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LAIS RAIANE FEITOSA MELO PAULINO

EFEITOS DO FATOR DE CRESCIMENTO EPIDERMAL, PROGESTERONA E MELATONINA NO DESENVOLVIMENTO, ULTRAESTRUTURA E EXPRESSÃO GÊNICA EM FOLICULOS SECUNDÁRIOS BOVINOS CULTIVADOS *IN VITRO*

FORTALEZA
2022

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Tese de Doutorado submetida a avaliação da banca examinadora do Programa de Pós-Graduação em Biotecnologia da Rede Nordeste de Biotecnologia da Universidade Federal do Ceará como parte dos requisitos para obtenção do título de Doutora em Biotecnologia. Área de concentração: Biotecnologia em Agropecuária

Orientador: Prof. Dr. José Roberto Viana Silva

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Aprovada em: 30/03/2022

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'Os rios não bebem sua própia água.
As arvores não comem os seus próprios frutos.
O sol não brilha para si mesmo.
As flores não espalham sua fragância para si.
Viver para os outros é uma regra da natureza (...)
A vida é boa quando você está feliz, mas a vida é muito melhor
quando os outros estão felizes por sua causa'.

Papa Francisco

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RESUMO

O presente estudo investiga os efeitos do fator de crescimento epidermal (EGF), progesterona (experimento 1) e melatonina (experimento 2) no desenvolvimento, viabilidade e expressão gênica em folículos secundários bovinos cultivados *in vitro* por 18 dias. Para este fim, folículos secundários (~ 0,2 mm) foram isolados do córtex ovariano e cultivados individualmente a 38,5 °C, com 5% de CO_2 no ar, por 18 dias, em TCM-199^{+} sozinho (meio de controle) ou suplementado com 10 ng/mL de progesterona, 10 ng/mL de EGF ou ambos EGF e progesterona (experimento 1). No experimento 2, os folículos foram cultivados em TCM-199^{+} sozinho ou suplementado com melatonina em diferentes concetrações 10^{-11}, 10^{-9}, 10^{-7} ou 10^{-5} M (fase 1), bem como em meio suplementado com 10^{-7} M melatonina, 10μM luzindol ou ambos (fase 2). Os efeitos desses tratamentos foram avaliados quanto ao crescimento, formação de antro, viabilidade, ultraestrutura e níveis de RNAm para H1FOO, GDF9, CCNB1, eIF4E e PARN (experimento 1) SOD, CAT, GPX1 e PRDX6 (experimento 2). No experimento 2 também foi investigado a expressão de receptores de melatonina tipo 1A/B em oócitos e células da granulosa em folículos primordiais, primários, secundários e antrais de bovinos. Os resultados do experimento 1 mostraram que os folículos cultivados em meio suplementado com EGF tiveram aumento significativo de diâmetro quando comparados aos folículos cultivados no meio controle e ainda a presença de progesterona no meio de cultivo aumentou a expressão de RNAms para GDF9 e ciclina B1 em oócitos. Além disso, os níveis de RNAms para cMOS e GDF9 aumentou significativamente com a adição de EGF. As análises ultraestruturais mostraram que os folículos cultivados em todos os tratamentos mantiveram a integridade das células da granulosa. Os resultados do experimento 2 mostraram a expressão de receptores de melatonina tipo 1A/B em oócitos e células da granulosa em folículos primordiais, primários, secundários e antrais em bovinos. Os estudos *in vitro* mostraram que folículos secundários cultivados em meio suplementado com melatonina na concentração de 10^{-7} M apresentaram diâmetros significativamente maiores do que aqueles cultivados em outros tratamentos. Além disso, a presença do antagonista do receptor de melatonina, luzindole, bloqueou os efeitos desse hormônio no crescimento folicular e na viabilidade. Os folículos cultivados em meio contendo apenas melatonina apresentaram taxas significativamente maiores de formação de antro. Folículos cultivados em meio contendo apenas melatonina apresentaram níveis mais elevados de RNAm para CAT e SOD do que aqueles cultivados com luzindol ou melatonina e luzindol. Luzindol sozinho ou melatonina e luzindol também reduziram os níveis de RNAm para GPX1

em folículos em cultivo. Em conclusão, o EGF promove o desenvolvimento de folículos secundários cultivados *in vitro* por 18 dias e aumenta a expressão de cMOS e GDF9, enquanto a progesterona isolada ou em associação com EGF não tem efeito positivo no crescimento folicular. A melatonina promove o crescimento e a formação de antro em folículos bovinos cultivados por meio de seus receptores acoplados à membrana.

Palavras-chave: Folículo secundário; expressão de RNAm; cultivo *in vitro*; bovino.

ABSTRACT

The present study investigates the effects of epidermal growth factor (EGF), progesterone (experiment 1) and melatonin (experiment 2) on development, viability and gene expression in bovine secondary follicles cultured in vitro for 18 days. For this purpose, secondary follicles (~0.2 mm) were isolated from the ovarian cortex and individually cultured at 38.5°C, with 5% CO_2 in air, for 18 days, in TCM-199^{+} alone (control medium) or supplemented with 10 ng/ml progesterone, 10 ng/ml EGF or both EGF and progesterone (experiment 1). In experiments 2, follicles were cultured in TCM-199^{+} alone or supplemented with melatonin in different concentrations at 10^{-11}, 10^{-9}, 10^{-7} or 10^{-5} M (phase 1), as well as in medium supplemented with 10 - 7 M melatonin, 10μM luzindole or both (phase 2). These treatments were developed for growth, antrum formation, viability, ultrastructure and mRNA levels for H1FOO, GDF9, CCNB1, eIF4E and PARN (experiment 1) SOD, CAT, GPX1 and PRDX6 (experiment 2). In experiment 2, the expression of type 1A/B melatonin receptors in oocytes and granulosa cells in primordial, primary, secondary and antral follicles of bovines was also investigated. GDF9 and cyclin B1 in oocytes increased RNA expression for GDF9 and cyclin B1 in oocytes. In addition, mRNA levels for cMOS and GDF9 increased significantly with the addition of EGF. The ultrastructures maintained in all follicles maintained the granulosa cells maintained. The results of experiment 2 showed the expression of type 1A/B melatonin receptors in oocytes and granulosa cells in primordial, primary, secondary and antral follicles in cattle. In vitro studies showed that secondary follicles cultured in medium supplemented with melatonin at a concentration of 10^{-7} M had significantly larger diameters than those cultured in other treatments. Furthermore, the presence of the melatonin receptor antagonist, luzindole, blocked the effects of this hormone on follicular growth and viability. Follicles cultured in medium containing only melatonin showed significantly higher rates of antrum formation. Follicles grown in medium containing only melatonin had higher levels of mRNA for CAT and SOD than those grown with luzindole or melatonin and luzindole. Luzindol alone or melatonin and luzindole also reduced mRNA levels for GPX1 in follicles in culture. In conclusion, EGF promotes the development of secondary follicles cultured in vitro for 18 days and increases the expression of cMOS and GDF9, while progesterone alone or in association with EGF has no positive effect on follicular growth. Melatonin promotes growth and antrum formation in cultured bovine follicles through its membrane-coupled receptors.

Keywords: Secondary follicle; mRNA stock; in vitro culture; bovine.

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µg	Micrograma
µM	Micromolar
5-HT	5-hidroxitriptamina
5-HTP	5-hidroxitriptofano
5-HTPD	5-hidroxitriptofano descarboxilase
AI	Anáfase I
Akt	Proteína quinase B
AMH	Hormônio antimülleriano
Ang II	Angiotensina II
AREG	Anfiregulina
ATP	Adenosina trifosfato
BMP-2	Proteína morfogenética óssea-2
BMP-4	Proteína morfogenética óssea-4
BMP-8B	Proteína morfogenética óssea-8B
BMP-15	Proteína morfogenética óssea-15
BIRC	Inibidores baculovirais da repetição de apoptose
BTC	Betacelulina
CAT	Catalase
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CCN	Rede de comunicação celular
CCNB1	ciclina B1
CCO	Complexo cumulus-oócito
CDK	Quinase dependente de ciclina
CE	Ceará
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
EREG	Epiregulina
FGF-2	Fator de crescimento fibroblástico-2
FOXO3a	Fator de transcrição *forkhead box 3a*
FSH	Hormônio folículo estimulante
GDF-9	Fator de crescimento e diferenciação-9
GnRH	Hormônio liberador de gonadotrofina
GPCR	Receptores acoplados à proteína G
GPX	Glutationa peroxidase
GR	Glutationa redutase
GSH	Glutationa reduzida
GSSG	Glutationa oxidada
H1FOO	Histona com ligante específico para oócito
H_2O_2	Peróxido de hidrogênio
HB EGF	Fator de crescimento de ligação à heparina
HIOMT	Hidroxindol-*O*-metiltransferase
IL-6	Interleucina-6
IGF-1	Fator de crescimento semelhante à insulina-1
kg	Kilograma
KGF	Fator de crescimento de queratinócitos
KL	*Kit* ligante
LH	Hormônio luteinizante
MI	Metáfase I
MII	Metáfase II
MEC	Matriz extracelular
MEM	Meio essencial mínimo
mg	Miligrama
MLKL	Proteína de linhagem mista semelhante a domínio de quinase
mm	Milímetro
mL	Mililitro
MOIFOPA	Manipulação de oócitos inclusos em folículos pré-antrais
mPR	Receptor de membrana de progesterona

MT_1	Receptor da melatonina do tipo 1
MT_2	Receptor da melatonina do tipo 2
MT_3	Receptor da melatonina do tipo 3
mTOR	Alvo mamífero da rapamicina
MZT	Transição materno zigótica
NAS	*N*-acetilserotonina
NRG 1-4	Neuregulina 1-4
$O_2^{-\bullet}$	Radical superóxido
$OH^{\bullet}$	Radical hidroxila
PARN	Ribonuclease específica poli (a)
P4	Progesterona
PDK1	Quinase-1 dependente de PI3K
pg	Picograma
pH	Potencial hidrogeniônico
PI3K	Fosfatidilinositol-3-quinase
PIP2	Fosfatidilinositol-4,5-bifosfato
PIP3	Fosfatidilinositol-3,4,5-trifosfato
PGRs	Receptores de progesternona
PR A/B/C	Receptores de progesterona A/B/C
Prdx6	Peroxiredoxina-6
PGRMC 1-2	Componente 1-2 da membrana do receptor de progesterona
PTEN	Fosfatase homóloga à tensina
RENORBIO	Rede Nordeste de Biotecnologia
RIPK1 / RIPK3	Receptor de interação com a proteina quinase 1/3
rpS6	Proteína ribossomal S6
RVG	Rompimento da vesícula germinativa
SMAD	Derivado de uma definição homóloga do gene Mad em drosófilas
SOD	Superóxido dismutase
TI	Telófase I
TCM-199	Meio de cultivo de tecido-199
TNF-α	Fator de necrose tumoral-alfa
TNFR-1	Receptor do tipo 1 da família TNF

TAZ	Coativador com afinidade ä PDZ
TPH	Triptofano hidroxilase
TGF-α	Fator de crescimento transformador-α
UFC	Universidade Federal do Ceará
VEGF	Fator de crescimento endotelial vascular
VG	Vesícula germinativa
YAP	Proteina associada a *Yes*

LISTA DE SÍMBOLOS

>	Maior
%	Porcentagem

SUMÁRIO

1 INTRODUÇÃO

Os avanços na produção *in vitro* de embriões estão relacionados com a utilização de oócitos competentes de grandes folículos antrais, que estão presentes no ovário em número relativamente reduzido (SUN *et al*., 2003). Assim, o desenvolvimento de sistemas de cultivo *in vitro* para explorar oócitos imaturos inclusos em folículos pré-antrais tem potencial para fornecer um grande número de oócitos para fertilização *in vitro* (FAUSTINO *et al*., 2013; TELFER *et al*., 2019). É importante considerar que os folículos pré-antrais representam 90 a 95% de toda a população folicular e armazenam a maioria dos oócitos presentes nos ovários de mamíferos (SÁ *et al*., 2020).

O cultivo *in vitro* de folículos pré-antrais tem se mostrado como uma valiosa ferramenta experimental para dar suporte a práticas clínicas e/ou zootécnicas no que se refere à reprodução e ao melhoramento genético (VO; KAWAMURA, 2021). No entanto, a obtenção de oócitos totalmente competentes fornecidos a partir de folículos pré antrais cultivados *in vitro* ainda é um grande desafio tendo em vista os diversos mecanismos moleculares envolvidos no controle do desenvolvimento folicular. Dessa forma, é importante melhorar os sistemas de cultivo adicionando fatores de crescimento e hormônios para manter a viabilidade, promover o crescimento folicular e oocitário e entender os mecanismos de controle da foliculogênese. Dentre essas substâncias, o fator de crescimento epidérmal (EGF), a progesterona (P4) e a melatonina são candidatos para melhorar a sobrevivência e o crescimento folicular *in vitro*.

O EGF tem importante papel na foliculogênese ovariana por regular a proliferação de células da granulosa, a expansão das células do cumulus, a maturação e a fertilização do oócito (AGUIAR *et al*., 2017; HSIEH *et al*., 2007). Mais recentemente, foi demostrado que o EGF melhora as taxas de sobrevivência de foliculos pré-antrais bovinos em um sistema de cultivo tridimensioal (JACHTER *et al*., 2022). Estudos mostraram que os efeitos do EGF na maturação oocitária podem ser potencializados pela P4, uma vez que esse hormônio aumenta a expressão de fatores semelhantes ao EGF que influenciam a maturação oocitária (CHOI *et al*., 2017). A P4 regula vários processos reprodutivos, incluindo ovulação, implantação e diferenciação sexual. A sinalização de P4 tem sido estudada principalmente por meio da ativação de receptores nucleares que atuam como fatores de transcrição para estimular a expressão de genes dependente de P4 (ELLMAN *et al*., 2009). Além disso, foi demonstrado que P4 desempenha um papel importante na regulação da maturação de oócitos bovinos tanto in vivo quanto *in vitro* pela sinalização por meio de receptores de P4 nucleares (PGRs) e de membrana (mPR) (O'SHEA *et al*., 2013).

A melatonina também é um fator que vem sendo intensamente estudado na área de reprodução. Devido ao seu tamanho pequeno e propriedades altamente anfifílicas (REITER *et al*., 2009; PARADIES *et al*., 2010), a melatonina pode atuar em meios aquosos e lipídicos, prevenindo danos ao DNA, a peroxidação de lipídios e preservando a função de organelas, em especial, das mitocôndrias. Consequentemente, a melatonina reduz eventos apoptóticos e a morte celular induzidos pelo estresse oxidativo (VENEGAS *et al*., 2012; TANABE *et al*., 2015; TAN; REITER, 2019; EL-RAEY *et al*., 2011). Em folículos pré-antrais, a melatonina melhorou o desenvolvimento de folículos primários e secundários em combinação com o hormônio folículo estimulante (FSH) (ROCHA *et al*., 2013). De modo geral, a atividade antioxidante da melatonina está relacionada à sua capacidade de doar elétrons (agente redutor) e, portanto, neutralizar as espécies realtivas de oxigênio (ERO) (ANJUM *et al*., 2011; BRAICU *et al*., 2013) ou de estimular a atividade do sistema antioxidante endógeno (LAMBERT; ELIAS, 2010; MONIRUZZAMAN *et al*., 2018). Além de atravessar as membranas celulares e atuar diretamente no citoplama, a melatonina também pode influenciar as funções celulares através da interação com receptores de membrana ou modulação de diferentes vias de sinalização celular (JANG *et al*., 2016; ZHANG *et al*., 2016; BARBERINO *et al*., 2017; NAM *et al*., 2018). Desta forma, é importante compreender os potenciais mecanismos moleculares pelos quais a melatonina regula o desenvolvimento folicular.

Muitos fatores inerentes ao oócito e o ambiente de cultivo *in vitro* determinam a chance de se ter o desenvolvimento folicular completo e a aquisição da competência oocitária *in vitro*. Pode-se destacar o aúmulos de vários tipos de RNAm (H1FOO, GDF9, CCNB1, eIF4E e PARN) durante o crescimento oocitário (BEZERRA *et al*., 2019), que diretamente relacionadas com o controle da maturação do oócito e do desenvolvimento embrionário inicial.

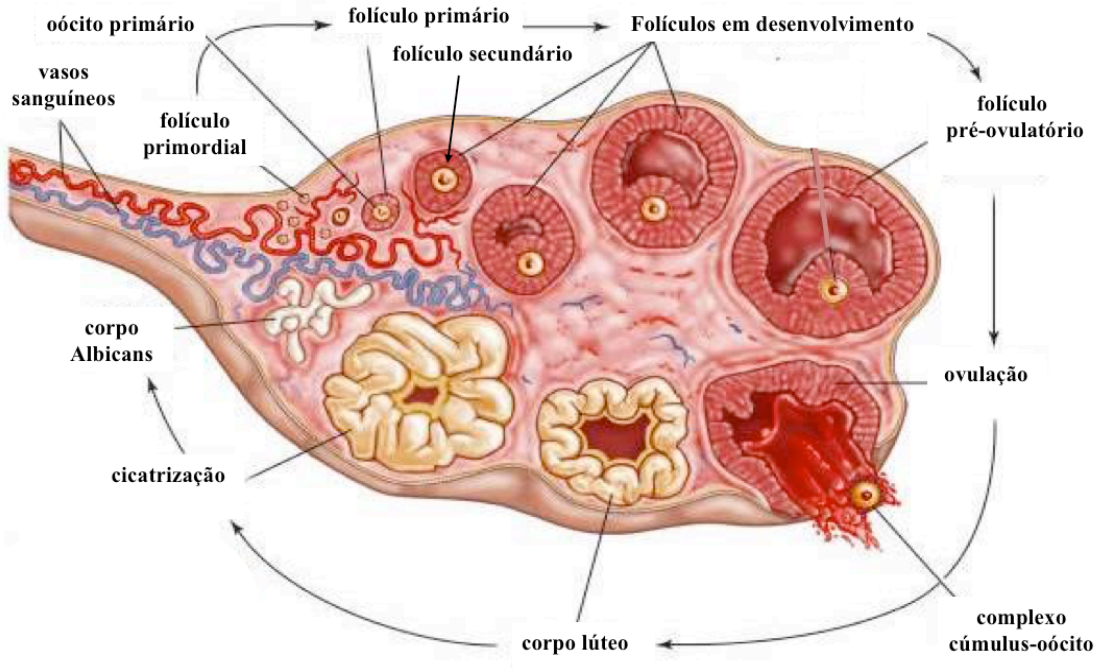
Para uma melhor compreensão da relevância desta tese, a revisão de literatura a seguir aborda aspectos relacionados aos processos envolvidos no crescimento e desenvolvimento *in vivo* e *in vitro* de folículos ovarianos, a importância de EGF, P4 e da melatonina para a foliculogênese, o estado atual do desenvolvimento *in vitro* de folículos pré-antrais e os principais desafios para obtenção de oócitos competentes a partir do cultivo *in vitro* de folículos pré antrais.

2 REVISÃO DE LITERATURA

2.1 Estrutura e função ovariana

O ovário dos mamíferos é um importante órgão do sistema reprodutor feminino, o qual exerce duas importantes funções fisiológicas: gametogênica, responsável pela liberação de oócitos maturos aptos a serem fecundados (ovulação) e endócrina, responsável por produzir hormônios, fatores de crescimento e peptídeos (MATSUDA *et al*., 2012; SHAH *et al*., 2018; ZHAO *et al*., 2021). O ovário é composto por duas porções, uma cortical e uma medular. O córtex é constituído de tecido conjuntivo, com células do estroma, folículos ovarianos e corpos lúteos. A medula é composta por tecido conjuntivo frouxo vascularizado, onde estão presentes fibroblastos, fibras elásticas, vasos sanguíneos, vasos linfáticos e fibras nervosas responsáveis pela sustentação e nutrição dos folículos ovarianos (MARTELLI *et al*., 2017; SHAH *et al*., 2018) (Figura 1). O folículo ovariano é constituído por um oócito circundado por células somáticas (células da granulosa e/ou da teca) e é considerado a unidade funcional e morfológica do ovário responsável pela manutenção da viabilidade oocitária durante o crescimento e a maturação dos oócitos (oogênese e foliculogênesse) (CORTVRINDT; SMITZ, 2001; FIGUEIREDO *et al*., 2008).

Figura 1. Estrutura ovariana ilustrando a presença de folículos ovarianos em diferentes estágios de desenvolvimento e corpos lúteos.



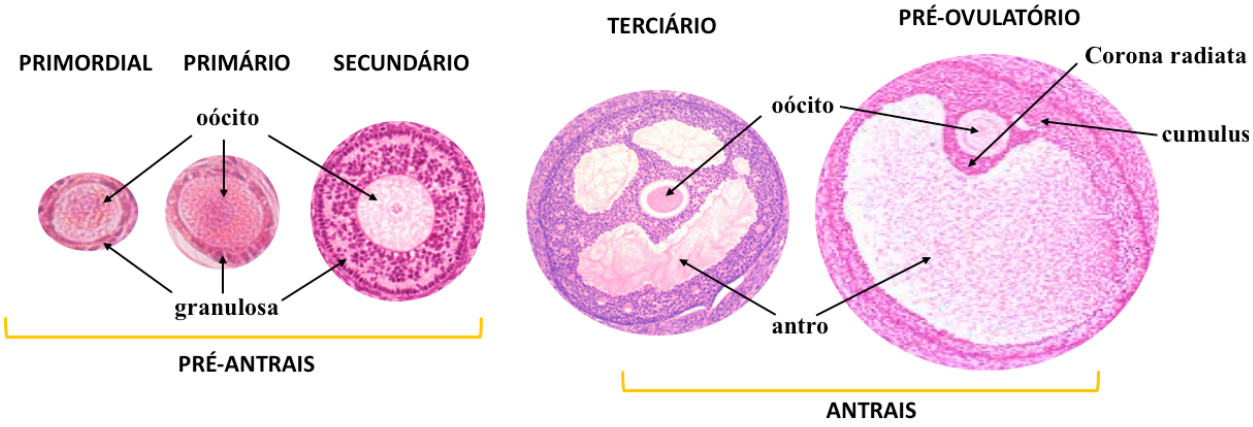
Fonte: adaptado de LUZ, 2018.

