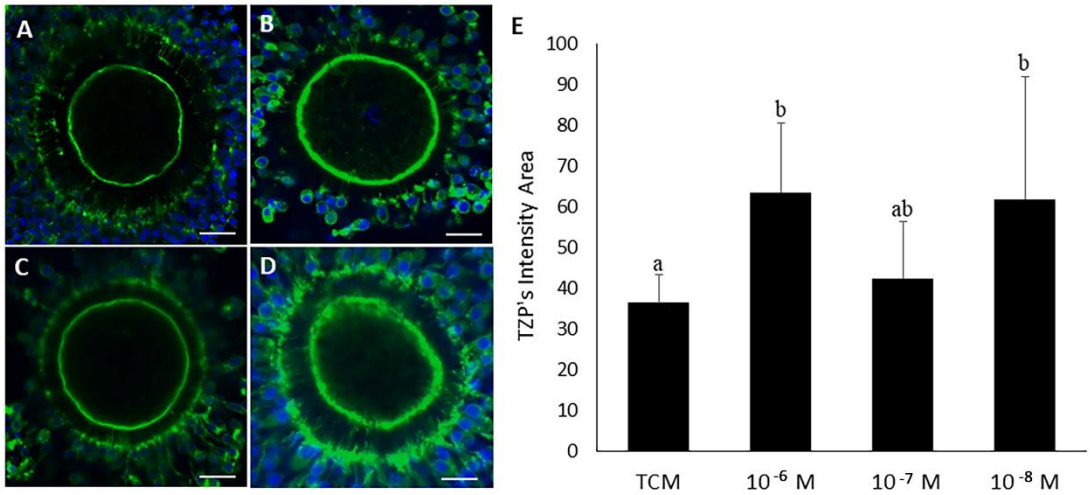


Figure 4. Ultrastructure of oocyte (A-B) and cumulus cells (C) of non-cultured COCs. Symbols: O: oocyte; ZP: zona pellucida; CC: cumulus cells; PVS: perivitelline space; V: vesicles; ER: endoplasmic reticulum; M: mitochondria; mv: microvilli; LD: electron-dense lipid droplets; CG: cortical granules; GC: Golgi complex; N: Nucleus; RER: rough endoplasmic reticulum. Bars: 10 µm (A); 2 µm (B); 5µm (C).

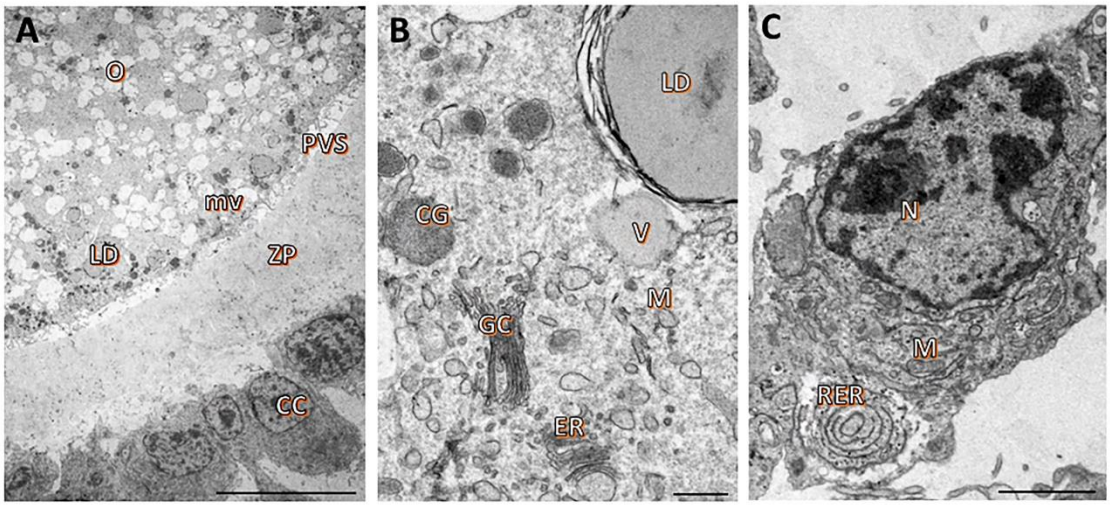


Figure 5. Electron micrograph of oocyte (A-B) and cumulus cells (C) after pre-maturation. Symbols: O: oocyte; ZP: zona pellucida; CC: cumulus cells; PVS: perivitelline space; V: vesicles; M: mitochondria; mv: microvilli; LD: electron-dense lipid droplets; GC: Golgi complex; N: Nucleus; RER: rough endoplasmic reticulum. Bars: 10 µm (A); 2 µm (B); 5µm (C).

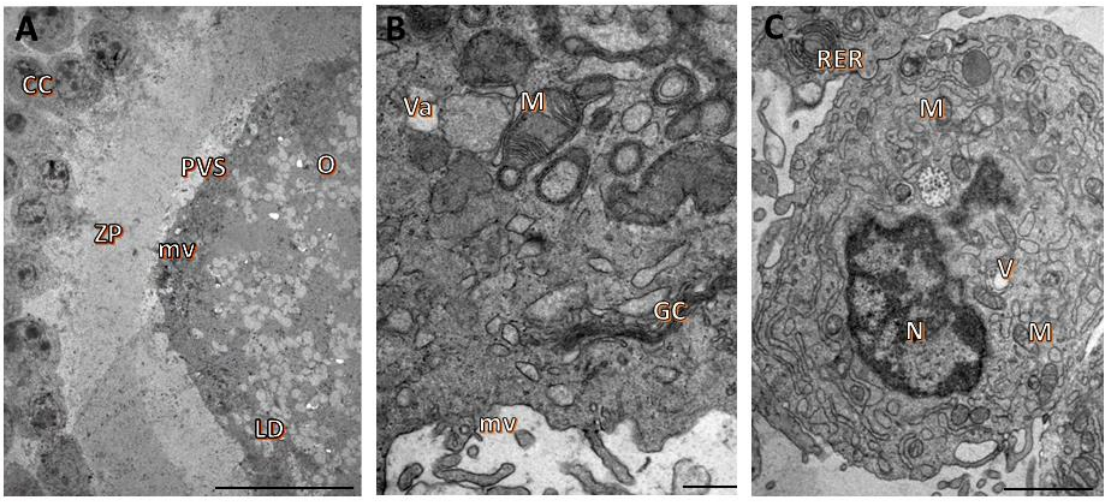


Figure 6. Electron micrograph of oocyte (A-B) and cumulus cells (C) matured in control medium. Symbols: O: oocyte; ZP: zona pellucida; V: vesicles; M: mitochondria; mv: microvilli; LD: electron-dense lipid droplets; GC: Golgi complex; N: Nucleus; ER: endoplasmic reticulum. Black arrow: transzonal projections (A), white arrow: isolated cortical granules (B). Bars: 10 µm (A); 2 µm (B); 5µm (C).