2.2 Crescimento e desenvolvimento de folículos ovarianos em mamíferos

2.2.1. Foliculogênese

Em mamíferos a foliculogênese tem início durante a vida fetal, quendo ocorre a formação de folículos primordiais que constituem a reserva ovariana. Logo após a sua formação, sob estímulos de fatores de crescimento locais, pequenos grupos de folículos primordiais são ativados e iniciam o desenvolvimento (Figura 2). Após esta ativação, os folículos se desenvolvem em folículos primários, secundários, antrais e pré-ovulatórios ou sofrem atresia durante este trajeto. Este processo de desenvolvimento folicular até a ovulação ou atresia é dependente da sinalização celular, fatores locais e hormonais (MONNIAUX *et al*., 2014; SKINNER, 2005).

Figura 2. Características morfológicas de folículos pré-antrais e antrais.



Fonte: elaborada pela autora em colaboração com COSTA et al., 2021.

A ativação dos folículos primordiais inicia-se durante a vida fetal e continua durante toda a vida fértil, até o esgotamento total da reserva ovariana. Esta etapa da folículogênese é regulada unicamente por fatores intra-ovarianos, não havendo ação hormonal (FORTUNE, 2003).

A ativação dos folículos primordiais caracteriza-se pela saída destes folículos do estágio de quiescência e entrada para o "pool" de folículos em crescimento. Esse processo resulta em alterações bioquímicas e funcionais nas células foliculares que levam ao aumento da atividade metabólica e transcricional destas células, levando à mudanças na morfologia das células da granulosa. Uma vez ativados, os folículos seguem um longo período de crescimento necessário

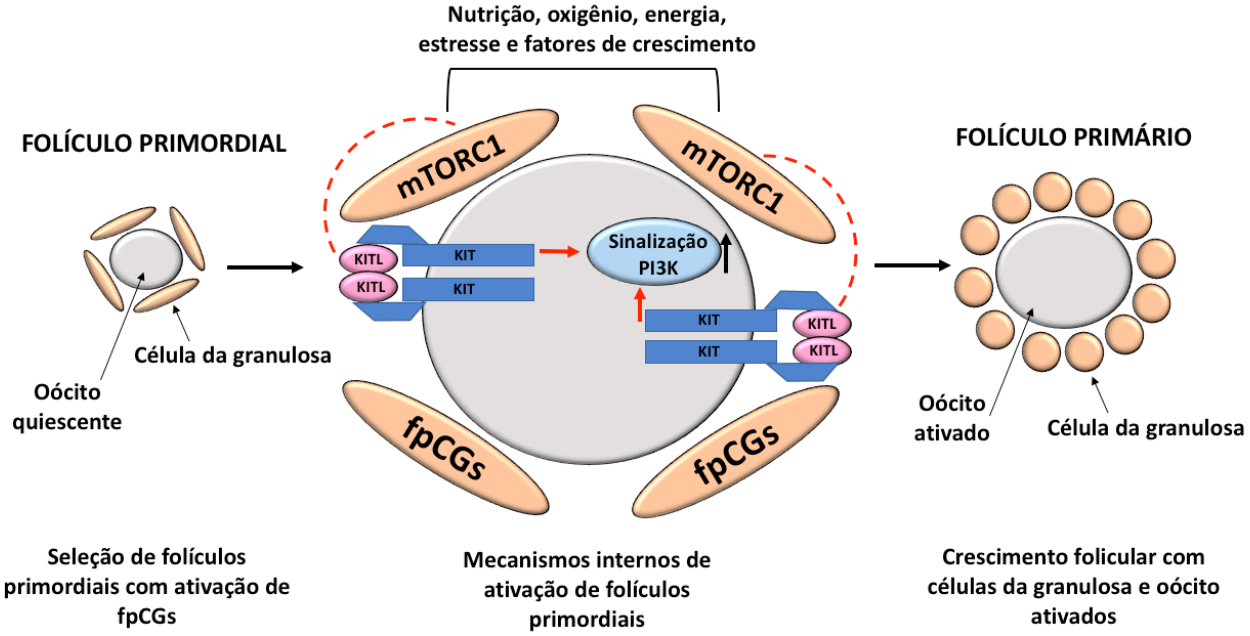
para o desenvolvimento de folículos pré-ovulatórios, ou, alternativamente, morrer por atresia (BRISTOL-GOULD, WOODRUFF, 2006). Em mamíferos o processo de ativação folicular é regulado por uma via de sinalização denominada de fosfatidilinositol 3-quinase (PI3K) – proteína quinase B (Akt) (REDDY *et al*., 2008; LIU *et al*., 2007a,b) iniciada pela ativação do gene *Mammalian Target of Rapamycin Complex* 1(mTORC1) nas células da pré-granulosa, resultando em sua diferenciação para células cuboides e no aumento de sua proliferação (ADHIKARI *et al*., 2010). A diferenciação promovida por mTORC1 leva a expressão do Kit Ligand (KL), dando inicio ao processo de crescimento oocitário e proliferação das células da granulosa. Em resposta à estimulação, o alvo mecanístico da sinalização do complexo 1 de rapamicina (mTORC1) é ativado nas CGs de folículos primordiais (CGs) selecionados, levando à diferenciação e proliferação dessas células. A sinalização de mTORC1 ativada em CGs também estimula uma regulação positiva da secreção do receptor KIT (KITL). KITL liga-se ao KIT na superfície do oócito dormente, e isso leva à ativação da sinalização intra-oócito PI3K. A sinalização de PI3K ativada em oócitos desperta os oócitos dormentes e estimula seu crescimento (Figura 3). Além disso, tem-se o início da expressão de fator de crescimento e diferenciação-9 (GDF9) e proteína morfogenética óssea-15 (BMP15), que mantém a proliferação e a diferenciação das células da granulosa através da sinalização de SMAD. Por intermédio desta via, muitos fatores de transcrição que agem na sobrevivência dos folículos e no crescimento oocitário são ativados (REDDY *et al*., 2008). Além disso, a ativação desta via dependerá do balanço entre as moléculas inibitórias e estimulatórias (SILVA *et al*., 2016).

Estudos em animais de laboratório também mostraram que a ativação e crescimento folicular através é regulado pela via de sinalização Hippo, que consiste em vários fatores que atuam como inibidores do crescimento, a partir de uma cascata de quinases que fosforilam e inativam as principais vias efetoras dasinalização: *Yes-associated protein* (YAP) e transcricional *coactivator with PDZ-binding motif* (TAZ) (HOLBOURN; ACHARYA; PERBAL, 2008). Quando a via de sinalização Hippo é interrompida, ocorre a desfosforilação de YAP e um aumento dos níveis nucleares de YAP. A proteína YAP atua em conjunto com fatores de transcrição TEAD que aumentam os níveis de expressão de fatores de crescimento da família CCN, bem como de fatores inibidores de apoptose – BIRC (*baculoviral inhibitors of apoptosis repeat containing*), estimulando o crescimento celular, sobrevivência e proliferação (PAN, 2007; HOLBOURN ACHARYA; PERBAL, 2008).

Após a ativação, quando uma camada completa de células da granulosa de morfologia cuboide circunda o oócito, são formados os folículos primários. Por mecanismos independentes de gonadotrofinas, os folículos primários se desenvolvem até chegar ao estágio de folículos

secundários pelo crescimento do oócito e pela atividade mitótica das células da granulosa (SILVA *et al*., 2014).

Figura 3**.** Mecanismos que regulam a ativação dos folículos primordiais.

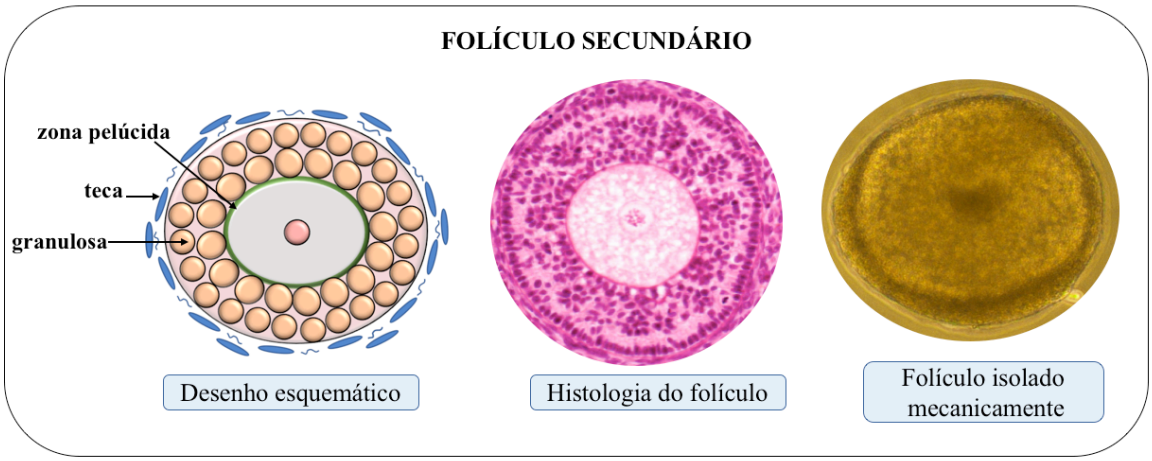


Fonte: Adaptado de ZHANG & LIU 2015 e ROSA, 2016.

Com o crescimento dos folículos primários e a multiplicação das células da granulosa, ocorre o desenvolvimento de duas ou mais camadas de células ao redor do oócito e, consequentemente, a formação dos folículos secundários (MCGEE; HSUEH, 2000; OKTEN; URMAN, 2010; RIMON-DAHARI *et al*., 2016). Os folículos secundários são caracterizados morfologicamente por um oócito com diâmetro aumentado, zona pelúcida visível rodeada por múltiplas camadas de células da granulosas cuboides (duas ou mais camadas) e formação de uma camada de células da teca em torno da membrana basal (figura 4) (HONDA *et al*., 2007). Além disso, a zona pelúcida pode ser identificada de forma evidente em bovinos (FAIR *et al*., 1997) e caprinos (LUCCI et al., 2001). As células da teca são recrutadas a partir do estroma ovariano sob o estímulo de fatores de crescimento parácrinos, tais como o KL e o Fator de Crescimento Fibroblástico básico (FGFb), os quais são sintetizados pelas células da granulosa e pelo oócito, respectivamente, e apresentam atuação mútua neste processo (KNIGHT E GLISTER, 2006).

Nos folículos secundários e estágios subseqüentes, a comunicação entre as células da granulosa e o oócito é realizada por junções intercomunicantes do tipo GAP que são formadas entre os dois tipos celulares. No ooplasma, o número de organelas e inclusões como complexo de Golgi, retículo endoplasmático liso, gotículas de lipídeos e vesículas ligadas à membrana são gradualmente formadas e sofrem deslocamento para a periferia. Estruturas específicas do oócito, como os grânulos da cortical e o espessamento progressivo da zona pelúcida podem ser evidenciadas nos folículos secundários em bovinos (HYTTEL *et al*., 1997)

Figura 4. Características morfológicas dos folículos secundários em desenho esquemático, estrutura histológica e morfologia de folículos mecanicamente isolados.



Fonte: elaborada pela autora sob colaboração de COSTA et al., 2021.

A transição do folículo secundário para antral geralmente é correlacionada com a aquisição da competência do oócito para retomar a primeira divisão meiótica. Dessa forma, o desenvolvimento de folículos pré-antrais compreende a fase mais longa de todo o processo de maturação, podendo durar meses. É nesse período que ocorre o maior crescimento oocitário, o qual por uma reorganização citoplasmática, novos produtos gênicos e organelas são sintetizados (VAN DEN HURK; ZHAO, 2005). Dessa forma, tem sido proposto que a competência de desenvolvimento do oócito é determinada, em parte, pela quantidade de transcritos armazenados durante a fase de transisão entre folículo secundário para folículo antral (SIRARD *et al*., 2006).

Com o avanço no crescimento dos folículos secundários e organização das células da granulosa em várias camadas, ocorre a formação de uma cavidade repleta de líquido rico em biomoléculas, caracterizando a classe de folículos antrais (MONNIAUX, 2016; FREITAS *et al.*, 2017; RYBSKA *et al*., 2018). Este fluido está presente no interior do antro sendo constituído

por água, proteínas séricas, eletrólitos e altas concentrações de estradiol e inibina, ambos produzidos pelas células da granulosa (MARTINS *et al*., 2008). Em adição, o fluido folicular fornece inúmeros fatores que suportam as funções foliculares apropriadas, incluindo a qualidade dos oócitos, tais como a interleucina-6 (IL-6), BMP2, AREG (anfiregulina) e GDF-9 (DUMESIC *et al*., 2015). Sua composição química contém uma mistura complexa de esteroides, metabólitos, polissacarídeos, proteínas e pequenos peptídeos, que, possivelmente, contribuem para sobrevivência, crescimento e maturação do oócito (humanos: KUSHNIR *et al*., 2016; FREITAS *et al*., 2017). Embora a formação do antro folicular não seja compreendida completamente, sabe-se que há a participação de componentes liberados pelas células da granulosa, fatores de crescimento e ainda a ação dos hormônios folículo estimulante (FSH) e luteinizante (LH) (NAGASHIMA *et al*., 2019).

Nos foliculos antrais, as células da granulosa sofrem diferenciação e são então classificadas em duas classes distintas de células: granulosa mural, que revestem a parede folicular e as célulasdo cúmulus, que estão associadas ao oócito. Geralmente, as células do cúmulos atuam no processo de desenvolvimento do oócito, enquanto as células murais estão relacionadas á função endócrina e arquitetura folicular (camundongo:WIGGLESWORTH *et al*., 2015).

Durante o desenvolvimento folicular, ocorrem três eventos principais, o recrutamento, a seleção e a dominância (VAN DEN HURK & ZHAO, 2005). A comparação dos padrões de expressão gênica observados por hibridização *in situ* em folículos antrais bovinos recrutados e selecionados indicou que a seleção está associada ao início da expressão do gene LHR em células da granulosa de bovinos (BAO & GARVERICK, 1998). Em algumas espécies a dependência de gonadotrofinas e o recrutamento ocorrem quando os folículos atingem 0,2 mm em camundongos, 2 mm em ovinos e primatas, 3 mm em caprinos e 4 mm em bovinos (RUBIANES; MENCHACA, 2003). Os receptores de LH (LHR) das células da granulosa parecem estar relacionados à dominância folicular.

Na espécie bovina, a partir de 4mm de tamanho, os folículos antrais tornam-se dependentes de hormônios gonadotróficos até o estágio de folículos pré-ovulatórios, que tem um diâmetro em torno dede 10 a 12 mm em *Bos indicus* e 12 a 15mm em *Bos taurus.* Os folículos antrais podem atingir o diâmetro de 8 mm independente do suporte do LH, mas o crescimento além de 9 mm requer LH endógeno ou FSH exógeno. O FSH e o LH são reguladores primários da foliculogênese durante a fase dependente de gonadotrofinas (GINTHER *et al*., 2003; DRIANCOURT, 2001).

Dos folículos que foram recrutados, somente um (espécies monovulatórias) ou grupo menor (animais poliovulatórios) será selecionado para continuar se desenvolvendo e, finalmente, ovular (DRIANCOURT, 2001; MATSUDA *et al*., 2012). Com a formação do folículo pré-ovulatório, o qual é caracterizado por um oócito circundado por células da granulosa especializadas, denominadas de células do cumulus, formando, assim, o complexo cumulus-oócito (CCO), tem-se o fim da foliculogênese (RYBSKA *et al*., 2018).

A maturação oocitária ocorre de maneira gradual e sincronizada com os eventos foliculares, visto que o desenvolvimento do folículo e seu oócito são eventos paralelos e relacionados funcionalmente (SILVA *et al*., 2016). Na maioria dos mamíferos, o oócito entra nos estágios iniciais da meiose durante a vida fetal e torna-se quiescente na fase de diplóteno da prófase I até que se tornem aptos a ovulação ou atresia (LONERGAN & FAIR, 2015). Em vacas, por volta de 72-82 dias de gestação, alguns oócitos do feto já iniciam a primeira prófase meiótica, passando pelos estágios de leptóteno, zigóteno, paquíteno e diplóteno, no qual ocorre a primeira parada da meiose, também denominado estágio de dictióteno ou de vesícula germinativa (VG) (RICHARDS, 1980).

Oócitos adquirem competência meiótica durante o estágio de vesícula germinativa (VG) através de uma intensa atividade transcricional, na qual estocam RNAm para estágios posteriores ao pico pré-ovulatorio de LH, tais como retomada da meiose, fecundação e embriogênese (GANDOLFI & GANDOLFI, 2001). Em bovinos, a ativação do genoma embrionário acontece relativamente tarde, durante o estágio de quatro células (MEMILI e FIRST, 1999). Assim, o oócito é o principal responsável por assegurar o desenvolvimento embrionário inicial, principalmente devido à presença de grande quantidade de RNAs mensageiros e proteínas que foram acumulados durante crescimento do oócito (FAIR *et al*., 2007, HAMATANI *et al*., 2008).

In vivo, com o objetivo de produzir um oócito haploide, o reinício da divisão meiótica ocorre simultaneamente ao pico de LH em oócitos que completaram o crescimento e adquirem competência meiótica (AYALON *et al*., 1972). Esta competência está correlacionada com o tamanho do oócito e o diâmetro do folículo. Na espécie bovina, oócitos com diâmetro em torno de 110-120 μm, inclusos em folículos de 2 a 3 mm de diâmetro, são considerados competentes para retomada da meiose (LEQUARRE *et al*., 2005). No entanto, somente folículos dominantes com diâmetro maior que 12 mm são responsivos ao estímulo do LH nas células da granulosa bovinas (SARTORI *et al*., 2001). Alguns estudos têm demonstrado que a angiotensina II (Ang II) é um dos fatores que faz a intermediação da retomada da meiose induzida pelo pico de LH em oócitos bovinos e que este evento é dependente prostaglandinas (BARRETA *et al*., 2008).

PORTELA *et al*. (2011) relataram que a Ang II aumenta a expressão de RNAm para anfiregulina e epiregulina e desencadeia a cascata ovulatória. SIQUEIRA *et al.* (2012) mostrou que, semelhante a Ang II e prostaglandinas, a progesterona (P4) também faz a intermediação da retomada da meiose após o estímulo das gonadotrofinas. NUTTINCK *et al*., (2011) reportaram que a produção de prostaglandina E2 pelas células do cumulus aumenta a atividade de proteínas quinases ativadas por mitógenos (MAPK) no oócito durante o reinício da meiose em bovinos.

O bloqueio meiótico no estágio de VG é mantido por elevadas concentrações de monofostato cíclico de adenosina (AMPc) que é produzido pelas células da granulosa e transferido para o oócito via junções comunicantes do tipo GAP. O pico pré-ovulatório de LH, leva a uma redução nos níveis de AMPc, em função da ruptura das junções GAP (entre oócito e células do cumulus) e posteriormente pela redução da sua produção pelas células da granulosa (KAWAMURA *et al.,* 2004). A redução nas concentrações de AMPc provoca a desfosforilação e consequente ativação do complexo fator promotor da meiose, que, por sua vez, está envolvido com a ruptura do envelope nuclear, condensação de cromatina, reorganização do citoesqueleto e bloqueio da atividade transcricional (MILLER; RUSSELL, 1992).

A maturação oocitária envolve duas etapas principais: a maturação nuclear e a maturação citoplasmática. A maturação nuclear inicia com o rompimento da vesícula germinativa (RVG), em virtude dos altos níveis de LH circulante, em que oócito progride para os estágios de metáfase I (MI), anáfase I (AI), telófase I (TI). A partir dessa etapa, ocorrerá a expulsão do primeiro corpúsculo polar, passando rapidamente pela fase de prófase II e resultando em um oócito em metáfase II (MII) (CURCIO *et al*., 2014). A meiose é então bloqueada novamente, e somente será retomada após fertilização (BEZERRA *et al*., 2016) quando o oócito passará pelos estágios de anáfase II e telófase II, seguidos da expulsão do segundo corpúsculo polar e formação do oócito haploide fertilizado. Em bovinos, a completa maturação nuclear ocorre entre 18 e 22 horas após remoção do ambiente folicular: a partir de 8 horas observa-se o rompimento da RVG e entre 12 e 15 horas os oócitos atingem o estágio de MI. Enquanto a transição entre os estágios de AI/TI ocorre rapidamente entre 15 e 18 horas (SIRARD *et al*., 1989).

Já a maturação citoplasmática envolve realocação de organelas, modificação da permeabilidade da membrana plasmática e a diferenciação da maquinaria de sinalização do cálcio, bem modificações transcricionais relacionadas ao perfil de expressão de proteínas de controle do ciclo celular responsáveis por tornar o oócito competente para ser fertilizado (TOSTI, 2006). Gonadotrofinas hipofisárias e a comunicação local bidirecional entre as células

do cumulus adjacentes e o oócito são importantes para maturação nuclear e citoplasmática (LONERGAN & FAIR, 2015).

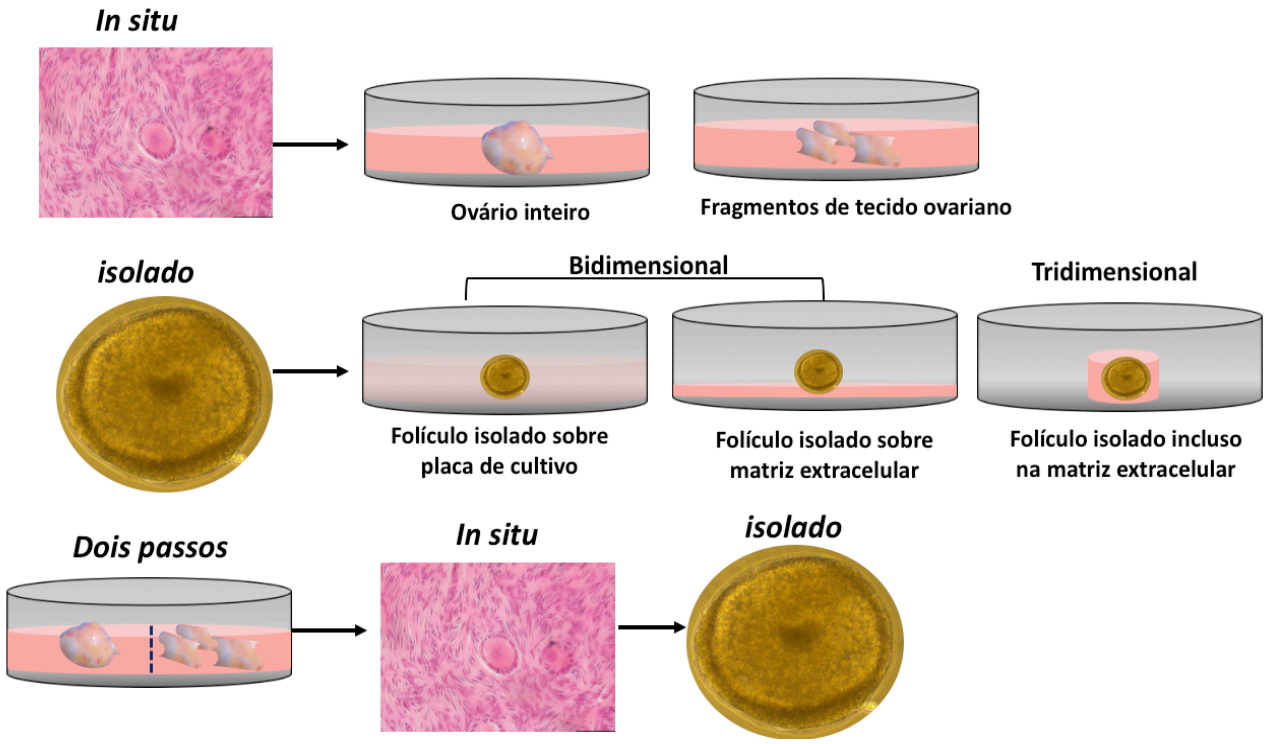
2.2.2. Desenvolvimento folicular in vitro

Para o desenvolvimento folicular *in vitro*, os folículos são resgatados do ambiente ovariano e cultivados até a sua completa maturação. A biotécnica de Manipulação de Oócitos Inclusos em Folículos Ovarianos Pré Antrais (MOIFOPA), também conhecida como Ovário Artificial, vem sendo desenvolvida na tentativa de elucidar os mecanismos envolvidos na regulação da foliculogênese inicial, realizar testes *in vitro* da ação de fármacos, radioatividade, vacinas imunoesterilizantes e nanopartículas sobre os oócitos, que se apresentam como uma opção ao uso de animais em experimentos. Além disso, visa produzir bancos genéticos (germoplasma) e aperfeiçoar a reprodução assistida de humanos e animais (FIGUEIREDO *et al.*, 2018).

A eficiência do cultivo *in vitro* pode ser avaliada por meio de diversos parâmetros, os quais são importantes para o entendimento da regulação da foliculogênese. Dentre eles destaca-se a ativação de folículos primordiais, sobrevivência, crescimento folicular e oocitário, formação de antro, expressão de RNAm para fatores chaves da foliculogênese, produção hormonal, maturação oocitária nuclear e citoplasmática, produção embrionária e de crias saudáveis (FIGUEIREDO *et al*., 2018).

Basicamente, os folículos pré-antrais (FPs) podem ser cultivados de duas formas: inclusos no tecido ovariano (*in situ*) ou na forma isolada. O cultivo *in situ* pode ser realizado utilizando o ovário inteiro (murinos) ou fragmentos de córtex ovariano. Já o cultivo de folículos isolados pode ser realizado diretamente na placa ou sobre uma matrix extracelular (MEC – sistema bidimensional - 2D) ou inclusos em MEC (sistema tridimensional - 3D) (figura 5). Em adição, pode ainda ser realizado um cultivo em dois passos, no qual é realizado primeiramente o cultivo *in situ*, que permite a ativação e o desenvolvimento do folículo primordial até estágio secundário, para posteriormente ser isolado e cultivado até o estágio antral (FIGUEIREDO *et al.,* 2018).

Figura 5. Diferentes sistemas de cultivo *in vitro* de folículos ovarianos.



Fonte: elaborada pela autora.

O cultivo de córtex ovariano permite a manutenção do contato intercelular e a integridade tridimensional dos folículos, fornecendo um complexo sistema de suporte que se assemelha ao ovário *in vivo* (ABIR *et al*., 2006), e objetiva estudar os fatores e mecanismos que controlam o início do crescimento de folículos primordiais quiescentes, ou seja, a ativação folicular (caprino: CELESTINO *et al*., 2011; ovino: CAVALCANTE *et al*., 2016; MONTE *et al.,* 2021; bovino: SILVA *et al*., 2013; RIBEIRO *et al*., 2015; PASSOS *et al.,* 2016). Em relação aos fatores que influenciam o crescimento dos folículos primordiais em bovinos, alguns estudos *in vitro* demonstraram que FSH (HULSHOF et al., 1995; RIBEIRO *et al*., 2015), jacalina (RIBEIRO *et al*., 2015), ativina A (HULSHOF *et al*., 1995; MCLAUGHLIN *et al*., 2010), testosterona (YANG; FORTUNE, 2007), VEGF (YANG; FORTUNE, 2006), IL-1ß (PASSOS *et al*., 2016), melatonina (CAVALCANTE *et al*., 2019) estão envolvidos. É importante destacar que o conhecimento dos mecanismos que controlam o desenvolvimento folicular, permite a manipulação *in vitro* da ativação de folículos primordiais e fornece basicamente duas abordagens científicas: 1) estimular a ativação, aumentando o *pool* de folículos em crescimento e, consequentemente, resultando em maior disponibilidade de oócitos competentes; 2) suprimir a ativação, preservando a reserva ovariana para uso futuro (DEVOS; GROSBOIS;

DEMEESTERE, 2020; VO; KAWAMURA, 2021). A possibilidade de manipular a ativação *in vitro* de folículos primordiais inclusos em tecido ovariano tem importância na prática clínica para tratar fêmeas com uma reserva folicular diminuída, buscando assim maximizar o número de oócitos maduros disponíveis (RESETKOVA *et al.,* 2013; VO; KAWAMURA, 2021).

No sistema de cultivo de folículos isolados, utiliza-se, em geral, folículos secundários para a obtenção de um maior número possível de oócitos meioticamente competentes (suínos: WU; EMERY; CARRELL, 2001; bubalinos: GUPTA *et al.,* 2008; ovino: ARUNAKUMARI; SHANMUGASUNDARAM; RAO, 2010; caprinos: SARAIVA *et al*., 2010; MAGALHÃES *et al*., 2011; bovinos: PAULINO *et al*., 2018, 2019; BEZERRA *et al.,* 2019, 2020; VASCONCELOS *et al.,* 2021).

A composição do meio é outro fator importante para a obtenção de sucesso durante o cultivo *in vitro* de folículos pré antrais. Uma variedade de meios de base tem sido usada para o *in vitro* de folículos pré antrais em muitas espécies, dentre eles o meio essencial mínimo (MEM); meio McCoy's e αMEM. Esses meios são ainda comumente suplementados com diferentes substâncias, bem como diferentes fatores de crescimento (TELFER *et al.,* 2008) e hormônios, tal como o FSH. Em bovinos, vários estudos mostraram que fatores como IGF1 (THOMAS *et al*., 2007), ativina A (MCLAUGHLIN *et al*., 2010; PASSOS *et al*., 2014), EGF (PAULINO *et al*., 2019), FGF2 (SUN *et al*., 2013) e GDF9 (VASCONCELOS *et al*., 2013) influenciam o desenvolvimento de folículos secundáros.

Embora os resultados a partir do cultivo *in vitro* de folículos pré-antrais sejam bastante promissores, é importante considerar que diversos fatores, pH, temperatura, luminosidade, tensão de oxigênio, excesso de manipulação, etc., podem afetar a qualidade celular e culminar na morte dos folículos (SOTO-HERAS; PARAMIO, 2020), o que pode reduzir, de maneira significativa, a eficiência dos sistemas de cultivo e dificultar a reprodutibilidade dos achados até então descritos, especialmente, em animais domésticos. Esses fatores, de algum modo, podem atuar induzindo a produção e acúmulo excessivo de ERO, que são uma das principais responsáveis pela atresia folicular.

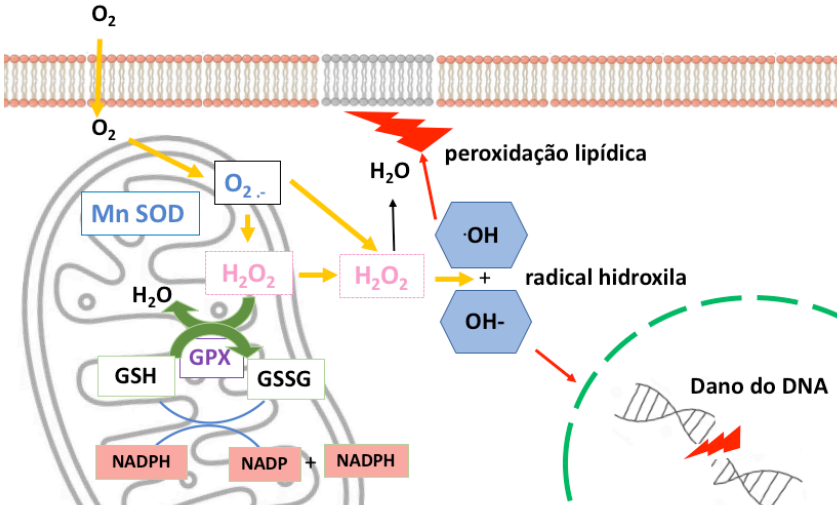
2.3 Estresse oxidativo durante o desenvolvimento folicular *in vitro*

As ERO são substâncias químicas instáveis formadas durante o metabolismo celular normal através das reações de óxido-redução do oxigênio, dentre as quais destacam-se o radical superóxido ($O_2^{-\bullet}$), o peróxido de hidrogênio (H_2O_2) e o radical hidroxila ($OH^{\bullet}$). Quando produzidas em proporções adequadas, as ERO desempenham um papel importante como mensageiros secundários em muitas reações fisiológicas (ALLEN; TRESINI, 2000; WANG *et*

al., 2017), incluindo alguns eventos no ovário (BEHRMAN *et al*., 2001; FUJII; IUCHI; OKADA, 2005). Dentre esses eventos destacam-se a retomada da meiose em oócito primários (TRIPATHI *et al*., 2009; PREMKUMAR; CHAUBE, 2015) e a ovulação do CCO pelo folículo pré-ovulatório (SHKOLNIK *et al*., 2011). A retomada meiótica de detenção de diploteno nos estágios M-I e M-II foi induzida pela geração tônica de H_2O_2 em ratas. (TRIPATHI *et al*., 2009). Já na ovulacão, durante o pico dos níveis de LH e a estimulação de seus receptores nas células murais e do cumulus, há a produção de ERO, que irão atuar como moduladores de moléculas relacionadas à inflamação (prostaglandinas, citocinas, enzimas proteolíticas) e mediar a apoptose das células da parede folicular, eventos que induzirão a eventual ruptura do folículo, permitindo a liberação do oócito (RIZZO *et al.,* 2012; PANDEY; CHAUBE, 2014). Além disso, as ERO são geradas pelas células luteais e estão envolvidas na síntese de progesterona e regressão natural do corpo lúteo (CARLSON *et al*., 1995; BEHRMAN *et al.,* 2001).

Nos sistemas aeróbicos, é essencial o equilíbrio entre as espécies reativas de oxigênio e o sistema de defesa antioxidante. Esses agentes oxidantes são gerados endogenamente, como consequência direta do metabolismo do O_2 e a produção em excesso de ERO, associado a mecanismos de defesa ineficientes, resulta em estresse oxidativo, que é responsável por efeitos prejudiciais às biomoléculas de células e tecidos, incluindo o DNA, as proteínas e os lipídios (VASCONCELOS *et al*., 2007; BARBOSA *et al.,* 2014). Ainda hoje, um dos fatores associados à baixa qualidade embrionária *in vitro* ainda é a geração de grande quantidade de ROS durante a maturação de oócitos *in vitro*, fertilização e desenvolvimento embrionário (WAIZ *et al*., 2016). Durante a maturação do oócito *in vitro*, o estresse oxidativo pode induzir erros cromossômicos, peroxidação lipídica e dano mitocondrial, que afetam seu potencial para formar um blastocisto (CHOI *et al*., 2007). O número de mitocôndrias no oócito é 100 vezes maior que o das células somáticas, o que mostra a necessidade de produção de energia para um drástico crescimento e diferenciação celular (MICHAELS *et al*., 1982). Portanto, a maioria das ROS é produzida a partir deste sistema, mesmo em condições fisiológicas. Velez-Pardo *et al*. (2007) revelaram os níveis mais elevados de ROS em embriões não competentes do que em embriões competentes, os quais têm correlação com dano mitocondrial (Figura 06).

Figura 6. Diagrama esquemático da geração de ROS e seus efeitos nas funções celulares.



Fonte: Adaptado de VELEZ-PARDO et al. (2007).

O equilíbrio entre a produção de ROS e sua desintoxicação é essencial para o sucesso da maturação oócito *in vitro*, fertilização e desenvolvimento embrionário. É importante ressaltar que a cultivo *in vitro* é realizada em incubadoras com 5% de CO_2 e aproximadamente 20% de O_2, devido ao menor custo em relação aos sistemas de incubadoras com baixa tensão de oxigênio atmosférico. No entanto, Fischer e Bavister (1993) relataram que a quantidade essencial era muito menos da metade do O_2 atmosférico, variando de aproximadamente 8,7% O_2 no oviduto de coelho, enquanto no útero de hamster e macaco é ainda menor (1,5% O_2). A exposição de células a altos níveis de O_2 atmosférico causa aumento na produção de ROS, que são altamente prejudiciais e desencadeiam danos celulares (CORRÊA *et al*., 2008; ROCHA-FRIGONI *et al*., 2016). Essas condições de cultivo têm sido associadas à baixa qualidade e viabilidade do embrião (TRUONG *et al*., 2016). Muitos outros fatores podem causar estresse oxidativo, como o manuseio de oócitos durante a coleta e maturação *in vitro*. Outros fatores associados ao estresse oxidativo incluem a exposição excessiva à luz, a ausência de uma combinação eficiente de antioxidantes, grandes volumes de meios de cultivo e a natureza estática dos sistemas de cultivo (SMITH E ROCHA, 2012; CASTILLO-MARTIN, 2014). Os componentes dos meios de cultivo também podem causar estresse oxidativo. Hashimoto et al. (2000) descobriram que uma alta concentração de glicose (> 20 mM) no meio IVM aumenta os níveis de ROS intra-oócitos, reduz GSH e prejudica a competência de desenvolvimento do oócito, provavelmente através da promoção da glicólise e fosforilação oxidativa. Além disso, a osmolalidade é outro fator que merece atenção. O meio hiperosmótico (310-330 mOsm / Kg)

prejudica o desenvolvimento do embrião (SWAIN *et al.,* 2011) e pode induzir alta produção de ROS, conforme relatado para outros tipos de células (DENG *et al*., 2015).

Como estratégia para minimizar os efeitos deletérios do estresse oxidativo, uma ampla variedade de antioxidantes tem sido utilizada durante a maturação, fertilização e desenvolvimento embrionário *in vitro* de oócitos. Chamados de antioxidantes não enzimáticos, eles são conhecidos como suplementos dietéticos naturais; incluindo vitamina E, vitamina A, vitamina C, flavonóides, carotenóides, glutationa, melatonina, entre outros (LU *et al*., 2018; XU *et al*., 2017; AGARWAL *et al*., 2005). Eles trabalham para impedir as reações em cadeia dos radicais livres (NIMSE E PAL, 2015; SANTOS-SÁNCHEZ *et al*., 2019; MOUSSA *et al.,* 2019). Muitos antioxidantes naturais podem ser encontrados em alimentos como frutas, vegetais, cereais, cogumelos, bebidas, flores, especiarias e ervas medicinais tradicionais (XU *et al*., 2018). Esses antioxidantes naturais não enzimáticos têm sido testados há décadas e vários estudos apontam para uma contribuição positiva desses suplementos durante a cultivo *in vitro* de embriões (CAJAS *et al*., 2020). Existem também antioxidantes enzimáticos que incluem o sistema de defesa antioxidante celular. Eles são superóxido dismutase (SOD), glutationa peroxidase (GPx), catalase (CAT), glutationa redutase (GSR) e peroxirredoxinas. Eles são capazes de converter produtos metabólicos oxidados em um processo gradual em H_2O_2 e posteriormente em água com a ajuda de cofatores (GOUGH *et al*., 2011; ZHAN *et al*., 2004). Essas enzimas são caracterizadas por reparar 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 na mitocôndria. Finalmente, existem os antioxidantes de defesa de quarta linha, que previnem a formação de radicais livres e suas reações. O sinal gerado pelos radicais livres induz a formação e transporte de um antioxidante apropriado para o local apropriado (STECKIEWICZ *et al*., 2019; AMJAD *et al.,* 2020). No ambiente *in vitro*, acredita-se que todo esse aparato de defesa, devido às adversidades do sistema *in vitro*, pode não ocorrer de forma eficaz. Recentemente, Von Mengden et al. (2020) demonstraram de forma mais específica os mecanismos de ação antioxidante em COC cultivados *in vitro*. Ambos os antioxidantes enzimáticos e não enzimáticos podem regular as reações causadas pelo estresse oxidativo e, assim, restaurar a integridade celular (AMJAD, *et al*., 2020).

A composição dos meios é um dos principais fatores que influencia no sucesso do cultivo *in vitro* e a presença de concentrações apropriadas de antioxidantes nestes meios, na tentativa de contrapor o aumento indiscriminado de ERO*,* tem favorecido a manutenção da

viabilidade e o desenvolvimento folicular (GOUVEIA *et al*., 2016; LINS *et al*., 2017; 2021). Assim, para escolher a melhor combinação de diversos fatores, antioxidantes para suplementar os meios de cultivo *in vitro*, é muito importante conhecer seus mecanismos de ação.

2.4 Melatonina, EGF, progesterona, e suas funções na foliculogênese

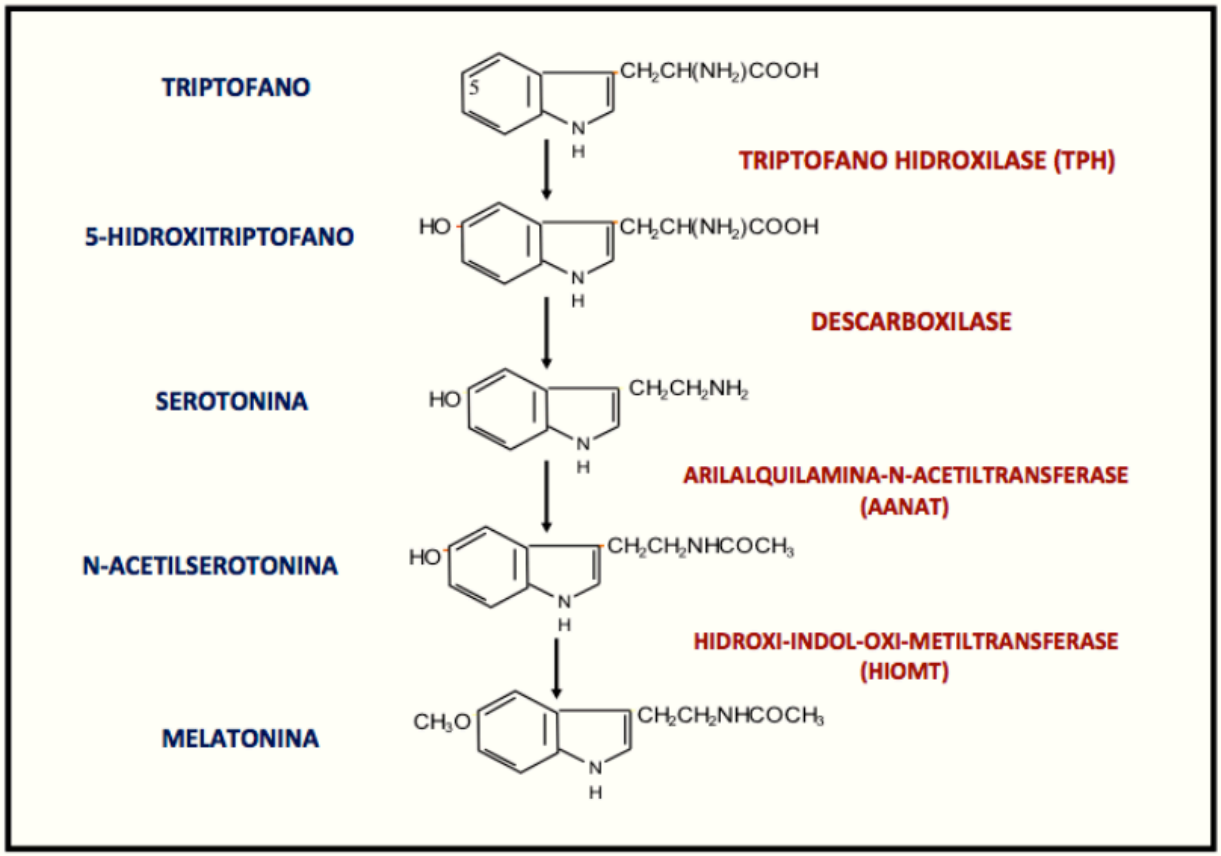
2.4.1. Melatonina e seus mecanismos de ação

A melatonina (N-acetil-5-metoxitriptamina; $C_{13}H_{16}N_2O_2$), descrita, pela primeira vez em 1958, é produzida e secretada endogenamente pela glândula pineal a partir do aminoácido triptofano (TAN *et al*., 2010). A melatonina regula ações centrais e periféricas importantes relacionadas aos ritmos circadianos, e também influencia as funções reprodutivas (TAMURA *et al.,* 2013; SUN *et al*., 2020). Este hormônio tem estado em evidência devido às suas variadas propriedades farmacológica (CLAUSTRAT; LESTON, 2015; HORNEDO-ORTEGA *et al*., 2016), dentre as quais, destaca-se a atividade antioxidante (KIM *et al.,* 2013; MONIRUZZAMAN *et al*., 2018).