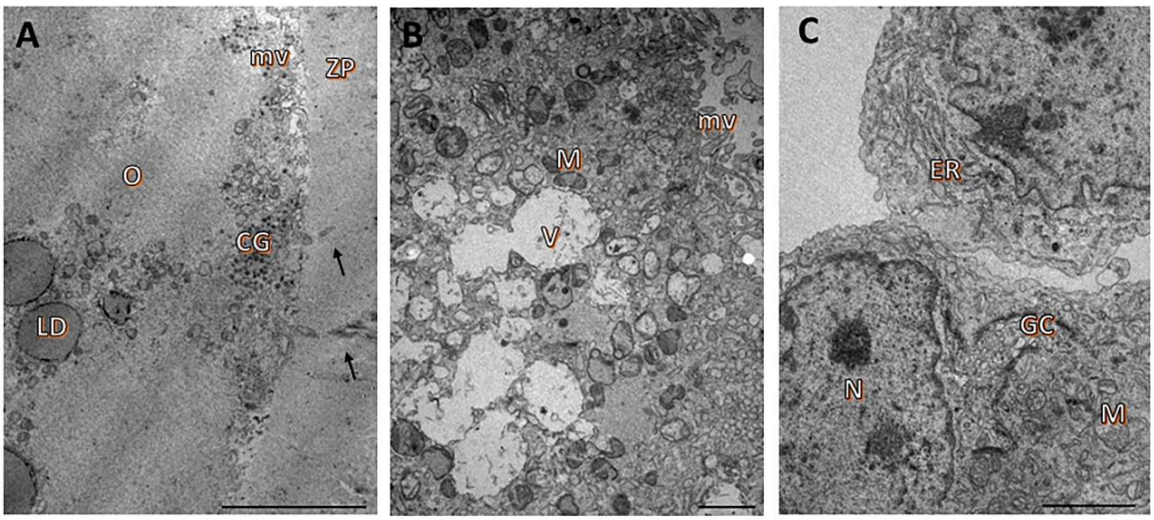


Figure 7. Electron micrograph of oocyte (A-B) and cumulus cells (C) matured with 10^{-6} M melatonin treatment. Symbols: O: oocyte; ZP: zona pellucida; CC: cumulus cells; V: vesicles; ER: endoplasmic reticulum; M: mitochondria; mv: microvilli; LD: electron-dense lipid droplets; GC: Golgi complex; N: Nucleus. Black arrow: transzonal projections (A), white arrow: isolated cortical granules (B), white star: dilated mitochondria (B). Bars: 10 µm (A); 2 µm (B); 5µm (C).

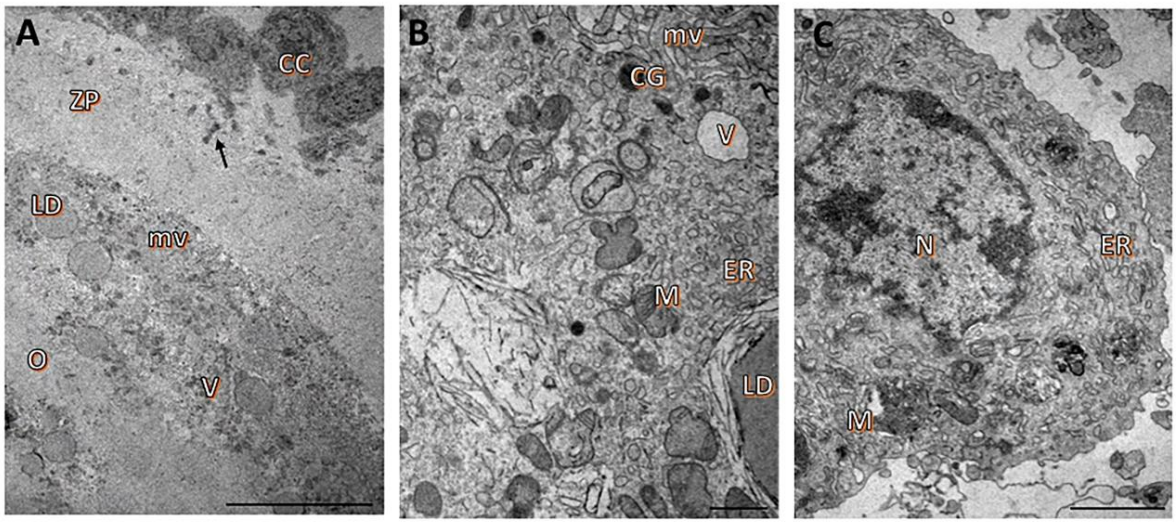


Figure 8. Electron micrograph of oocyte (A-B) and cumulus cells (C) matured with 10^{-7} M melatonin treatment. Symbols: O: oocyte; ZP: zona pellucida; CC: cumulus cells; V: vesicles; ER: endoplasmic reticulum; M: mitochondria; mv: microvilli; LD: electron-dense lipid droplets; GC: Golgi complex; N: Nucleus. Bars: 10 µm (A); 2 µm (B); 5µm (C). White arrow: clustering of cortical granules (A).