A síntese da melatonina se inicia na ausência da luz, quando o triptofano é convertido em 5-hidroxitriptofano (5-HTP) por ação da enzima triptofano hidroxilase (TPH). A enzima 5-hidroxitriptofano descarboxilase (5-HTPD) remove o grupo alfa-carboxila terminal do 5-HTP e o transforma em 5-hidroxitriptamina (5-HT), conhecido como serotonina, que se converte em *N*-acetilserotonina (NAS) pela ação enzima arilalquilamina-*N*-acetiltransferase (AANAT). Em seguida, a NAS, após oximetilação pela enzima hidroxindol-*O*-metiltransferase (HIOMT), origina a melatonina (Figura 7) (DUBOCOVICH *et al.,* 2003).

Além da atividade sistêmica da melatonina, estudos relatam a produção local deste hormônio no ovário e atuação direta na fisiologia ovariana, uma vez que foram detectadas altas concentrações de melatonina no fluido folicular (RÖNNBERG *et al*., 1990; ITOH *et al*., 1999). No ovário, a melatonina é produzida pelas células da granulosa e do cumulus e a concentração intrafolicular aumenta conforme o crescimento foluclar (TAMURA *et al.,* 2009). A melatonina regula a produção de progesterona pelas células da teca, e auxilia no rompimento folicular, que ocorre na ovulação (REITER *et al.,* 2014).

Figura 7. Síntese da melatonina. A melatonina é sintetizada a partir do aminoácido triptofano que, por ação da enzima triptofano hidroxilase, é convertido em 5-hidroxitriptofano. Este, por sua vez, é 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 enzima hidroxindol-*O*-metiltransferase (HIOMT), origina a melatonina.

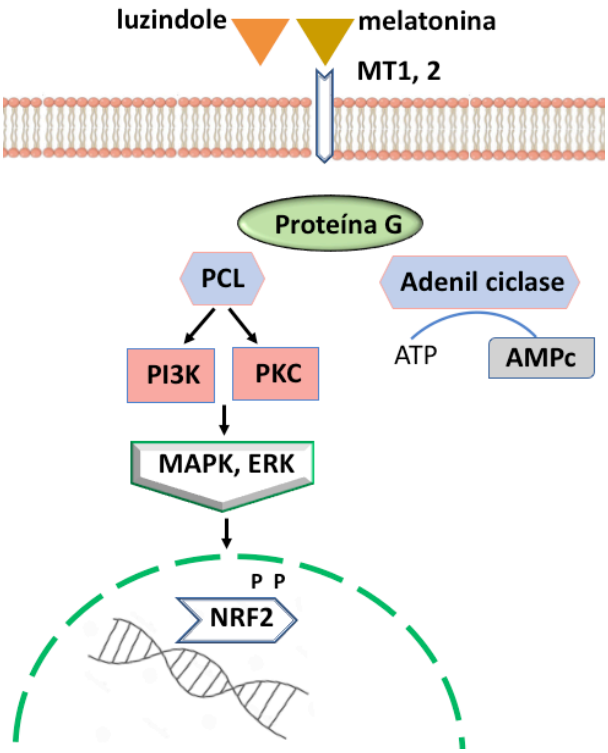


Fonte: adaptado de CIPOLLA-NETO E AFECHE, 2012.

A melatonina pode atuar nas células por meio de processos independentes e dependentes do receptor. Os receptores de melatonina 1A (MT_{1A}) e 1B (MT_{1B}) pertencem à classe dos receptores acoplados à proteína G (GPCR) (STAUCH *et al*., 2020). A melatonina também pode atuar ligando-se a um terceiro sítio encontrado em animais, que é a enzima citosólica quinona redutase 2 (QR2), capaz de neutralizar os radicais livres (ČERNYŠIOV *et al*., 2015; BOUTIN, 2016). O receptor MT_{1A} tem uma afinidade aproximadamente três vezes maior para a melatonina do que o receptor MT_{1B} e é considerado responsável pelos efeitos circadianos da melatonina e modulação da transdução de sinal no sistema reprodutivo (PALA *et al*., 2013; JOCKERS *et al*., 2016). Em células foliculares, estudos sugeriram que o receptor MT_{1A} parece estar mais envolvido na regulação da atividade da melatonina do que o MT_{1B} (BARBERINO *et al*., 2017; TALPUR *et al*., 2017). A proteína para o receptor MT_{1A} já foi imunolocalizada em folículos pré-antrais e antrais caprinos (BARROS *et al*., 2013) e de camundongos

(BARBERINO *et al*., 2017). Além disso, autores avaliaram e demonstraram a imunoexpressão da proteína para o receptor MT_{1A} apenas em CCO ovinos (XIAO *et al*., 2019). No entanto, em bovinos, não há dados sobre a expressão da proteína para o receptor MT_{1A} nos diferentes estágios de desenvolvimento folicular (folículos pré-antrais e antrais). No que diz respeito ao receptor MT_{1B}, já foi demonstrado que por meio da interação com a melatonina, que ele aumenta a produção de trifosfato de adenosina (ATP), reduz a apoptose e eleva as enzimas antioxidantes (HE *et al.,* 2016a). Estudos comprovam que o bloqueio das vias de receptores de melatonina pode acontecer com um inibidor específico (luzindol) (TIAN *et al*., 2014; TIAN *et al*., 2017). O Luzindol (N-acetil-2-benziltriptamina) é um antagonista competitivo do receptor da melatonina amplamente utilizado para estudar as ações da melatonina nas células (WANG *et al*., 2021).

Figura 8. Mecanismos de ação da melatonina dependente dos receptores MT1 e MT2. Os mecanismos de ação da melatonina para regular o estresse oxidativo sob indução da ativação do Nrf2.

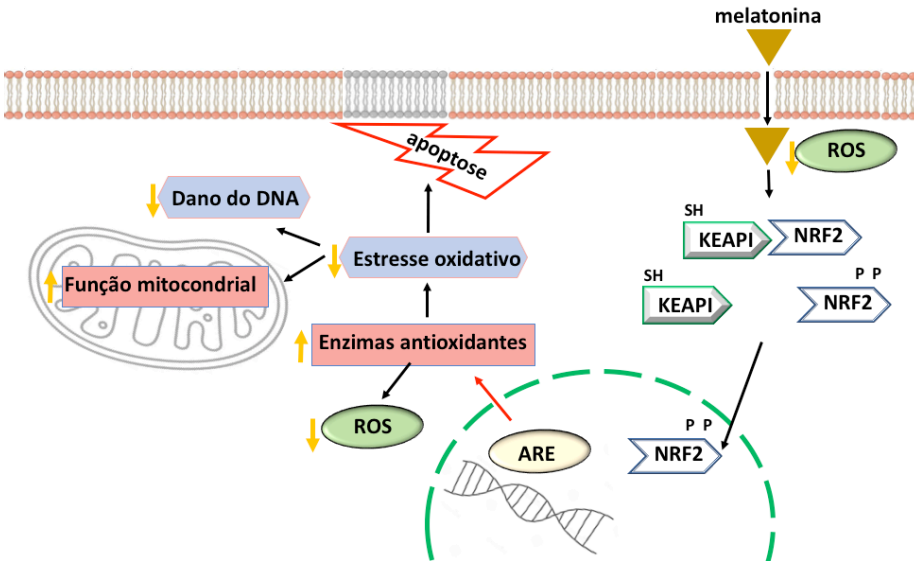


Fonte: elaborada pela autora.

Recentemente, foi relatado que o mecanismo antioxidante da melatonina pode ser regulado pela sinalização do Nrf2 (KIM *et al*., 2019; SHAH *et al*., 2017; TRIVEDI *et al.,* 2016). Já se sabe que o Nrf2 medeia sua atividade antioxidante por translocação para o núcleo, onde

se liga a elementos de resposta antioxidante (ERA) para regular a expressão de genes relacionados à desintoxicação e mecanismos antioxidantes (KANSANEN *et al.,* 2013; MA, 2013). Portanto, observa-se que a melatonina pode regular muitas funções fisiológicas por meio de diferentes vias de sinalização dependentes do receptor. A melatonina também pode atuar independentemente dos receptores (WANG *et al*., 2021). Junto com seus metabólitos, que agem como antioxidantes, ele cria uma cascata antioxidante que produz produtos de eliminação de radicais (REITER *et al*., 2016) (Figura 09), minimizando o estresse oxidativo por meio de uma variedade de mecanismos que está relacionada à sua capacidade de doar elétrons (agente redutor) e, portanto, neutralizar as ERO, especialmente $OH^{\bullet}$ (REITER *et al*., 2003; ANJUM *et al*., 2011). Além disso, devido ao seu tamanho pequeno e propriedades altamente anfifílicas (REITER *et al*., 2009; PARADIES *et al*., 2010), a melatonina pode atuar em meios aquosos e lipídicos, acumulando-se nas mais diversas estruturas celulares, prevenindo danos ao DNA e a peroxidação de lipídios e preservando a função de organelas, em especial, das mitocôndrias reduzindo, assim, eventos apoptóticos e a morte celular induzidos pelo estresse oxidativo (VENEGAS *et al*., 2012; TANABE *et al*., 2015; TAN; REITER, 2019; EL-RAEY *et al*., 2011).

Figura 09. Mecanismos de ação da melatonina independentemente dos receptores. A melatonina consegue passar livremente na membrana das células e com seus metabólitos, induz a fosforilação do NRF2 fator que ativa a proteína ARE que por sua vez cria uma cascata antioxidante reduzindo as ERO e consequentemente o estresse oxidativo influenciando o potencial antioxidante da membrana mitocondrial, transferindo elétrons dentro da célula.



Fonte: Elaborada pela autora.

No cultivo *in vitro* de tecido cortical ovariano bovino, a melatonina promoveu a ativação de folículos primordiais e manteve a densidade de células estromais (CAVALCANTE *et al*., 2019). Em folículos secundários, a melatonina melhorou o desenvolvimento folicular em combinação com FSH (ROCHA et al., 2013), induziu a formação de antro, o crescimento oocitário e a retomada da meiose, aumentou os níveis oocitários de GSH e mitocôndrias ativas e diminuiu os níveis oocitários de ERO (BARROS et al., 2020a). Em oócitos ovinos de folículos antrais iniciais cultivados *in vitro*, a melatonina promoveu o desenvolvimento, diminuiu os níveis de ROS, aumentou a atividade mitocondrial e promoveu a aquisição da competência meiótica desses oócitos (BARROS *et al*., 2020b). Durante a maturação de COC (padronizar, tem local que é CCO), a melatonina proporcionou uma proporção maior??? de oócitos que extrusaram o corpo polar, com níveis de ROS relativamente baixos em porcos (SHI *et al*., 2009) e aliviou os defeitos meióticos em oócitos de camundongos (TAMURA *et al*, 1998).

A melatonina também tem efeitos relevantes no desenvolvimento embrionário *in vitro*. Em suínos, a melatonina teve um efeito positivo nas taxas de clivagem e aumentou o número total de células dos blastocistos (RODRIGUEZ-OSORIO *et al*., 2007). Além disso, a suplementação de melatonina no meio de cultivo *in vitro* de oócitos bovinos envelhecidos aumentou significativamente a velocidade de desenvolvimento até o estágio de blastocisto após a fertilização *in vitro* e diminuiu a taxa de apoptose (LIANG *et al.,* 2017). Sánchez-Ajofrín *et al.* (2020) relataram recentemente que a adição de melatonina ao meio de transporte do ovário aumentou as taxas de clivagem e blastocisto e teve um efeito positivo na expressão de genes essenciais relacionados ao desenvolvimento embrionário. A melatonina também tem a capacidade de proteger os embriões dos efeitos nocivos de diferentes fatores de estresse, como o calor (RODRIGUEZ-OSORIO *et al.,* 2007; LI *et al.,* 2015; CEBRIAN-SERRANO *et al*., 2013; CAVALLARI *et al.,* 2019) e H_2O_2 (HE *et al*., 2016). Esses achados abrem perspectivas para o uso seguro e eficiente da melatonina como potencial efeito sobre o desenvolvimento *in vitro* de folículos pre-antrais inclusos em tecido ovariano bovino. É importante compreender também os possíveis mecanismos pelos quais a melatonina pode desempenhar suas ações no ovário.

2.4.2. EGF e seus mecanismos de ação

O EGF é um membro da família EGF que promove o crescimento e a diferenciação celular pela ligação ao receptor do fator de crescimento epidérmal (EGFR). A família EGF

compreende aproximadamente 10 proteínas, que compartilham características estruturais e funcionais semelhantes, incluindo EGF, fator de crescimento transformante-α (TGF-α), neuregulinas 1–4 (NRG 1-4), fator de crescimento semelhante ao fator de crescimento de ligação à heparina (HB-EGF), anfirregulina (AREG), epirregulina (EREG) e betacelulina (BTC) (ASHKENAZI *et al.,* 2005; HARRIS *et al*., 2003).

Já é sabido que o EGF estimula o crescimento do oócito durante a transição do folículo primordial para o folículo primário *in vitro* (SILVA *et al.,* 2004). Os receptores de EGF parecem estar localizados principalmente nos oócitos de folículos primordiais e primários (GARNETT;WANG;ROY, 2002). Além disso, o EGF em concentrações semelhantes ou superiores a 10ng / mL atua indiretamente na inibição da ativação de folículos primordiais (DOS SANTOS *et al*., 2018) bem como na produção de ROS (AGUIAR *et al*., 2017).

Em folículos secundários, por outro lado, os receptores de EGF são observados predominantemente nas células da granulosa e têm sido implicados na proliferação das células da granulosa (REEKA;BERG;BRUCKER, 1998; WANDJI; EPPIG; FORTUNE; 1996). Recentemente um estudo monstrou que tanto a sobrevivência como o diâmetro de foliculos secundários bovinos, foram significativamente influenciados pela suplementação de EGF sistema de cultura 3D sem matriz (JACKTER *et al*., 2022). Outros estudos mostram que, em folículos pré-antrais, a suplementação de EGF no cultivo *in vitro* promove o desenvolvimento e sobrevivência folicular (equinos: MAX *et al*., 2018; AGUIAR *et al*., 2017; bovinos: DOS SANTOS *et al*., 2018).

Durante os estágios finais do desenvolvimento folicular, o EGF e seus receptores realizam a mediação do sinal de LH, afetando assim a maturação do oócito e o desenvolvimento folicular (RCHANI & GILCHRIST, 2018). Observa-se que o LH se liga a seus receptores nas células da granulosa da parede folicular durante a onda de LH, levando a um aumento acentuado na concentração de cAMP intracelular e ativação de p38MAPK-CREB (proteína de ligação ao elemento de resposta a cAMP proteína quinase ativada por mitógeno PKA-p38), que regula positivamente a expressão de membros da família EGF (SHIMADA *et al*., 2016). Após a ligação ao seu receptor, o EGF pode inibir a expressão do RNAm de Nppc (o gene que codifica o peptídeo natriurético tipo C [CNP]), levando ao nível reduzido de cGMP nas células da granulosa (SHUHAIBAR *et al.,* 2015), o que promove o desenvolvimento de oócitos primários.

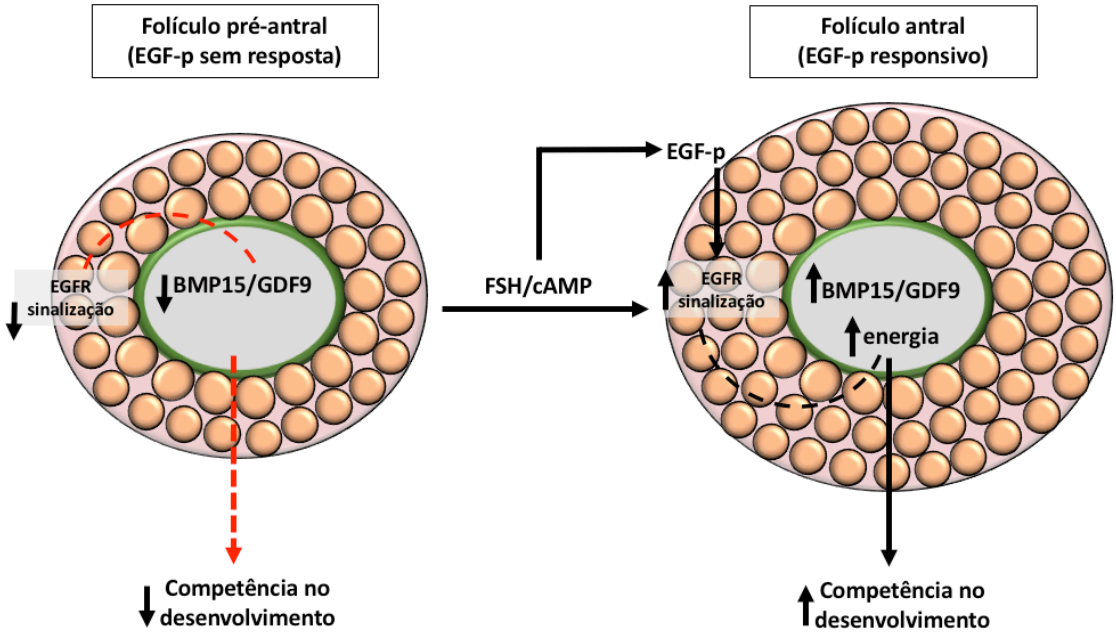
Dessa forma, o EGF e três peptídeos principais de EGF (ou seja, AREG, EREG e BTC) são indicados para induzir a maturação de oócitos, incluindo maturação nuclear (YANG *et al*., 2017), maturação citoplasmática (AMBEKAR *et al*., 2015), expansão do cúmulus

(SETYAWAN, & LEE, 2019) e processo de ovulação (SHIMADA *et al*., 2016). A ativação do EGFR, em cooperação com os fatores secretados pelo oócito (OSFs), estimula a expressão gênica que permite a expansão do cúmulus e a ovulação (MOTTERSHEAD *et al.,* 2015).

EGF e EGFR distribuíram-se principalmente em células da granulosa, células do cumulus, oócitos, fluido folicular e teca externa de folículos pré-antrais e folículos antrais (bovino: YURU LUO *et al.,* 2020; camundongo: ZHANG *et al.,* 2015). Vários estudos mostraram que a expressão de RNAm de EGFR é menor em CCs de pequenos folículos antrais do que em grandes folículos antrais homólogos (SINGH *et al*., 1995; PROCHAZKA *et al*., 2003; CAIXETA *et al*., 2009; EL-HAYEK *et al.,* 2014). No entanto, em um modelo porcino, foi observado que CGs de pequenos folículos antrais (<4 mm) exibem expressão igual de transcritos de EGFR como aqueles de folículos antrais maiores (> 4 mm), porém a produção de proteína EGFR e a fosforilação e a atividade subsequente de ERK1/2 foram prejudicadas (RITTER *et al.,* 2015).

Os complexos cumulus-oócitos derivados de pequenos folículos antrais, que têm baixa competência de desenvolvimento, exibem sinalização de EGFR subdesenvolvida, pois não respondem aos peptídeos de EGF (EGF-p), dessa forma, a aquisição da capacidade de sinalização do EGFR pelas células cumulus é uma marca registrada do desenvolvimento do COC (RICHANI E GILCHRIST, 2018). À medida que a foliculogênese progride, a funcionalidade do EGFR é induzida em COCs pelas ações combinadas de FSH / cAMP e fatores secretados pelo oócito (dos quais BMP15 e GDF9 / cumulina foram identificados) e está associada ao aumento da competência de desenvolvimento do oócito. A estimulação do peptídeo EGF induz a glicólise nas células do cúmulus e fornecimento de metabólitos (por exemplo, NADPH) para o oócito, facilitando a atividade mitocondrial do oócito e a produção de energia necessária para o desenvolvimento (figura 10).

Figura 10. Mecanismo hipotético de cooperação entre o FSH endócrino e sinais parácrinos de oócitos para promover a sinalização de EGFR em células do cúmulus.



Fonte: figura adaptada de RICHANI E GILCHRIST, 2018.

Nesse contexto, torna-se evidente a grande importância do EGF para a foliculogênese ovariana, uma vez que o EGF exerce papel importante na regulação de diversos processos, incluindo a ativação de folículos primordiais, proliferação de células da granulosa, redução da taxa de atresia e manutenção da viabilidade folicular. Com base nos estudos realizados, é notório o envolvimento positivo do EGF para o desenvolvimento folicular *in vitro*, porém, mesmo com os avanços, esse fator sozinho ainda não é capaz de promover a competência oocitária a partir de foliculos pré-antrais cultivados.

2.4.3. Progesterona e seus mecanismos de ação

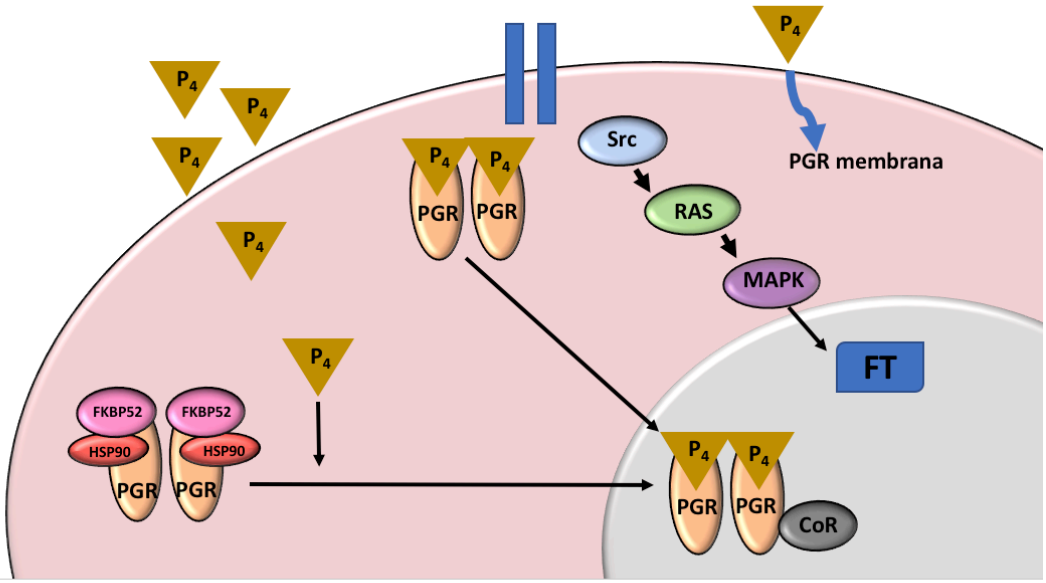
A progesterona (P4) é um hormônio esteroide endógeno que é comumente produzido pelo córtex adrenal, bem como pelas gônadas, que consistem nos ovários e nos testículos. A molécula P4 é um derivado do colesterol, composta exclusivamente de 21 átomos de carbono e tem inúmeras funções no corpo humano, especialmente no sistema reprodutor (GOLETIANE *et al*., 2007). A P4, por sua vez, é um hormônio essencial na oogênese e desempenha um papel no crescimento folicular, ovulação e luteinização (KIM *et al.,* 2017). P4 também é necessária para o apoio materno da sobrevivência e desenvolvimento do concepto a gestaçõ em mamíferos (HAYASHI *et al*., 2012).