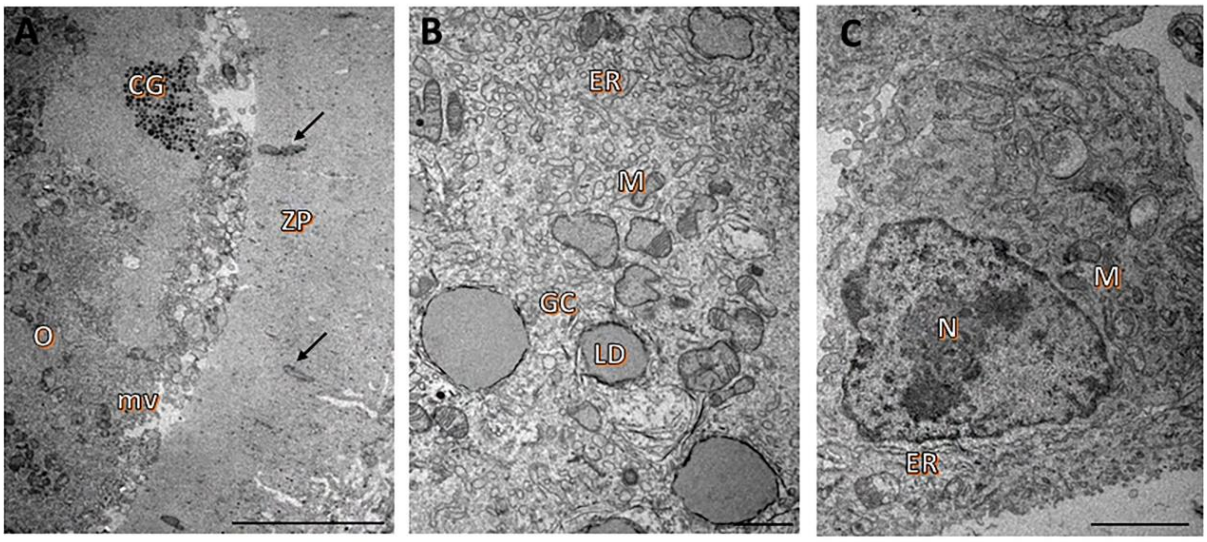


Figure 9. Electron micrograph of oocyte (A-B) and cumulus cells (C) matured with 10^{-8} M melatonin. Symbols: O: oocyte; ZP: zona pellucida; V: vesicles; ER: endoplasmic reticulum; M: mitochondria; mv: microvilli; Va: vacuoles; LD: electron-dense lipid droplets; GC: Golgi complex; N: Nucleus. Bars: 10 µm (A); 2 µm (B); 5µm (C). Black arrow: transzonal projections (A), white arrow: isolated cortical granules (B), white star: dilated mitochondria (C), black star: reduced perivitelline space (A).