Embora a via biossintética de produção de P4 seja concluída de maneira independente do órgão produtor de esteroides, o subconjunto específico e a quantidade de qualquer hormônio

esteroide são dependentes da expressão enzimática de cada órgão respectivo. Por exemplo, enquanto as gônadas os ovários realizam esteroidogênese, esses órgãos diferem na expressão de enzimas específicas, resultando na produção de níveis únicos e variados de P4 (TARABORRELLI *et al*., 2015).

Os efeitos da P4 no ovário são mediados indiretamente, via eixo hipotalâmico-hipofisário, ou diretamente, via interações com os seus receptores no ovário (SLOMCZYNSKA *et al.,* 2000). Ao se ligar ao citoplasma, a P4 sofrerá dimerização seguida de translocação para o núcleo, onde poderá se ligar a uma fita de DNA. A ligação da P4 ao DNA dentro do núcleo permite a regulação subsequente da expressão gênica. Esse hormônio esteróide se liga principalmente ao receptor de progesterona (PGR) localizado em todo o corpo, comumente encontrados como três isoformas dos receptores de progesterona: PR-A, PR-B e PR-C. Existe uma relação antagônica entre os receptores PR-A e PR-B. PR-A pode inibir a transcrição de DNA que é induzida por PR-B, bem como o receptor de estrogênio (VALADEZ-COSMES *et al.,* 2016; TARABORRELLI *et al*., 2015). A sinalização canônica da P4 requer a presença do ligante da P4 e a liberação do PGR pelas proteínas chaperonas. Após a liberação e ligação do ligante de progesterona, o PGR dimerizado entra no núcleo e se liga aos elementos de resposta dentro das regiões promotoras e governa o recrutamento de co-reguladores da transcrição. Este método de sinalização genômica é o mais lento dos mecanismos de sinalização da P4. O PGR também pode funcionar em um contexto não genômico por meio da ligação de proteínas contendo o domínio SH3, como a quinase Src, em receptores de membrana específicos. A ligação do PGR à Src quinase induz a rápida ativação da via RAS/RAF/MAPK dentro da célula. Além disso, os receptores de P4 integrados na membrana induzem uma rápida ativação das vias de sinalização celular após a ligação do ligante extracelular (Figura 11). Em células luteais, a P4 intraovariano parece funcionar como uma "luteotropina universal", conforme evidenciado por sua capacidade de promover sua própria secreção (STOUFFER, 2003).

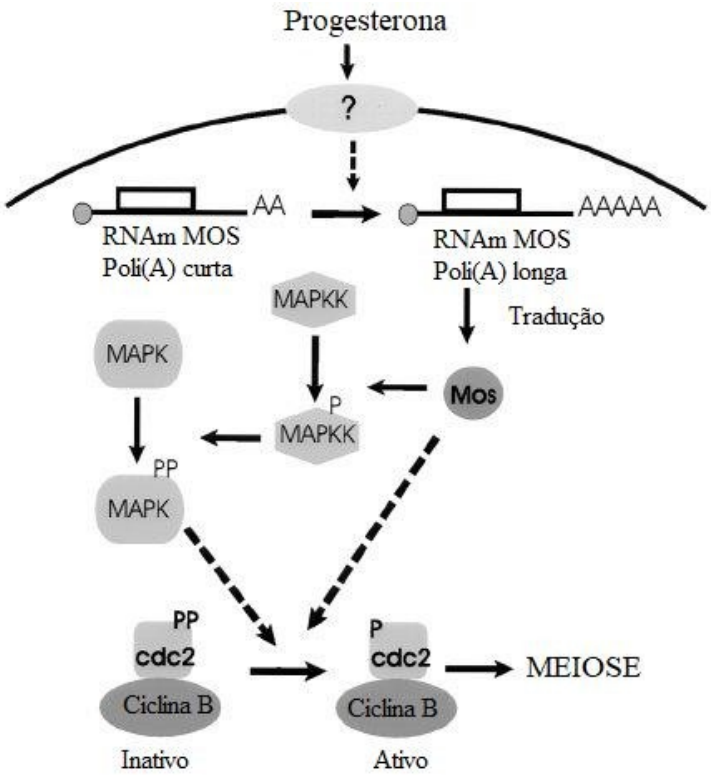
Figura 11**.** O PGR opera através de múltiplas vias de sinalização dentro da célula. Entra no núcleo e se liga aos elementos de resposta dentro das regiões promotoras que governa o recrutamento de co-reguladores da transcrição. E também pode funcionar por meio da ligação de proteínas contendo o domínio SH3, em receptores de membrana específicos induzindo a rápida ativação da via RAS /MAPK dentro da célula para promover o fator de transcrição.



Fonte: elaborada pela autora.

Em bovinos, a P4 induz a transcrição de genes e afeta a competência oocitária, sinalizando através de PGRs que cumina na retomada da meiose (APARICIO *et al.,* 2011; O´SHEA *et al.,* 2012) (figura 12). Os COCs bovinos expressam receptores nucleares (PGR-A, PGR-B) e P4 ligados à membrana (mPRa, mPRb, componente da membrana do receptor de progesterona (PGRMC 1 e 2) (APARICIO *et al.,* 2011). Além disso, a expressão proteica desses receptores muda dinamicamente após a maturação *in vitro* em resposta a LH, FSH ou P4 (LUCIANO *et al*., 2010). Em bovinos, também foi demonstrado que a quantidade de PR aumenta conforme o folículo se desenvolve, indicando que a P4 pode regular o crescimento folicular durante seus estádios iniciais do desenvolvimento (D´HAESELEER *et al.,* 2007). Também foi verificado que os PR atuam mediando os efeitos protetores da P4 contra a apoptose em células da granulosa de folículos pré-ovulatórios bovinos (VALADEZ-COSMES *et al*., 2016). No entanto, em alguns casos, o efeito de fatores pró-apoptóticos supera o efeito protetor da P4, e a degeneração folicular acontece.

Figura 12. Mecanismo de atuação de progesterona, que influencia a poliadenilação de cMOS e fosforilação de quinases que irão ativar a ciclina B culminando na retomada da meiose.



Fonte: D´HAESELEER et al., 2007.

A sinalização de P4 tem sido estudada principalmente por meio da ativação de receptores nucleares que atuam como fatores de transcrição para estimular a expressão do gene dependente de P4 (BEZERRA *et al*., 2020). Estudos anteriores indicaram que ações colaborativas de P4 e EGF são necessárias para induzir aumento na produção de hCG e potencialização da sinalização de EGF em células da granulosa de folículos periovulatórios em humanos (CHOI *et al*., 2017). CARVAJAL et al. 2015, também demonstraram que o tratamento com P4 potencializou a sinalização da via do EGF em linhagens de células de câncer de mama cultivadas *in vitro*. Em foliculos pré-antrais bovinos ainda é desconhecido as ações colaborativas do EGF e da P4 no cultivo *in vitro.*

2.5 A importância do acúmulo de RNAm nos oócitos durante a foliculogênese

Durante o crescimento de oócitos bovinos imaturos ocorrem mudanças globais do perfil transcriptômico para assegurar a maturação meiótica. Foi identificado um grande número de diferentes genes em oócitos bovinos. Dos quais aproximadamente 75% foram mais expressos em oócitos imaturos, reforçando a ideia de que a maioria dos transcritos é acumulada quando os oócitos se encontram no estágio de vesícula germinativa. Estes transcritos irão coordenar o subsequente desenvolvimento oocitário até a ativação do genoma embrionário

(MAMO *et al*., 2011). Sabe-se que alguns RNAm são traduzidos em oócitos MI e MII, enquanto outros são traduzidos durante a fertilização ou durante os estágios iniciais da embriogênese (MAMO *et al*., 2011).

Durante o período de bloqueio meiótico, apesar da aparente quiescência, os oócitos aumentam expressivamente de volume e sofrem importantes transformações citoesqueléticas e moleculares, que conferem potencial para suportar os demais estágios de desenvolvimento. Tais transformações envolvem não somente a diferenciação e o deslocamento de organelas citoplasmáticas, mas, principalmente, a transcrição e o estoque de RNAm, que serão mobilizados em momentos específicos para síntese proteica (BREVINI-GANDOLFI E GANDOLFI, 2001; SIRARD *et al.,* 2006). Concomitantemente, as células da granulosa se proliferam, diferenciam-se e estabelecem comunicação com os oócitos por meio das junções GAP, com transferência bidirecional de fatores de baixo peso molecular, constituindo o complexo cumulus-oócito. A possibilidade de transcrição gênica durante o estágio diplóteno da prófase I confere aos oócitos capacidade de síntese e de estoque de transcritos essenciais para suportar todo o processo de maturação oocitária até a embriogênese inicial (BREVINI-GANDOLFI E GANDOLFI, 2001).

A primeira grande transição de desenvolvimento em embriões de vertebrados é a transição materno-zigótica (MZT), RNAms maternos são degradados e a transcrição zigótica começa. O splicing alternativo (AS) pré-mRNA permite que um único transcrito primário sintetize vários RNAms, estendendo o conteúdo de informação e as possibilidades regulatórias de genomas eucarióticos superiores (CHENG *et al.,* 2020).

Os zigotos de animais herdam um número considerável de transcritos maternos armazenado no citoplasma durante a oogênese (HIGUCHI *et al*., 2018). Mais precisamente, a maturação molecular trata da transcrição, armazenamento e processamento dos RNAms transcritos que serão, posteriormente, traduzidos em proteínas pelos ribossomos. As proteínas derivadas desses RNAm estão envolvidas tanto na maturação quanto nos eventos celulares subsequentes: fertilização, formação de pronúcleos e embriogênese inicial, devendo, portanto, ser estocadas até sua utilização. Como o consumo desses transcritos será feito antes da ativação do genoma embrionário, o armazenamento correto deles no citoplasma do oócito é de fundamental importância. Após a retomada da meiose, não haverá mais expressão gênica e, portanto, tudo o que foi produzido durante a fase de crescimento deverá ser metabolizado no momento adequado. As maiores causas de falha na maturação oocitária são o armazenamento, processamento e recrutamento inapropriado do RNAm materno (CAMPOS *et al.*, 2011). Do

mesmo modo, a ação de algumas moléculas implicadas na mucificação e do cumulus também é regulada por RNAm maternos (LIN *et al.,* 2014).

Durante o crescimento, os oócitos sintetizam grandes quantidades de RNAm que se acumulam para formar um grande estoque de RNAm materno, dentre esses, estão o GDF9, a quinase cMOS, a ciclina B1 (CCNB1) e a histona com ligante específico para oócito (H1FOO). Quando os oócitos atingem o tamanho máximo, a redução do nível de AMPc no interior do oócito (KAWAMURA *et al.,* 2011) induz alterações na morfologia nuclear resultando na condensação da cromatina (VIEUX; CLARKE, 2018) e consequente retomada da meiose. Bezerra *et al.* (2019) identificou aumento na expressão de H1FOO, cMOS, GDF9 e CCNB1 durante o cultivo *in vitro* de folículos secundários, pré-maturação e maturação de oócitos de folículos antrais.

Alguns pesquisadores afirmam que o crescimento do oócito já está finalizado no estágio de folículo antral, antes da conclusão do desenvolvimento folicular (LI; CHIAN, 2017), pois perto do momento da formação da cavidade antral, há um aumento superior a 100 vezes no volume do oócito. Esse aumento está associado ao acúmulo de mitocôndrias e outras organelas, RNAm e proteínas que apoiarão o desenvolvimento embrionário precoce após a fecundação. O oócito em crescimento também passa por outras mudanças, incluindo o acúmulo de grânulos corticais, que desempenharão um papel importante e esse evento é chamado de maturação citoplasmática do oócito (SÁNCHEZ; SMITZ, 2012; EL-HAYEK; CLARKE, 2016).

No que diz respeito à aquisição de competência oocitária, o oócito não é uma estrutura passiva. Pelo contrário, através da síntese de fatores de crescimento, o oócito controla inúmeras funções das células da granulosa ao seu redor como proliferação, diferenciação, expressão de receptores, esteroidogênese, expansão do cumulus e, consequentemente, determina seu próprio desenvolvimento. Dentre os fatores parácrinos secretados pelo oócito considerados importantes peptídeos reguladores intraovarianos estão o GDF-9 e a BMP15 os quais pertencem à família dos fatores de crescimento transformante beta (TGFβ) e são codificados por genes específicos de mesma designação (CROCOMO *et al.* 2019).

A histona H1oo está localizada no núcleo dos oócitos do estágio da GV e se liga aos cromossomos nos estágios subsequentes. A H1foo é sintetizada se acumula durante a oogênese, e sua presença é mantida até algum estágio do embrião inicial, no qual se segue uma rápida degradação (TANAKA *et al.,* 2001; FU *et al*., 2003). Ao contrário das histonas ligantes somáticas, H1foo provou ser indispensável para a maturação meiótica de oócitos em GV de camundongo (FURUYA *et al*., 2007). Em oócito bovino, foi observado que expressão de H1foo após a GVBD ainda é essencial para a progressão meiótica de oócitos bovinos. Dessa forma,

estes achados demonstram de forma conclusiva que o H1foo é essencial para o processo de maturação de oócitos bovinos (YUN *et al*., 2015). Além disso, sabe-se que alguns RNAms são traduzidos em oócitos entre a metáfase I (MI) e MII, enquanto outros são traduzidos durante a fertilização ou durante os estágios iniciais da embriogênese. Em relação aos mecanismos que regulam o armazenamento e a tradução dos RNAm, um que é particularmente importante é a poliadenilação citoplasmática (RICHTER; LASKO, 2011). Após a fertilização, os RNAm armazenados sofrem extensas modificações pós-transcricionais que resultam na tradução de proteínas e/ou degradação até a ativação da transcrição do genoma embrionário (XIE *et al*., 2018).

Acredita-se que a ativação da tradução de RNAm dormentes não é importante apenas para montar o maquinário que impulsiona o ciclo celular. Também é necessário para a reprogramação nuclear, ativação da transcrição no zigoto e, paradoxalmente, para montar a maquinaria envolvida na desestabilização e degradação do RNAm materno (CONTI; FRANCIOSI, 2018). A substituição dos transcritos maternos por zigóticos é necessária para o desenvolvimento normal e deve ser realizada de forma estritamente regulamentada (ROSA; BRIVANLOU, 2017). Além disso, o potencial de desenvolvimento embrionário e a qualidade dos blastocistos são determinados pela adequada concentração oocitária de RNAm (BIASE *et al.*, 2014). Assim, as etapas críticas na maturação do oócito e no desenvolvimento embrionário inicial dependem exclusivamente de um programa de tradução de RNAm materno.

2.6 Fatores que dificultam a obtenção de oócitos competentes a partir de folículos pré antrais cultivados *in vitro*

ARTIGO 1

Why is it so difficult to have competent oocytes from *in vitro* cultured preantral follicles?

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Why is it so difficult to have competent oocytes from *in vitro* cultured preantral follicles?

Competent oocytes from preantral follicles?

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Author contribution statement

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Competing interest statement

The authors declare that they have not competing interests.

Abstract

The developmental competence of oocytes is acquired gradually during follicular development, mainly through oocyte accumulation of RNA molecules and proteins that will be used during fertilization and early embryonic development. Several attempts to develop in vitro culture systems to support preantral follicle development up to maturation are reported in the literature, but oocyte competence have not yet been achieved in woman and domestic animals. The difficulties to have fertilizable oocytes are related to thousands of mRNAs and proteins that need to be synthesized, long term duration of follicular development, size of preovulatory follicles, composition of in vitro culture medium and the need of multi-step culture systems. The development of a culture system that maintains bidirectional communication between the oocyte and granulosa cells and that meets the metabolic demands of each stage of follicle growth is the key to sustain an extended culture period. This review discusses the physiological and molecular mechanisms that determine acquisition of oocyte competence in vitro, like oocyte transcriptional activity, follicle and oocyte sizes, and length and regulation of follicular development in murine, human and domestic animal species. The state of art of in vitro follicular development and the challenges to have complete follicular development in vitro are also highlighted.

Keywords: Follicle growth. Oocyte maturation. Culture systems. Preantral follicles

Introduction

For most mammalian species, formation of primordial follicles takes place pre-natally and constitutes the basic reserve of gametes, which are recruited for growth throughout the reproductive age. Follicular development involves growth of preantral follicles, i.e., activation of primordial follicle and development to primary and secondary follicle stages; as well as formation of antral and Graafian follicles to release a cumulus-oocyte complex (COC) at ovulation [1-2]. During follicle growth, oocytes have great transcriptional activity and store a large amount of RNA to support the processes of fertilization, embryonic development and activation of embryonic genome [3]. Additionally, changes in carbohydrate and lipid metabolism, mitochondrial function and location, reduction of oxygen radicals, epigenetic reprogramming, and bidirectional communication between cumulus cells and oocytes are observed during oocyte cytoplasmic maturation [4]. All these events are crucial for a proper acquisition of oocyte competence.

Current advances in *in vitro* embryo production are still dependent on a supply of oocytes from large antral follicles, which are present in the ovary in relatively small numbers [5]. Thus, the development of *in vitro* culture systems to explore immature oocytes enclosed in preantral follicles have a great potential to provide a large number of oocytes for *in vitro* fertilization in human [6] and domestic animal species [7-10]. It is important to consider that preantral follicles represent 90 to 95% of the entire follicular population and store the majority of oocytes present in mammalian ovaries [11]. The complex mechanisms controlling follicular development are, however, obstacles to have competent oocytes *in vitro*. Over time, many studies have been conducted to optimize culture systems to support three crucial stages, i.e., primordial follicles activation; growing of primary and secondary follicles up to large antral follicles; and COC maturation (mice [12], human [13], and domestic animals: caprine [14], ovine [15]; bovine [7], swine [16]).

Complete oocyte and follicle development *in vitro* were, however, reported only in mice [12], while oocyte competency was not completely reached in human and domestic animals, despite decades of work. The conditions that influence acquisition of oocyte competence *in vitro* are dependent on follicular and oocyte status. Growing follicles contain oocytes that are producing RNAs and proteins; while oocytes of preovulatory follicles stop transcription and resume meiosis [17]. Attempts to optimize *in vitro* maturation of oocytes (IVM) have been reported, but various factors, like the source of oocytes and the size of follicles from which the COCs are collected are determinant for the success of oocyte competency *in vitro* [18-19]. Evidences have shown that oocyte gene expression profile, length of follicular development and sizes of oocytes and preovulatory follicles in different species are directly related to the chances of having successfully competent oocytes after long term *in vitro* culture of preantral follicles [20].

This review aims to show the characteristics of oocyte transcriptional activity in different species, the length of follicular development, the sizes of oocytes and preovulatory follicles and to explain how these factors can determine the chance of having competent oocytes after long term *in vitro* culture of preantral follicles in mice, human and domestic animals species.

Transcriptional activity in oocytes of murine, human, and domestic animal species

Since the sequencing of the first genome to the present day, the scientific community has relied on a single genome that is used as a reference for each species, and thus used as the basis for a wide range of genetic analyzes, including studies of variation within and between the species [21]. The number of genes is quite variable between species (Table 1) and all ovarian functions from primordial follicle development to luteal phase are probably related to differences in gene expression. Oocytes have high transcriptional activity and store a great

amount of RNA to support the processes of fertilization, early embryonic development and activation of genome [3]. Moreover, transcriptional activity in the oocyte is discontinuous throughout follicular development and it is inactivated as the oocyte resumes meiosis. The ultrastructure of oocyte nucleolus and the location of proteins indicate that synthesis and maturation of ribosomal RNA does not occur continuously [70]. Oocytes at germinal vesicle (GV) stage are highly transcriptionally active, accumulating large amounts of messenger RNA (mRNA) that are subsequently needed for the transition from oocyte to zygote. A large proportion of transcripts, about 30% in mice, is stored in GV oocytes and used later during oocyte nuclear maturation and early embryonic development, when the transcription is silent [71]. Total RNA content differs importantly among species (Table 1), and these differences can influence the chances of having fully competent oocytes from *in vitro* cultured follicles. This RNA accumulation through endogenous transcription and/or transfer from surrounding somatic cells and oocytes makes the oocyte the most RNA rich cell of the body [72]. A fully-grown mice oocyte accumulates up to ~27 million copies of mRNA molecules [73], while somatic cells contains up to 300,000 RNAs [74].

During growth, oocytes undergo extensive morphological changes and expression of maternal genes is important in determining oocyte quality and directing early embryo development [75]. Table 1 shows the number of genes expressed in oocytes of mice, human and domestic animals. Variation in the number of genes expressed is certainly related with the length of follicular development, and with successfully *in vitro* follicular growth.

It is also important to consider that mRNA alternative splicing can influence acquisition of oocyte competence [76]. Alternative splicing is a versatile regulatory mechanism that allows individual genes to generate more than one mRNA isoform, which in many cases encode functionally distinct proteins [77]. For example, in cow oocytes, a total of 10,494 genes were expressed in the oocyte [78], and a total of 39,014 transcripts were identified [79]. Recent

analyses have shown that the abundance of alternative splicing events reaches > 95-100% in human genes and 63% in mouse genes [80]. Human genes also have higher frequency of alternative splicing events than mice genes (1.92 versus 1.22) [81]. Thus, variation in alternative splicing among species is certainly linked with the complex mechanisms that regulate oocytes growth and are directly related with the difficulties to establish culture conditions to enable oocytes to produce thousands of mRNAs and proteins in proper levels during their growth *in vitro*.

Regulation of mRNA stability also plays a key role during oocyte growth and early stages of embryo development and is closely linked to translational regulation and localization of mRNA [82]. To facilitate the transition from maternal to embryonic control, mammalian oocytes need to store and then selectively use these mRNAs. There is a period of intense metabolic activity resulting in synthesis of large amounts of mRNAs, proteins and macromolecular structures. Unlike the mRNA of most somatic cells, the oocyte mRNA is extremely stable [83]. The half-life of most mice oocyte mRNAs is long (~2 weeks) resulting in accumulation of transcripts in oocyte cytoplasm. After fertilization, stored mRNAs undergo extensive post-transcriptional modifications which results in either protein translation and/or degradation until activation of transcription from embryonic genome [84]. Maternal mRNAs normally undergo decomposition in embryos of all animals during the maternal-zygotic transition, in which developmental control changes from the maternal to the zygotic genome, including degradation of maternal components (mRNAs and proteins) and activation of zygotic genome [85-86].

Micro-RNAs (miRNAs) have recently been identified as potent regulators of gene expression that act through post-transcriptional modification of mRNA to adjust the production of proteins involved in cell signaling pathways. There is growing literature demonstrating that miRNA regulates biologically important pathways for follicular growth and oocyte health in

women [87]. As the ovarian follicles grow, the antral cavity is formed by fluid secretion from granulosa cells. This follicular fluid is rich in nutrients and also in signaling molecules, which include miRNA, allowing communication and nutritional support between mural granulosa cells, cumulus cells and growing oocytes [49]. Many miRNAs can act at different stages of mammalian oocyte development, regulating the proliferation and survival of cumulus cells or directly affecting the oocyte itself [88], as shown in Table 1. Differences in the number of miRNA controlling granulosa cell and oocyte development reflect the complexity of follicular development in each species.

RNA-seq analysis showed similar maternal miRNA counts among cow and pig, while smaller rodent oocytes (mice and hamsters) have lower miRNA counts than cow and pig oocytes [74]. Thousands of miRNA, mRNAs and proteins regulate follicle growth and oocyte competence [89] in each species and this complex control is certainly related with chance of having competent oocyte after long term *in vitro* culture of preantral follicles. However, a comprehensive gene expression analysis is still needed to determine the main targets of each miRNA and how they influence protein expression in *in vitro* grown oocytes. Table 1 shows various miRNA that have important roles in the control of granulosa cell and oocyte development in different species. The function of these miRNA was recently discussed by Wondim et al. [88].

It is important to consider that human and domestic animals have higher number of genes expressed in their oocytes than mice and that mice oocytes have lower miRNA than bovine and porcine oocytes. Alternative splicing events is also lower in mice genes than in human [80]. Thus, it is clear that *in vitro* development of primordial to preovulatory follicles in human and domestic animals is a far more complex process than in mice, since the oocytes need to accumulate much more mRNA, miRNA and proteins. This fact helps to explain why is much

more difficult to have competent oocytes from *in vitro* cultured preantral follicles in human and domestic animals than in mice.

Follicle and oocyte sizes in murine, human and domestic animal species

The size of preovulatory follicles may also be a key marker for the chance of having complete follicle development *in vitro*. In all species, follicle growth has different patterns, since preovulatory follicles of some species reach a diameter up to a thousand times the size of the oocyte [90-91] (Table 2). During follicular growth, oocyte reaches its maximum size and become competent at the antral phase, while the total volume of the follicle continues to expand until the preovulatory stage [102]. Near the time of antrum formation, oocytes show a great increase in their volume, which is associated with the accumulation of mitochondria and other organelles, mRNA and proteins that will support early embryonic development after fertilization [103-104]. Oocytes of mice secondary follicles have already reached 80.5% of their final diameter, while those of bovine and human species reached 55.2% and 60.8 of their final volume, respectively (Table 1). Throughout the development of ovarian follicle there is a positive relationship between follicular size and oocyte diameter, however the changes in oocyte diameter are not directly proportional to follicular size [90, 105]. Follicular and oocyte diameters show great differences among species, especially when mice follicles are compared to those of large animal and human species (Table 2).

It is important to consider that human and domestic animal species have preovulatory follicles with higher diameters than those of mice. Similarly, oocytes have larger size than those of mice. Mice secondary follicles have oocytes that reached 80.5% of their final diameter, which is very different from human and domestic animal species (Table 2). These characteristics reinforces that the development of *in vitro* culture systems to support acquisition of oocyte competency in human and domestic animal species is a very difficult task. It is clear

that a multi-step culture system, with different combinations of many hormones and growth factors in each step of growth, is needed to allow follicles to reach proper size and to accumulate mRNA and proteins to assure fertilization and early embryo development. Large volume of follicular fluid is accumulated during the various stages of antral follicle development and it is important to consider that microvesicles can transport microRNAs from granulosa cells to oocytes, which can determine oocyte fate [106].

Length of follicular development in murine, human and domestic animals

The interval of time between the activation of primordial follicle and its development to preovulatory follicle is around 21 days in mice (Table 1). Antral follicular growth and the final stages of maturation in rodents take around 48 hours [28]. O´Brien *et al.* (2003) reported a mice offspring after culturing, for 22 days, primordial follicles up to ovulatory stages [12], but this *in vitro* oocyte culture period was associated with various health problems during his short lifespan [107].

In human species, follicular development takes around 180 days for a recruited primordial to grow and develop to the tertiary follicles (Table 1) [37, 38]. The duration of development from primordial to secondary follicles is around 90 days, and the development from secondary follicles to antral follicles take approximately 71 days. Small antral follicles requires about 15 days to grow and develop to the preovulatory stage, while these latter follicles requires about 15 to 20 days to develop to the ovulatory stage [37-38,108]. In a long-term culture system (32 weeks) of human preantral follicles, Fabbri *et al.* [109] reported follicle growth and survival in cultured ovarian tissue, but the oocytes were not able to be fertilized. Human preantral follicles have grown in a multi-step culture system and their oocytes reached MII stage, but cytoplasmic and molecular maturation of these oocytes were still not ideal [13]. This data indicate that long term *in vitro* culture period is needed to support follicle

development, which makes this task very difficult. Some authors reported the consequences of the relatively short period *in vitro* to produce human embryos from IVF oocytes, i.e., increased risks of preterm birth, low birth weight, congenital anomalies, and perinatal mortality [110-112].

In domestic animals, the duration of follicular development is approximately 180 days in the cow, while in sheep and pig; primordial follicles take 3 to 5 months to reach the dominant follicle stage (Table 1) [51,61,113], with much of this time spent in the preantral stages of development. The development of early antral follicles until preovulatory stage is also very long, and around 42 days [114-116]. A follicle progress from the preantral to small antral stage in 27 days in sheep species [117], while in goats, this time is approximately 75 days [118]. In goats, culture of early antral follicles *in vitro* for 18 days resulted in pregnancy after oocyte IVF, but early embryo loss was reported [14], probably due to consequences of the 18-day *in vitro* culture period. In cow, even short *in vitro* culture period during oocyte maturation, fertilization and embryo development is associated with increased neonatal mortality and perinatal deaths [119]. This data indicate that much research is still needed to obtain fully competent oocytes after *in vitro* culture of preantral follicles for human and domestic animal species [120].

Preantral follicles from human and domestic animal species requires the development of a tightly regulated culture system with adequate nutrients, cytokines, growth factors, and developmental stage-dependent hormones to support they development across several months. Epigenetic changes in oocytes throughout the process of folliculogenesis can affect pregnancy maintenance and health of offspring [121-123]. Additionally, in a long-term *in vitro* culture system, the follicles are kept in conditions that are different from their natural environment and need adequate conditions to survive and to develop. Consequently, stress conditions can elevate the levels of reactive species of oxygen (ROS). *In vitro*, preantral follicles are exposed to supra

physiological concentrations of oxygen (up to 20%) [92], which can result in excessive formation of ROS [124]. Oxidative stress arises when excessive ROS production reacts with cellular lipids, proteins, and nucleic acids, resulting in DNA fragmentation, and lipid peroxidation, which alters membrane structure and function, and cause protein damage [93]. Various other factors are associated with oxidative stress *in vitro*, i.e., high concentration of glucose, variation in medium osmolality, excessive exposure to light, absence of efficient combination of antioxidants, large volumes of culture media, variation in temperature, and static nature of culture systems [125]. In this sense, rigorous control of culture conditions and specific combination of antioxidants in each step of follicular development are needed to reduce oxidative stress during long-term *in vitro* culture of preantral follicles in human and domestic animal species.

Regulation of follicular development in murine, human and domestic animal species

Considering that a fully-grown oocyte accumulates up to ~27 million of mRNA molecules (mice: [73]), we can speculate that many thousands of proteins control oocyte growth and follicle development. In recent years, an increasing number of new proteins involved in the regulation of follicular development have been identified [94]. Previously, Wang *et al.* [95] identified 2,781 proteins in immature mice oocytes, while Pfeiffer *et al.* [96] identified 3,699 proteins in mice oocytes at metaphase II. In human species, 2,154 proteins were found in the oocytes [99], while in domestic animals, Memili *et al.* [97] revealed 4,395 proteins expressed in cumulus cells and 1092 proteins expressed in bovine oocytes. Recently, Walter *et al.* [98] detected 2,226 proteins in bovine oocytes and this number was highly influenced by maturation condition. Another important aspect linked to follicular and oocyte development is the molecules present in follicular fluid [100, 126]. A study in pigs revealed a total of 2,321 proteins in oocytes of small and large follicles, while 2,876 proteins have been detected in pig follicular

fluid [127]. These data shows that acquisition of oocyte competence, regardless of species, depends on thousands of proteins for a proper regulation of this process. The *in vitro* environment can alter the synthesis of many of these proteins, and also their levels, which can have a negative impact on follicle growth and oocyte competence. Considering the range of proteins that controls oocyte growth, a comprehensive survey of the proteome of follicles and oocytes developed *in vitro* is still needed to allow the development of an efficient multi-step culture system.

State of art of *in vitro* follicle development

Great progress in the in vitro growth of preantral follicles has been described in different species (Table 3). The best results were obtained in mice, i.e., primordial follicles developed up to maturation in a two-step culture system, and birth of live offspring was reported after oocyte fertilization [12]. In human species, Laronda et al. [128] reported the transition from primordial to secondary follicles after in vitro culture of ovarian cortex, and pointed out that the early stages of in vitro human follicle development require the support of native ovarian cortex. More recently, MII oocytes were obtained after in vitro culture of human unilaminar follicles in a multi-step culture system, but their developmental potential is unknown [13].

For domestic animals, in vitro growth of ovine primordial follicles up to primary and secondary follicles was described [15]. Other studies showed that in vitro culture of secondary follicles resulted in production of MII oocytes, but low number of embryos reach the morula stage after oocyte in vitro fertilization or parthenogenetic activation [15]. In goat, various studies showed in vitro growth of primordial follicles up to primary and secondary follicles [92, 131-132], while a few 2 to 16-cell embryos have been produced after in vitro culture of secondary follicles and fertilization of their oocytes [133-135]. More recently, Sá et al. [14] reported the first pregnancy after IVF of goat oocytes from in vitro cultured secondary follicles. In bovine species, various studies also demonstrated activation and growth of primordial

follicles up to primary and secondary follicles in vitro [136-139]. Mclaughlin et al. [129] reported antrum formation after in vitro culture of bovine primordial and intermediate follicles (< 40 μm diameter) in a two-step culture system. After culture ovarian tissues rich in primordial follicles, these authors isolated the in vitro grown secondary follicles (~110 μm in diameter) and demonstrated antrum formation after in vitro culture. Various other studies showed in vitro development of antral follicles after culturing bovine secondary follicles [10, 140-143]. In pig, oocytes from in vitro cultured secondary were fertilized and gave rise to embryos after their fertilization. The main barriers to have competent oocytes, embryos and offspring in human and domestic animal species after in vitro follicle culture are shown in Figure 13. The need of the oocyte to store a very large number of transcripts and proteins during a very long culture period is also a limiting factor (Table 1).

Challenges to have follicular development and oocyte competence in vitro

The development of *in vitro* conditions to reproduce follicular development is a challenge for researchers in the field. It is not only necessary to have the best combination of hormones, growth factors and antioxidants, in each stage of a multistep culture system, to support the synthesis and accumulation of thousands of RNAs and proteins in the oocyte. It is important to consider that *in vitro* cultured follicles are not in their natural environment and need adequate conditions to survive and to develop. Surrounding cells, extracellular matrix (ECM) and their byproducts are important to support successfully follicular development in the ovaries [144]. There are increasing evidences that mechanical signaling plays a crucial role in development of follicles and oocytes throughout folliculogenesis [145]. An appropriately rigid ovarian extracellular environment can be a necessary requirement for follicle survival and growth [146]. Thus, understanding the complexities of mechanotransduction and the biomechanics of oocytes and follicles is very important to develop proper *in vitro* culture systems. To reinforce this, Hayashi et al. (2012) differentiated mice primordial germ cell–like

cells *in vitro* and re-aggregated them with female gonadal somatic cells for further culture [147]. They reported follicle development *in vitro*, but fully functional germinal vesicle oocytes were obtained only upon transplantation under ovarian bursa, confirming that, besides hormones and growth factors, ovarian environment is important for successful development of oocytes.

Besides the aforementioned challenges to have an efficient culture system for preantral follicles, it is important to appreciate how this *in vitro* environment can affect the various levels of epigenetic regulation in oocytes and embryos. Because the epigenome is responsive to the environment, it is not surprising that *in vitro* grown oocytes and embryos have altered gene expression and concomitant changes in chromatin states [148]. The mammalian oocytes acquire its epigenome during their growth and there is a general concern that long-term *in vitro* culture of preantral follicles might interfere with the process of genomic imprinting [149-150]. Correct establishment and maintenance of genomic imprinting during oocyte growth is necessary for normal embryo development. Studies in various animal models have revealed a link between *in vitro* cultured oocytes and altered genomic imprinting [151-152]. Drastic reprogramming of the epigenome occurs during gametogenesis and initial embryogenesis, leading to a reset of epigenetic changes and conversion of differentiated gametes into a totipotent embryo [153]. The dynamics of this epigenome reprogramming has been extensively studied in rodent and woman models, with fewer studies focusing on domestic species. Deficiencies in epigenetic remodeling during this period can cause serious developmental defects, especially in domestic species where assisted reproductive technologies are widely used to accelerate the genetic selection of superior animals [154]. Abnormalities in embryo, fetus, placenta and offspring produced *in vitro* can to be due to the inadequate establishment and / or maintenance of epigenetic changes during the *in vitro* period [155, 76].

The mice born after culturing primordial follicles *in vitro* for 22 days [10] had obesity and hyperplasia of islets of Langerhans, lipidosis in the liver, lymphosarcoma of the small

intestine, and hydrocephaly after 14 months of age. This mouse was not old; males of his genotype generally survive for 2 to 3 years. Probably, his health condition was associated with the circumstances of his origin, i.e., from *in vitro* cultured oocytes [107]. It is important to highlight that in human and domestic animal species, primordial follicles need approximately 180 days of *in vitro* culture to complete their development and the consequences of this long period *in vitro* for the offspring need to be considered. Despite the relatively short period *in vitro* to produce human embryos from oocytes recovered from antral follicles, increased frequency of hypertension in pre-adolescent and adolescent children born using this technology has been observed [156]. In domestic animals, calves derived from IVP embryos have also been reported to experience increased neonatal mortality and perinatal deaths, in large part due to abnormal offspring syndrome which is often manifested as calves born with abnormally large body size [157].

Future directions to have competent oocytes from in vitro cultured preantral follicles

Extensive research is still required to develop a proper *in vitro* system to support long-term culture of preantral follicles from human and domestic animal species. Once isolated from the ovaries, the follicles lose the natural support structure promoted by the adjacent extracellular matrix (ECM), which can detrimentally alter their development *in vitro* [158]. Thus, the development of an artificial ovary to provide an adequate follicular environment capable of recreating a three-dimensional structure can be an alternative to improve *in vitro* follicular development [159]. Several three-dimensional culture systems using alginate, fibrin and polyethylene glycol, have been proposed to recapitulate the mechanical properties of the ovary. These matrices, however, lack the biological functionality of the ECM, do not have all the components present in native ovarian ECM and lack the ability to sequester growth factors [160-161]. Currently, there is growing interest in the use of scaffolds based on decellularized ECM to have an structure that resemble an ovary after seeding ovarian follicles and stromal

cells *in vitro* [162-164]. Ovarian ECM is known to play an essential role not only as a framework that provides architectural support, but also as a specialized microenvironment capable of regulating cell activities and development, including communication, migration, proliferation, survival, steroidogenesis, growth, aggregation and morphology [165]. Thus, the development and improvement of scaffolds based on decellularized ECM may represent a powerful tool to support follicular and oocyte growth under *in vitro* conditions. Furthermore, cell-cell and cell-matrix interactions can create cellular mechanochemical processes that can have a beneficial influence on *in vitro* follicular development [145].

Another alternative to improve follicular development *in vitro* is to supplement culture medium with microvesicles and exosomes, which are naturally present in follicular fluid [166]. Microvesicles and exosomes are extracellular vesicles that play paracrine roles during *in vitro* follicle development by delivering cytoplasmic components of proteins, non-coding RNA, and growth factors [167]. Recently, Zhang et al. (2021) reported that exosomes from stromal cells improves early follicular development and inhibits apoptosis in vitro [168].

Conclusions

Many oocyte inherent factors and the *in vitro* culture environment determine the chance of having successfully complete follicle development and acquisition of oocyte competence *in vitro*. Differences in oocyte transcription levels, in alternative mRNA splicing, in number of proteins, in sizes of oocyte and preovulatory follicles among species are directly related to the complex mechanisms that regulate follicle growth and oocyte maturation, and consequently impairs the establishment of efficient culture system. Additionally, the *in vitro* environment can alter the synthesis of mRNA and proteins, and also their levels, which certainly impact follicle growth and oocyte competence. A multi-step culture system, with different combinations of many hormones, growth factors and antioxidants, in each step of growth, is needed to allow

follicles to reach proper size and to accumulate mRNA and proteins in a long-term culture period, especially for woman and domestic animals. Because thousands of proteins are involved in the control of follicle development, definition of the right combination of factors, in proper concentrations, is not an easy task. It is very important to identify the conditions that promote epigenetic changes that can impair acquisition of oocyte competent in *in vitro* cultured follicles. Identifying specific deleterious epimutations can prevent the associated adverse consequences to embryos and offspring produced from *in vitro* grown oocytes. Many advances in the knowledge of the complex mechanisms that control follicle development in different species are still needed to allow the development of an efficient long-term multi-step culture system for preantral follicles of human and domestic animal species.

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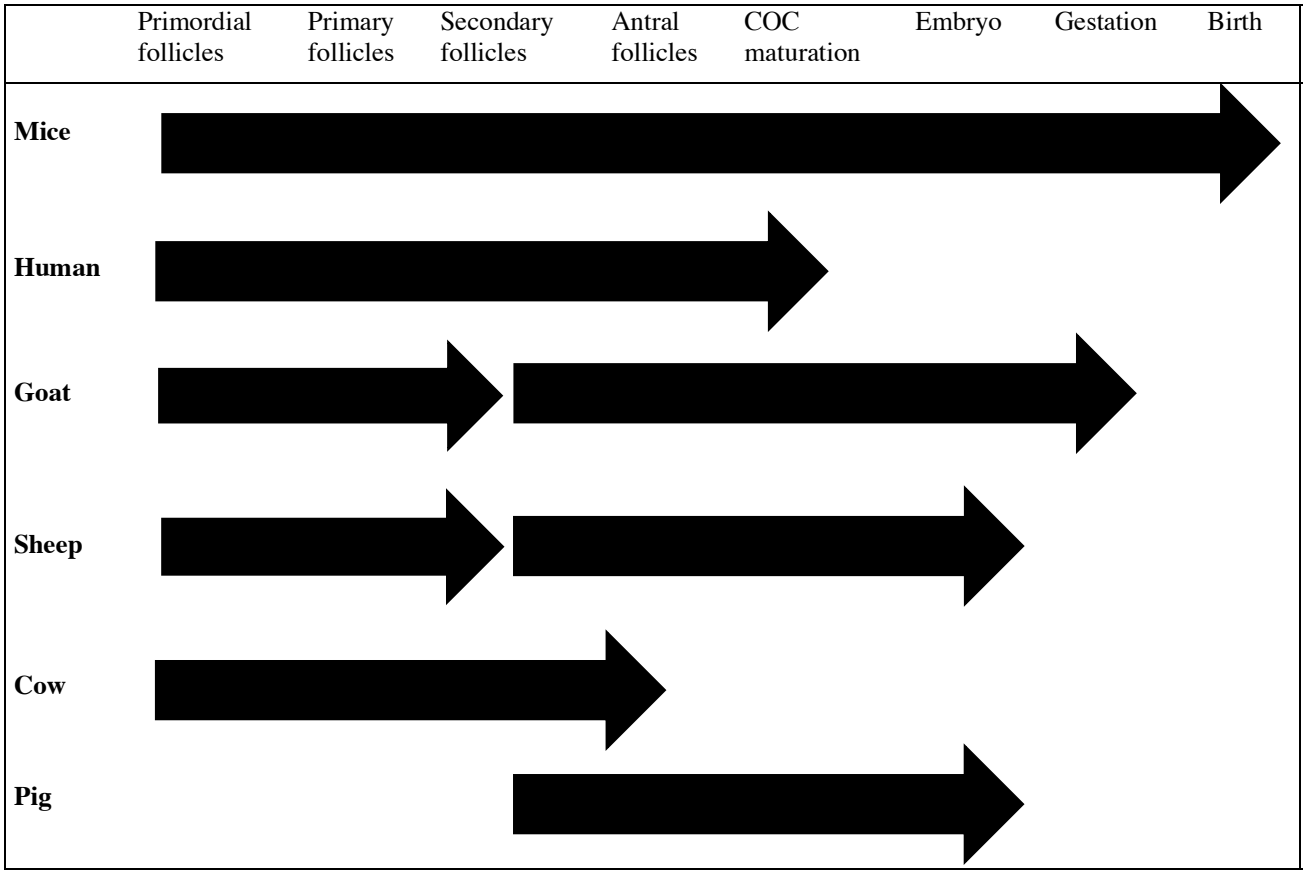
List of figures and tables

Figure 13



Main Barriers
Epigenetic changes in oocytes throughout the folliculogenesis. Offspring with health problems.
MII oocytes with low developmental competence. Abnormal large polar body in matured oocytes.
Primordial follicles do not develop beyond secondary stage. Embryo loss at early stages of pregnancy
Primordial follicles do not develop beyond secondary stage. Embryos fail to develop up to blastocyst stage
Oocyte degeneration after 18 days of culture, inability to resume meiosis and low developmental competence
No reports on primordial to secondary follicle development *in vitro*, embryos fail to develop up to blastocyst stage

References: mice: [12]; human: [13, 128]; goat: [14]; sheep [15]; cow [129]; pig: [16,130].