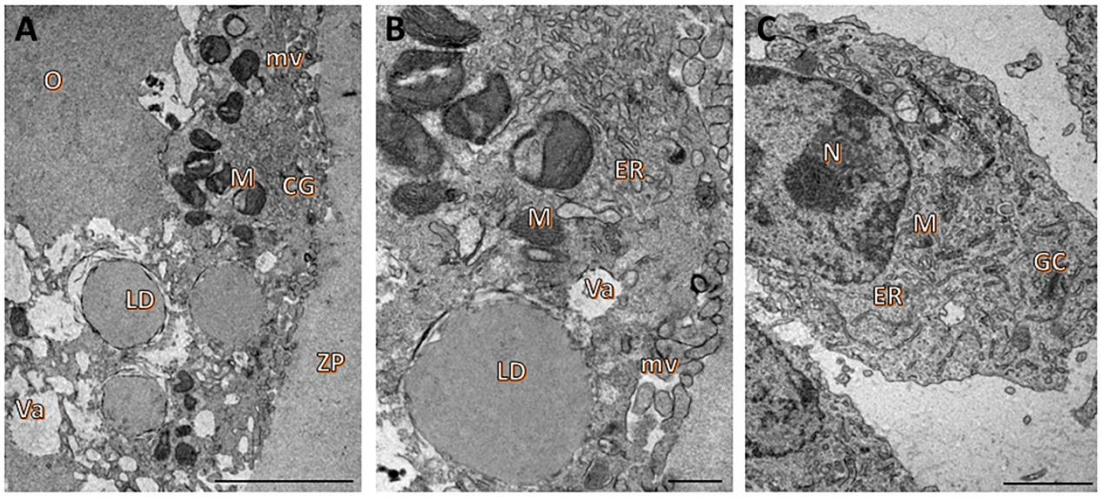
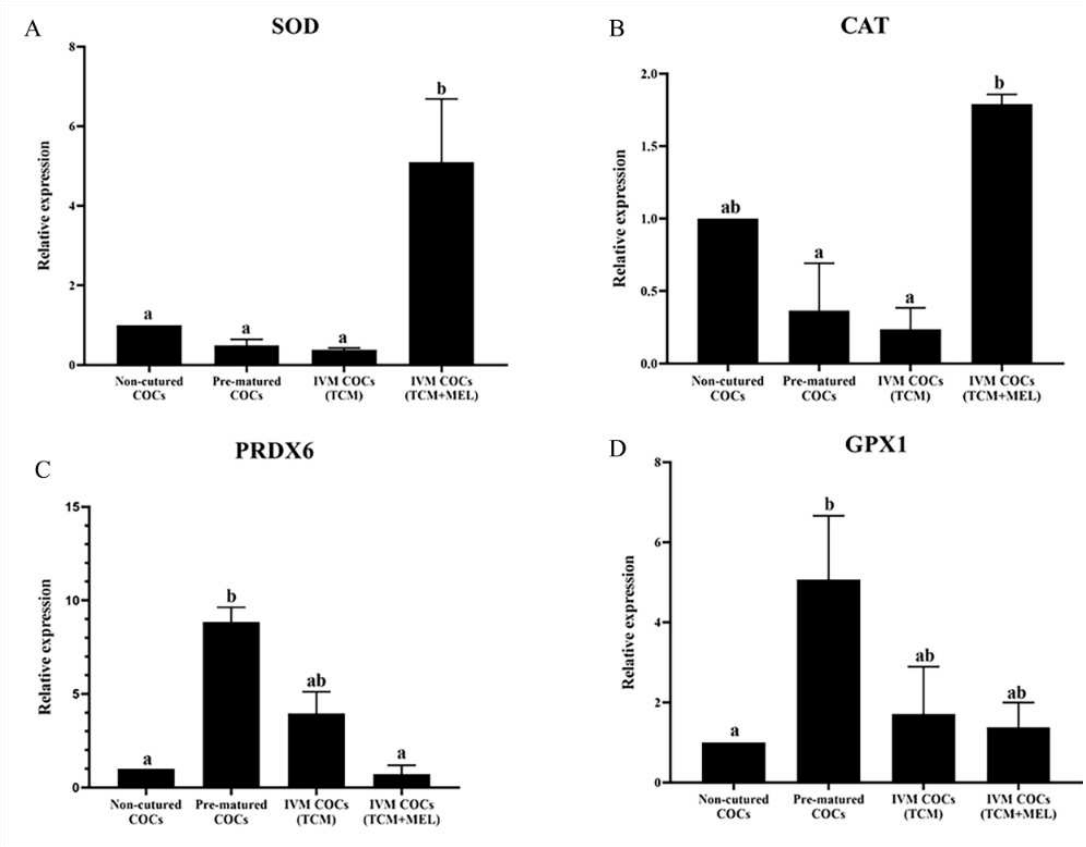