Tables

Table 1. Genome, number genes expressed in oocytes, total RNA stored in oocytes, miRNA associated with granulosa cell and oocyte development, length of folliculogenesis and successfully in vitro folliculogenesis in mice, domestic animals and woman.

	Genome	**Nº genes expressed in oocytes**	**Total RNA stored in oocytes**	**miRNA associated with granulosa cell and oocyte development**	**Length of folliculogenesis**	**Successfully in vitro folliculogenesis**
Mice	24,408	6,126	0.6 ng	miR-378, miR-383, miR-224 and miR-322-5p	21 days	Yes
Human	46,831	15,165	330 pg	miR-335-5p, miR-99a, miR-143, miR-15a, let-7b-5p and *miR-21-5p*	180 days	No
Goat	22,175	20,020	--	miR-101-3p, miR-1271, chi-miR-4110, miR-10b, miR-206 and miR-1	150 days	No
Sheep	21,160	11,693	0.76 ng	oar-miR-485-3p, oar-miR-377-3p, oar-miR-150 and miR-143	150 days	No
Cow	22,000	10,494	2.4 ng	miR-144, miR-1603, miR-190b, miR-29b, miR-29c, miR-29e, miR-412 and miR-449b, miR-208a, miR-2317, miR-2320, miR-365-5p, miR-584, miR-628, miR-876, let-7a, miR-375, cluster miR-183-96-182, mir-130b, cluster mir-17-92 and miR-424/503	180 days	No
Pig	21,630	12,258	0.65 ng	let-7a, miR-17, miR-5p, miR-92a, miR-145, miR-224, miR-378, miR-274, miR-126-3p, miR-125b, let-7d-5p, miR-200b and miR-26a	-	No

mice: [22-28]; human: [29-38]; goat: [39-45]; sheep: [46-51]; cow: [52-61]; pig: [47,55,62-69]

Table 2. Follicular and oocyte diameters at various stages of development in mice, domestic animals and woman.

Follicle diameters	**Mice**	**Woman**	**Goat**	**Sheep**	**Cow**	**Pig**
Primordial follicles	17.0µm	40.0µm	30.0µm	35.0µm	40.0µm	34.0µm
Primary follicles	52.0µm	54.0µm	50.0µm	50.0µm	100.0µm	40.4µm
Secondary follicles	130.0µm	200.0µm	200.0µm	240.0µm	200.0µm	142.0µm
Preovulatory follicles	0.42mm	20.0mm	7.0mm	5.0mm	18.0mm	10.0mm
Oocyte diameters	**Mice**	**Woman**	**Goat**	**Sheep**	**Cow**	**Pig**
Primordial follicles	13.0µm	30.0µm	26.6µm	22.0µm	30.0µm	26.0µm
Primary follicles	29.0 µm	42.0µm	38.2µm	31.0µm	49.0µm	27.3µm
Secondary follicles	63.6µm	73.0µm	63.0µm	70.0µm	69.0µm	66.0µm
Oocyte growth *	(80.5%)	(60.8%)	(57.2%)	(63.6%)	(55.2%)	(61.8%)
Competent oocyte	79.0 µm	120.0µm	110.0µm	110.0µm	125.0µm	120.0µm

* Percentage of growth of oocytes enclosed in secondary follicles in relation to competent oocytes.

mice [92-93]; woman [63,94]; goat [94,95]; sheep [96,97]; cow [98-100]; pig [101-102];

Table 3. State of art of in vitro preantral follicle development in in mice, domestic animals and woman.

Species	Main advances after in vitro culture of preantral follicles	Main Barriers
Murine (mice)	Birth of live offspring after oocyte fertilization derivatives of primordial follicles.	Epigenetic changes in oocytes throughout the folliculogenesis. Offspring with health problems.
Human	Mature oocytes obtained from primordial follicles.	MII oocytes with low developmental competence. Abnormal large polar body in matured oocytes.
Caprine	Pregnancy after IVF of oocytes from secondary follicles.	Primordial follicles do not develop beyond secondary stage. Embryo loss at early stages of pregnancy
Ovine	Production of embryos from secondary follicles.	Primordial follicles do not develop beyond secondary stage. Embryos fail to develop up to blastocyst stage.
Bovine	Development of antral follicles from primordial follicles.	Oocyte degeneration after 18 days of culture, inability to resume meiosis and low developmental competence.
Swine	Production of embryos from secondary follicles.	No reports on primordial to secondary follicle development *in vitro*, embryos fail to develop up to blastocyst stage.

mice: [12]; woman: [13, 128]; goat: [14]; sheep [15]; cow [129]; pig: [16,130]

3 JUSTIFICATIVA

A escolha do modelo experimental justifica-se pelo fato de o Brasil possuir o segundo maior efetivo bovino do mundo, destacando a produção de carne a partir desta espécie que tem contribuído de forma crescente com o produto interno bruto (PIB) brasileiro (IBGE, 2020). Desta forma, a realização de pesquisa que possibilitem uma melhor compreensão do processo da folículogênese ovariana pode contribuir para a multiplicação de bovinos geneticamente superiores e, consequentemente, para o desenvolvimento da economia nacional. Além disso, a foliculogênese na espécie bovina tem muitas similaridades com a espécie humana, o que pode contribuir para a evolução das pesquisas reprodutivas aplicadas aos humanos.

Neste sentido, o presente estudo busca estratégias para potencializar os meios de cultivo e evitar os danos foliculares causados pelo estresse oxidativo com o uso de hormônios, fatores de crescimento e potenciais agentes antioxidantes. O estudo do crescimento *in vitro* de folículos pré-antrais é de grande importância tanto para a pesquisa fundamental, quanto para a reprodução animal. Além disso, o presente trabalho fornece base para novas pesquisas que tem como objetivo entender os eventos moleculares que ocorrem durante o cultivo *in vitro*, aumentando assim a eficiência da técnica de produção *in vitro* de embriões no Brasil.

O ineditismo deste trabalho destaca-se pela avaliação dos efeitos da interação entre EGF e progesterona durante o cultivo de folículos secundários bovinos. Anteriormente, CHOI *et al*. (2017) demonstraram que os efeitos do EGF podem ser potencializados pela progesterona durante a maturação oocitária, uma vez que esse hormônio aumenta a expressão de fatores semelhantes ao EGF. Outros estudos mostraram que o EGF regula vários processos, como proliferação de células da granulosa, expansão das células do cúmulus, maturação e taxa de fertilização do oócito (CELESTINO et al., 2009; AGUIAR *et al.*, 2017; HSIEH *et al*., 2007). Além disso, este estudo também é pioneiro por avaliar os mecanimos de ação da melatonina e do bloqueio dos seus receptores durante o cultivo de folículos secundários bovinos. O bloqueio dos receptores de melatonina pelo luzindole é de grande importância de para se compreender os mecanismos de ação deste hormônio.

De um modo geral, este trabalho também contribui para o desenvolvimento de condições de cultivo favoráveis para os folículos secundários bovinos, o que é de grande importância, pois os principais avanços obtidos nesta espécie estão relacionados com a formação de antro durante o cultivo folicular (SILVA *et al*., 2018, PAULINO *et al*., 2018, 2019; BEZERRA *et al.,* 2019, 2020; VASCONCELOS *et al.,* 2021).

4 HIPÓTESES CIENTÍFICAS

- A adição de EGF e P4, bem com a interação entre esses fatores ao meio de cultivo *in vitro* proporcionam o crescimento, formação de antro, mantém a viabilidade e ultraestrutura de folículos secundários bovinos.
- EGF e P4 aumentam os níveis de expressão de RNAm de GDF9, c-MOS, H1foo, ciclina B1, PARN e eIF4E em oócitos de folículos secundários cultivados *in vitro.*
- Os receptores de melatonina estão presentes nas células da granulosa, células da teca e oocito de folículos pré antrais e antrais da espécie bovina.
- A adição de melatonina ao meio de cultivo *in vitro* proporcionam o crescimento, mantém a viabilidade e a ultraestrutura de folículos secundários bovinos.
- Os efeitos *in vitro* da melatonina no crescimento, formação de antro, viabilidade e ultraestrutura de folículos secundários bovinos são bloqueados pelo antagonista de receptor luzindole.
- A melatonina aumenta os níveis de expressão de RNAm de SOD, CAT, GPX1 e PRDX6 em oócitos de folículos secundários cultivados *in vitro*.

5 OBJETIVOS

5.1 Objetivo geral

Avaliar a influência do EGF, P4 e melatonina sobre o desenvolvimento, ultraestrutura e os níveis expressão de mRNAs envolvidos durante o cultivo *in vitro* de folículos secundários bovinos.

5.2 Objetivos específicos

- Avaliar os efeitos do EGF e P4 no crescimento, viabilidade e ultraestrutura de folículos secundários bovinos após 18 dias de cultivo.
- Investigar a influência do EGF e P4 nos níveis de expressão de RNAm de GDF9, c-MOS, H1foo, ciclina B1, PARN e eIF4E em oócitos de folículos secundários cultivados *in vitro*.
- Caracterizar a expressão imunohistoquímica da proteína para o receptor da melatonina do tipo 1 A/B nas estruturas celulares das diferentes categorias de folículos bovinos.
- Investigar o efeito de diferentes concentrações de melatonina (10^{-11}, 10^{-9}, 10^{-7} e 10^{-5} M) no crescimento e viabilidade de folículos secundários bovinos cultivados *in vitro* por 18 dias.
- Avaliar os efeitos de melatonina (10^{-7}M) e do bloqueio do receptor de melatonina pelo luzindole (10μM) no crescimento, formação de antro e viabilidade de folículos secundários bovinos após 18 dias de cultivo.
- Investigar a influência antioxidante da melatonina e luzindole nos níveis de expressão de RNAm de SOD, CAT, GPX1 e PRDX6 em oócitos de folículos secundários cultivados *in vitro*.

6 CAPÍTULO I

Effects of epidermal growth factor and progesterone on development, ultrastructure and gene expression of bovine secondary follicles cultured in vitro

[Efeitos do fator de crescimento epidérmico e da progesterona no desenvolvimento, ultraestrutura e expressão gênica de folículos secundários bovinos cultivados *in vitro*]

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Effects of epidermal growth factor and progesterone on development, ultrastructure and gene expression of bovine secondary follicles cultured in vitro

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Abstract

The aims of this study were to investigate the effects of epidermal growth factor (EGF) and progesterone on the development, viability and the gene expression of bovine secondary follicle culture *in vitro* for 18 days. Secondary follicles (~0.2 mm) were isolated from ovarian cortex and individually cultured at 38.5°C, with 5% CO_2 in air, for 18 days, in TCM-199^{+} (n=63) alone (control medium) or supplemented with 10 ng/ml progesterone (n=64), 10 ng/ml EGF (n=61) or both EGF and progesterone (n=66). The effects of these treatments on growth, antrum formation, viability, ultrastructure and mRNA levels for *GDF-9*, *c-MOS*, *H1foo* and *cyclin B1* were evaluated, significantly different ($p < 0.05$). The results showed that there was a progressive increase in follicular diameter in all treatments, but only follicles cultured in medium supplemented with EGF had increased significantly in diameter when compared to

follicles cultured in the control medium at the end of the culture period, significantly different ($p < 0.05$). A positive interaction between EGF and progesterone was not observed. In addition, the presence of EGF, progesterone or both in culture medium did not influence the rate of follicle survival and antrum formation. However, the presence of only progesterone in cultured medium increased the expression of mRNAs for *GDF9* and *cyclin B1* in oocytes. EGF also significantly increased the levels of mRNAs for *cMOS* and *GDF9* when compared to follicles cultured in control medium. Ultrastructural analyzes showed that cultured follicles in all treatments maintained the integrity of granulosa cells. In conclusion, the EGF promotes the development of secondary follicles cultured in vitro for 18 days and increases the expression of *cMOS* and *GDF9*, while progesterone alone or in association with EGF have not a positive effect on follicular growth. However, progesterone increases the expression of *GDF9* and *cyclin B1* in oocytes.

Keywords: *GDF9*; *cyclin B1*; *cMOS*; oocytes; viability; ultrastructure.

1. Introduction

Preantral follicles are the majority of the ovarian follicle population and their use as a source of homogeneous oocytes for bovine reproductive biotechnologies could result in a substantial advance in this field [1]. *In vitro* culture of secondary follicles of ruminants have already been performed in an attempt to obtain oocytes suitable for fertilization (caprine [2], bovine [3]) and production of embryos have been reported for caprine [4] and ovine species [5]. However, in the bovine species, the results are limited to the formation of early antral follicles during secondary follicle culture in vitro [3,6,7,8,9]. In this way, in vitro culture of secondary follicles is necessary to provide information on the follicular needs during development, which can help to make possible the use of their oocytes in assisted reproduction techniques. Thus, it is important to improve culture systems by adding growth factors and hormones to understand

its mechanisms and to optimize the *in vitro* development of bovine secondary follicles. Among these substances, epidermal growth factor (EGF) and progesterone (P4) are candidates to improve follicular survival and growth *in vitro*.

Previous studies have shown that EGF has an important role in ovarian folliculogenesis, by regulating several processes, like proliferation of granulosa cells and atresia (caprine [10], equine [11]). In cattle, the addition of EGF increases cumulus cells expansion and improved maturation and oocyte fertilization rate [12]. More recently, Choi et al. [13] showed that the effects of EGF on goat oocyte maturation can be potentiated by progesterone, since this hormone increases the expression of EGF-like factors that influences oocyte maturation. However, it is still not yet known if EGF and progesterone interacts positively to promote in vitro development of bovine preantral follicles.

Progesterone regulates various reproductive processes in females, including ovulation, implantation and sexual differentiation. Signaling downstream of P4 has been studied primarily through the activation of nuclear receptors that act as transcription factors to stimulate P4-dependent gene expression [14]. In addition, P4 has been shown to play an important role in regulating bovine oocyte maturation both in vivo and in vitro by signaling through both nuclear (PGRs) and membrane P4 receptors (mPR). Inhibition of P4 synthesis and blockage of the receptors (PRs) have negative effects on oocyte maturation and/or subsequent embryonic development [15,16]. Moreover, blocking PGR activity in cumulus-oocyte complexes (COCs) during in vitro maturation also decreased BCL-xL and increased active caspase 3 expression, showing that P4 signaling through the PGRs is involved in preventing apoptosis and promoting cell survival in bovine COCs [15].

In general, follicular and oocyte development is orchestrated by the expression of a wide variety of genes. Svoboda et al. [17] reported that during follicular growth, oocytes

accumulate large amounts of RNAs and proteins that play important roles during oocyte maturation and early embryonic development. Transcripts for growth and differentiation factor 9 (GDF9), oocyte-specific linker histone *(H1FOO*), cyclin B1 and c-MOS kinase are among the messenger RNAs that are stored during oocyte development [18]. Products of these genes have various functions, for example, GDF9 stimulates oocyte and follicular growth *in vitro* [19], H1Foo is essential for the maturation of bovine oocytes [20], and cyclin B is one of the main regulators of the changes that occur during oocyte maturation [21]. The c-MOS kinase plays a crucial role in the control of meiosis in oocytes in addition to regulating cell survival and apoptosis [22]. Most of these mRNAs are stored during oocyte growth and are translated into proteins at oocyte maturation, emphasizing the importance of mRNA storage. In this sense, factors such as poly(A) ribonuclease *(PARN)* and initiation factor 4E (*eIF4E)* are described in the literature for their action in the control of the RNAs stock and for their role in the translation of these RNAs into proteins, respectively [23]. Thus, it is important to evaluate the influence of EGF and P4 on the expression of these factors during the in vitro growth of bovine secondary follicles.

The aims of this study were to evaluate the effects of EGF, P4, and both EGF and P4 on growth, viability and ultrastructure of bovine secondary follicles after 18 days of culture. In addition, the influence these substances on the levels of mRNA expression for *GDF9, c-MOS, H1foo, cyclin B1, PARN* and *eIF4E* in oocytes of secondary follicles cultured *in vitro* was investigated. These mRNAs are stored in the oocyte and their proteins are involved in the control of follicle development [GDF9: 18-19], oocyte maturation [c-MOS, H1foo and cyclin B1: 20-22] and early embryonic development [PARN and eIF4E: 23]. Thus, increased expression of these markers is indicative of oocyte competence to assure its maturation and embryo development.

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, USA) unless otherwise indicated in the text.

2.2. Source of ovaries

Bovine ovaries (n=40) from adult cycling cows were collected from a local slaughterhouse immediately after slaughter. After collection, the ovaries were washed in 70% ethanol for about 10 sec, followed by two rinses in TCM-199 buffered with HEPES and supplemented with penicillin (100 mg/ml) and streptomycin (100 mg/ml). Later, the ovaries were transported within 1h to the laboratory in TCM-199 at 4°C. This study is in accordance with procedures approved by the Ethics and Animal Welfare Committee of the Federal University of Ceara (Nº 16/16).

2.3. Follicle isolation and in vitro culture

In the laboratory, the ovaries were subjected to fragmentation of the ovarian cortex (1-2 mm) with a sterile scalpel blade and placed in TCM-199 medium supplemented with Hepes. Secondary follicles with approximately 200 µm in diameter were manually dissected from the strips of the ovarian cortex using 26-gauge needles under a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan). After isolation, follicles with a visible oocyte surrounded by granulosa cells, an intact basement membrane, and no antral cavity were selected for culture. Then, follicles were individually cultured in 100 µL of culture medium under mineral oil in petri dishes (60 x 15 mm, Corning, USA). The control culture medium, called TCM-199^{+}, consisted of TCM-199 (pH 7.2-7.4) supplemented with 10 µg/mL insulin, 5.5 µg/mL transferrin and 5 ng/mL selenium

(ITS), 3.0 mg/mL bovine serum albumin (BSA), 2mM glutamine, 2mM hypoxanthine, 50 μg/mL of ascorbic acid and 100 ng/mL FSH. The secondary follicles were randomly cultured in TCM-199^{+} alone or supplemented with 10 ng/mL EGF, 10 ng/mL P4 or both EGF and P4. Concentrations of EGF and P4 were chosen according to results of previous studies [24,25].

In each treatment, from 74 to 79 follicles were cultured. The follicles were cultured at 38.5°, which is the physiological temperature of cow [26], with 5% CO_2 in air, for 18 days to evaluate follicle development. Every two days of culture, 60 μL of medium was replaced by fresh medium. On days 0, 6, 12 and 18, the percentage of morphologically normal follicles was assessed. Follicles with an opaque and/or extruded oocyte and opaque granulosa cells were considered degenerated. In addition, two perpendicular measurements were performed in the normal follicles using an inverted microscope with NIS Elements 2.4 software (Nikon, Nikon Instruments Inc., Japan).

2.4. Assessment of preantral follicle viability by fluorescence microscopy

After culture, follicles (n=20/treatment) were incubated in 100 μL droplets of TCM-199 containing 4mM calcein-AM and 2mM ethidium homodimer-1 (Molecular Probes, Invitrogen, Karlsruhe, Germany) at 37°C for 15 min. Afterwards, the follicles were washed three times in TCM-199 and examined under fluorescence microscope (Nikon, Eclipse, TS 100., Japan). Oocytes and granulosa cells were considered viable if the cytoplasm was stained positively with calcein-AM (green) and if the chromatin was not labeled with ethidium homodimer-1 (red) [27].

2.5. Expression of mRNA for GDF9, c-Mos, H1foo, cyclin B1, PARN and eIF4E in cultured oocytes

To evaluate the levels of mRNAs specifically in the oocytes, morphologically normal follicles that were cultured in each treatment had their basement membrane ruptured and the oocytes were removed from the follicles. For each treatment, four groups of denuded oocytes were then stored at -80°C until extraction of the total RNA for further analysis of the levels of mRNAs for *GDF9, C-Mos*, *H1foo, Cyclin B1, PARN and EIF4E.*

Total RNA extraction was performed using a Trizol® purification kit (Invitrogen, São Paulo, Brazil) in accordance with the manufacturer's instructions. Quantification of mRNA was performed using SYBR Green. PCR reactions were composed of 1 µl cDNA as a template in 7.5 µl of SYBR Green Master Mix (PE Applied Biosystems, Foster City, CA, USA), 5.5 µL of ultra-pure water, and 0.5 µM of each primer. The primers were designed by using the Primer Quest SM program (http://www.idtdna.com) to perform amplification of *GDF-9, c-Mos, H1foo, Cyclin B1, PARN, EIF4E* and *β-tubulin*. The specificity of each primer pair was confirmed by melting curve analysis of PCR products. The thermal cycling profile for the first round of PCR was initial denaturation and activation of the polymerase for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30s at 58°C, and 30 s at 72°C. The final extension was for 10 min at 72°C. All reactions were performed in a Step One Plus instrument (Applied Biosystems, Foster City, CA, USA). The ΔΔCt method was used to transform the Ct values into normalized relative expression levels [28].

2.6. Ultrastructural analysis of cultured preantral follicles

For qualitative analysis of organelles in the cytoplasm of oocytes and granulosa cells, isolated and cultured follicles (n= 6 to 10 per treatment) were fixed in Karnovsky solution (4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer - pH 7.2) for at least 4h at room temperature (approximately 25°C). After fixation, follicles were embedded in drops of 4% low melting agarose and kept in sodium cacodylate buffer. Specimens were post-

fixed in 1% osmium tetroxide, 0.8% potassium ferricyanide and 5 mM calcium chloride in 0.1 M sodium cacodylate buffer for 1h at room temperature, washed in sodium cacodylate buffer and counterstained with 5% uranyl acetate [29]. The samples were then dehydrated through a gradient of acetone solutions and thereafter embedded in epoxy resin. Thereafter, semi-thin sections (2µm) were cut and stained with toluidine blue. Subsequently, ultra-thin sections (60nm) were counterstained with uranyl acetate and lead citrate, and examined under a Morgani-FEI transmission electron microscope.