Figure 10. Relative mRNA levels for SOD (A), CAT (B), PRDX6 (C), and GPX1 (D) in non-cultured COCs, prematured and matured COCs in control medium alone or supplemented with 10-8 M melatonin. a,b Differ between treatments ($P < 0.05$).



9 CONCLUSÕES GERAIS

Diante dos resultados obtidos foram formuladas as seguintes conclusões:

- A suplementação de melatonina durante o cultivo *in vitro* de tecido ovariano bovino promove a ativação de folículos primordiais e aumenta as fibras de colágeno na MEC através de seus receptores transmembranares e mTORC1.

- A melatonina aumenta a sobrevivência dos folículos agindo por outras vias, regulando diretamente o estresse oxidativo.

- A suplementação de 10^{-8} M de melatonina durante o cultivo *in vitro* de folículos antrais iniciais bovinos melhora a viabilidade oocitária e preserva a ultraestrutura das organelas, zona pelúcida e membranas celulares do oócito e da granulosa.

- A associação entre o peptídeo natriurético tipo C e a forscolina ou a combinação de peptídeo natriurético tipo C, forscolina e IBMX aumentam as porcentagens de oócitos no estágio VG durante a pré-maturação *in vitro* de CCOs bovinos.

- A presença de 10^{-8} M de melatonina no meio de maturação melhora as taxas de retomada meiótica, preserva TZPs, aumenta a atividade mitocondrial e a expressão relativa de RNAm para SOD e CAT em CCOs maturados *in vitro*.

10 PERSPECTIVAS

A suplementação de melatonina aos meios de cultivo possui um grande potencial de utilização, afim de otimizar os índices de desenvolvimento folicular e a aquisição de competência de oocitária *in vitro*. Esta intervenção é particularmente relevante para o aprimoramento das biotécnicas aplicadas a reprodução animal, visando a maximização de gametas femininos aptos a fertilização *in vitro* com subsequente produção *in vitro* de embriões. No entanto, são necessários mais estudos visando investigar os efeitos da adição de melatonina em associação com outros antioxidantes e fatores de crescimento chaves envolvidas na competência oocitária. Mais investigações são imprescindíveis para a compreensão mais detalhada dos mecanismos de ação da melatonina durante o cultivo *in vitro* de folículos, na maturação *in vitro* e em etapas subsequentes da produção *in vitro* de embriões. Uma vez elucidados, tais mecanismos de ação podem alicerçar a utilização pivotal da melatonina como suplemento na composição do meio de base durante o cultivo folicular e na maturação oocitária *in vitro*.

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ANEXO A - ARTIGOS PUBLICADOS

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Review article

Mechanisms of action of non-enzymatic antioxidants to control oxidative stress during in vitro follicle growth, oocyte maturation, and embryo development

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Melatonin acts through different mechanisms to control oxidative stress and primordial follicle activation and survival during *in vitro* culture of bovine ovarian tissue

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Reproduction in Domestic Animals

ORIGINAL ARTICLE

Melatonin improves the viability and ultrastructure of bovine oocyte-granulosa complexes of in vitro cultured early antral follicles

Bianca R. Silva, Danisvânia R. Nascimento, Francisco C. Costa, Antônia V. Azevedo, Laís R. F. M. Paulino, Francisco L. N. Aguiar, Ana L. P. S. Batista, Mariana A. M. Donato, José R. V. Silva

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Animal Reproduction Science
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Effects of cyclic adenosine monophosphate modulating agents during oocyte pre-maturation and the role of melatonin on in vitro maturation of bovine cumulus-oocyte complexes

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