2.7. Statistical analysis

The data of follicular diameters were subjected to analysis using the Kolmogorov-Smirnov test to evaluate normal distribution. Then, these data were subjected ANOVA. Comparisons among treatments were performed using the Student-Newman-Keuls (SNK) test (GraphPad Prism software, version 5.0). Data concerning follicular survival and antrum formation after in-vitro culture in each treatment were compared by Chi-square test, and the results were expressed as percentages. The levels of mRNA were analyzed by Kruskal-Wallis test, followed by the post hoc non-parametric test: the Dunn's Multiple Comparison Test. Differences were considered significant when $P<0.05$.

3. Results

3.1. Effects of EGF and P4 on follicular growth, viability and antrum formation

A progressive and significant increase in follicular diameters was observed with the increase of culture period from 0 to 6, 12 and 18 days in all treatments. After 6 and 12 days of culture, follicles cultured in medium supplemented with EGF had higher diameters than those

cultured in control medium alone or supplemented with P4 and both EGF and P4. At the end of culture time, the diameter of follicles cultured in presence of EGF were significantly higher than those cultured in control medium ($p < 0.05$), but did not differ from follicles cultured with P4 or both EGF and progesterone ($p > 0.05$) (Table 4). The percentage of normal follicles after 18 days of culture in medium supplemented with EGF (93.44% [57/61]), P4 (93.75% [60/64]) and both (96.96% [64/66]) was higher than that of follicles cultured in control medium (74.60% [47/63]), but the differences were not significant.

The fluorescence microscopy assessment using calcein-AM labeling showed that follicles cultured for 18 days in medium supplemented with EGF, P4 and both were viable, since they had positive staining for calcein-AM, but not for ethidium homodimer-1. Follicles cultured with control medium were also stained with calcein-AM, except peripheral stromal cells, that were positive for ethidium homodimer-1 (Figure 14).

The percentage of antrum formation in secondary follicles after 18 days of culture in medium supplemented with EGF or progesterone was higher than that of follicles cultured in control medium (42.55% [20/47]) alone or supplemented with both EGF (58.33% [35/60]) and P4 (54.38%[31/57]), but the differences were not significant.

3.2. Levels of mRNA for GDF9, c-Mos, H1foo, cyclin B1, PARN and eIF4E in cultured oocytes

The levels of mRNA for *GDF9, cMOS, H1foo, cyclin B1, PARN* and *eIF4E* in oocytes of bovine secondary follicle after 18 days of culture in TCM 199 alone or supplemented with EGF, P4 or both EGF and P4 are showed in Figure 15. The presence of both EGF and P4 did not influence the levels of mRNA for *H1Foo, eIF4E* and *PARN*, when compared with control medium. However, EGF significantly increased the levels of mRNA for *cMOS* and *GDF9*. In

addition, when compared to control medium, higher levels of *cyclin B1* and *GDF9* mRNA were seen oocytes of follicles cultured in presence of only P4 ($P < 0.05$).

3.3. Ultrastructural analysis of cultured secondary follicles

Granulosa cells (Figure 16A) and oocytes (Figure 16B) of uncultured secondary follicles had well preserved ultrastructure. In the oocyte, the organelles were well distributed, while the vacuoles were minimally observed. In follicles that have been cultured for 18 days in TCM-199^{+}, an increase in the number vacuoles and multivesicular bodies in the ooplasm were observed (Figure 16D), but granulosa cells (Figure 16C) were similar to those of uncultured follicles (Figure 16A). The follicles cultured in the presence of the EGF, P4 or both EGF and P4 also had increased number of vacuoles in the ooplasm, but the granulosa cells were well preserved and both oocyte and granulosa cells were well connected (Figure 16E-J).

4. Discussion

This study shows that EGF promotes bovine secondary follicle growth in vitro and increases the levels of mRNA for *cMOS* and *GDF9* (Figure 2). Recently, Wee and Zhixiang [30] reported that EGF modulates activation of second messengers that are responsible for regulate cellular growth and differentiation. EGF production and activity are very important for granulosa cell proliferation in growing preantral follicles [31]. This factor binds to its receptor tyrosine kinase, and stimulates the survival of cells through the intracellular signaling, such as phosphatidylinositol 3-kinase, as well as mitogen-activated protein kinase and Janus-activated kinase/signal transducers and activators of transcription [32]. EGF have reduced the levels of apoptosis in preantral follicles cultured in vitro (porcine [24]) and inhibited spontaneous DNA cleavage through the stimulation of anti-apoptotic genes, such as BCL [33], survivin and kappa

nuclear factor B (NFkB) [34,35]. Various studies have confirmed that EGF can inhibit follicular apoptosis and promote granulosa cells proliferation and differentiation in different species (bovine [10], caprine [36], ovine [37], equine [11]). Regarding the positive effect of EGF on expression of *GDF9* and *cMOS*, previous studies reported that EGFR functionality in granulosa cells depends on GDF9 secreted by the oocyte, emphasizing the importance of oocyte and granulosa cells cross-talk [38]. This is consistent with the findings that mouse oocytes mediate cumulus cell EGFR expression, via GDF9 [39]. In addition, cross-talk between EGFR-ERK1/2 and SMAD2/3 signalling in granulosa and cumulus cells also appears important to elicit GDF9 expression [40]. Increasing in expression of cMos mRNA after culturing bovine secondary follicles with EGF can have a positive impact for acquisition of oocyte competency, since previous studies showed an important role of cMos in the maintenance of MII arrest and oocyte maturation [41].

Progesterone did not influence the growth of bovine secondary follicles, but did increase the levels of mRNA for *GDF9* and *cyclin B1*. Previous studies have already reported that progesterone did not stimulate proliferation of granulosa cells [42]. However, the positive effects of progesterone on the levels of mRNA for *GDF9* and *cyclin B1* in the oocytes of cultured secondary can be important for acquisition of their competence, since GDF9 stimulates oocyte growth *in vitro* [19], and cyclin B is one of the main regulators of the changes that occur during oocyte maturation [21].

This study shows that there is no interaction between EGF and progesterone during culture of bovine secondary follicles. However, previous studies indicated that collaborative actions of progesterone and EGF were required for hCG-induced increases in production and potentiation of EGF signaling in human periovulatory granulosa cells [13]. CARVAJAL et al. [43] also demonstrated that progesterone treatment potentiated the signaling of the EGF

pathway in breast cancer cell lines cultured in vitro. Thus, a positive interaction between EGF and progesterone depends on follicular stage of development and cell type.

Regarding the ultrastructural features of follicles, independently of the presence of EGF, P4 or both, cultured follicles had oocytes with signs of degeneration, but the granulosa cells remained intact. According to Driancourt and Thuel [44], the oocyte is the first cell within the follicle to be affected by atresia, and whether oocyte atresia is related to improper dialogue between the oocyte and its surrounding granulosa cells remains unclear. In addition, we showed in previous study that the oocyte of goat preantral follicles were more sensitive to degeneration than granulosa cells [45]. In addition, preantral follicles cultured for 14 days had high condensation of the nuclear material and presented vacuoles suggesting the occurrence of a degenerative process in the oocyte, but not in granulosa cells [46].

In conclusion, EGF promotes a follicular growth and enhances the expression of mRNA for *GDF9* and *cMOS* in cultured bovine follicles, while progesterone does not influence follicular growth in vitro, but increases the expression of *GDF9* and *cyclin B1* in oocytes. However, EGF and progesterone have no synergic effect during bovine preantral follicle growth in vitro.

Conflict of interest statement

There was no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Acknowledgments

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List of tables and figures

Table 4.

	Day 0	Day 6	Day 12	Day 18
TCM-199^{+}	198.1±3.6^{aA}	233.4±6.7^{bA}	253±7.9^{cA}	266.2±8.1 ^{dA}
P4	197.9±3.1^{aA}	230.8±5.9^{bA}	252.8±7.3^{cA}	288.1±11.3 ^{dAB}
EGF	194.6±3.1^{aA}	245.3±9.1^{bB}	273.6±10.4^{cB}	300.2±11.3 ^{dB}
P4+EGF	189.8±2.9^{aA}	229.5±6.1^{bA}	255.3±8.5^{cA}	282.6±10.3 ^{dAB}

Significantly different ($p < 0.05$)
a, b, c, d Lower case letters represent statistically significant differences between days of culture for each treatment (between columns)
A, B, C, D Capital letters represent statistically significant differences between treatments at the same day of culture (same columns)

Figure 14

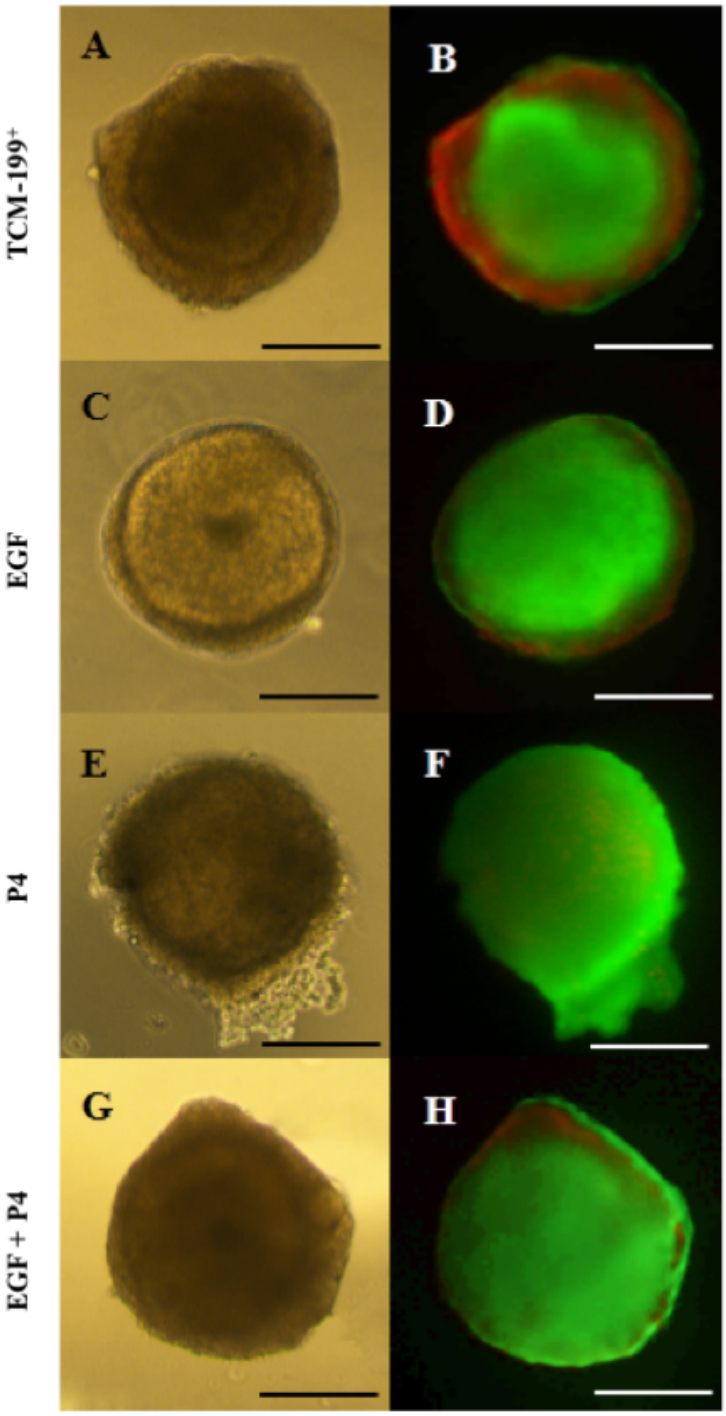
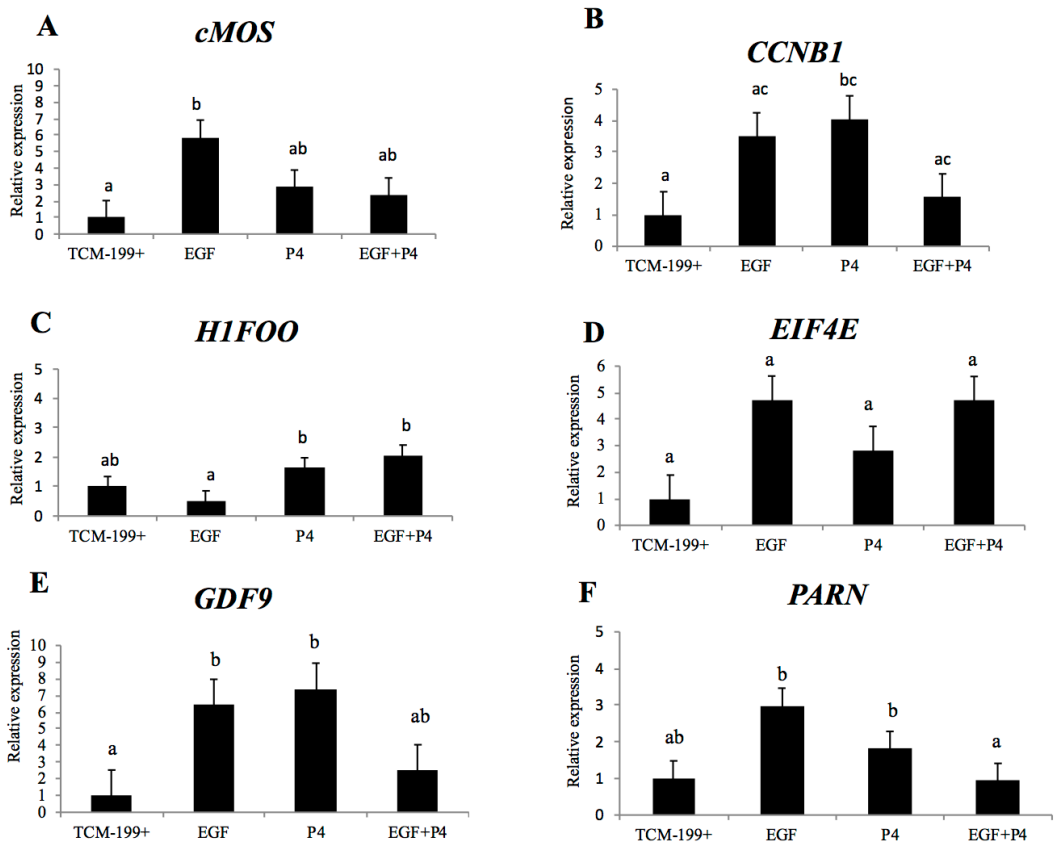


Figura 15.


A
cMOS
Relative expression
a
b
ab
ab
TCM-199+
EGF
P4
EGF+P4
B
CCNB1
a
ac
bc
ac
C
H1FOO
ab
a
b
b
D
EIF4E
a
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GDF9
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b
ab
F
PARN
ab
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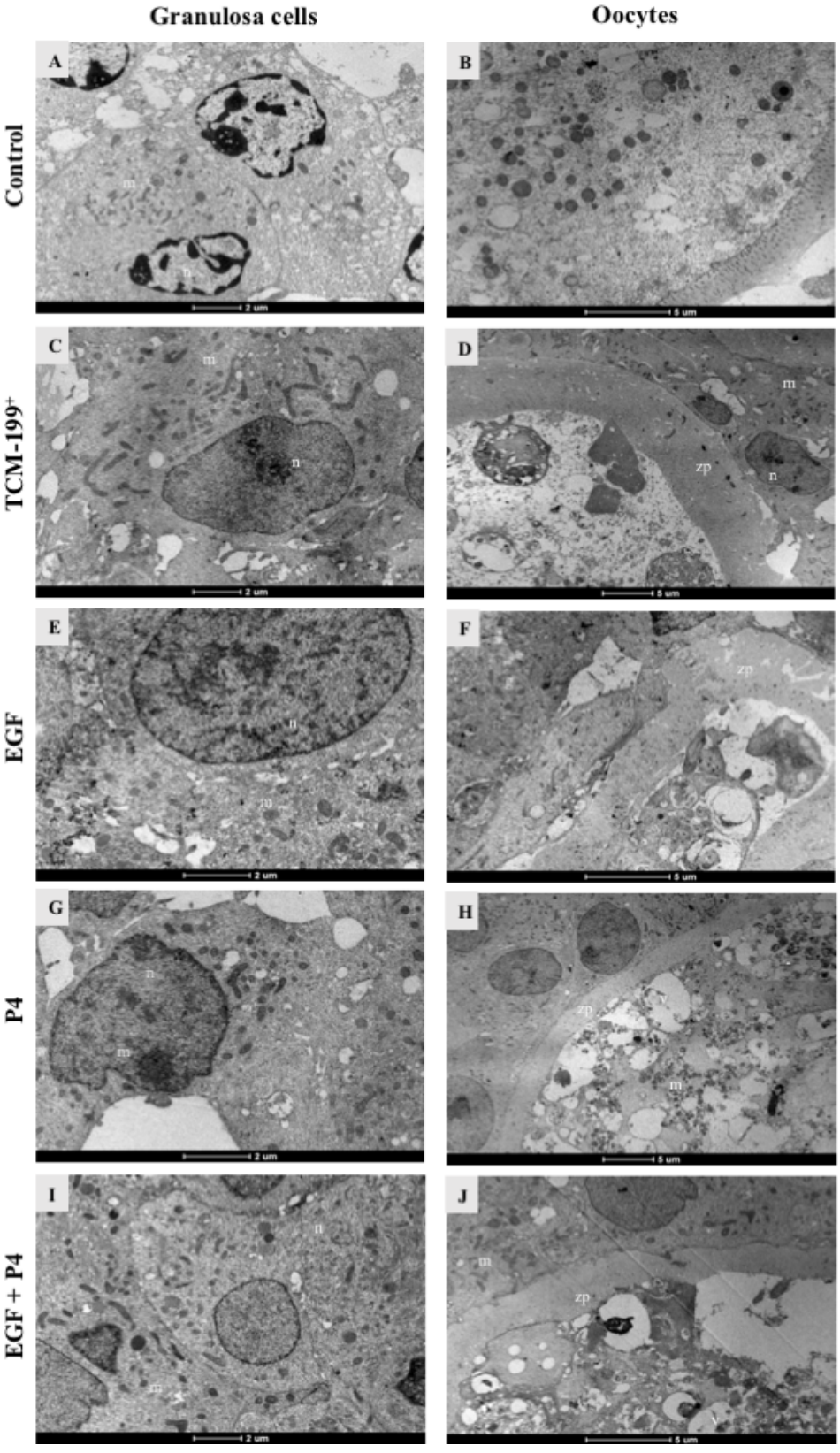
a

Figura 16.


Granulosa cells
Oocytes
Control
TCM-199+
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P4
EGF + P4
A
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7 CAPÍTULO II

Immunolocalization of melatonin receptors in bovine ovarian follicles and in vitro effects of melatonin on growth, ultrastructure and gene expression in secondary follicles

[Imunolocalização de receptores de melatonina em folículos ovarianos bovinos e efeitos *in vitro* da melatonina no crescimento, ultraestrutura e expressão gênica em folículos secundários]

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Immunolocalization of melatonin receptors in bovine ovarian follicles and *in vitro* effects of melatonin on growth, viability and gene expression in secondary follicles

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Abstract

This study aims to investigate the (1) expression of type 1A/B melatonin receptors in bovine ovaries and (2) the *in vitro* effects of melatonin on secondary follicle development, antrum formation, viability, and expression of mRNA for *SOD*, *CAT*, *GPX1* and *PRDX6*. In experiment 1, the expression of melatonin receptors 1 A/B in bovine ovarian follicles was demonstrated by immunohistochemistry. In addition, to choose the most effective concentration of melatonin on follicular growth and viability, isolated secondary follicles were cultured individually at 38.5°C, with 5% CO_2 in air, for 18 days in TCM-199^{+} alone or supplemented with 10^{-11}, 10^{-9}, 10^{-7} or 10^{-5} M melatonin. Then, in experiment 2, melatonin receptor antagonist, luzindole, was tested to further evaluate the mechanisms of actions of melatonin, i.e., the follicles were

cultured in control medium alone or supplemented with 10^{-7} M melatonin, 10μM luzindole and both 10^{-7} M melatonin and 10μM luzindole. Follicular growth, morphology and antrum formation were evaluated. At the end of culture, viability of secondary follicles was analyzed by calcein-AM and ethidium homodimer-1, and the levels of mRNA for *SOD, CAT, GPX1* and *PRDX6* were evaluated by real time PCR. In experiment 1, immunohistochemistry results showed expression of melatonin receptors type 1A/B in oocyte and granulosa cells of primordial, primary, secondary and antral follicles. Secondary follicles cultured in medium supplemented with melatonin at different concentrations had well preserved morphology follicles after 18 days of culture. Furthermore, follicles cultured in presence of 10^{-7} M melatonin presented significantly higher diameters than those cultured in other treatments. In experiment 2, the presence of melatonin receptor antagonist, luzindole, blocked the effects of melatonin on follicular growth and viability. In addition, follicles cultured in medium containing only melatonin had significantly higher rates of antrum formation. Follicles cultured in medium containing only melatonin had higher levels of mRNA for CAT and SOD than those cultured with luzindole or both melatonin and luzindole. Luzindole alone or both melatonin and luzindole also reduced the levels of mRNA for *GPX1* in cultured follicles. In conclusion, melatonin promotes growth of bovine secondary follicles through its membrane-coupled receptors, while luzindole blocks the effects of melatonin on follicle growth and expression of antioxidant enzymes in cultured follicles.

Keywords: Follicular development. Ovary. Cow. Melatonin. Luzindole.

2. Introduction

Melatonin (N-acetyl-5-methoxytryptamine) is a neuroendocrine hormone secreted by the pineal gland that influences the reproductive processes in several species [human [1]; swine [2], ovine [3]; bovine [4]; caprine [5] and murine [6]]. Melatonin can act by two types of

receptors, i.e., type 1A and type 1B, which are G-protein-coupled membrane bound receptors [7, 8]. In the ovaries, mRNA for melatonin receptors type 1A were identified in mouse granulosa cells from primary follicle stage onward [9], while in rats they were expressed in granulosa cells from secondary and antral follicles [10]. The expression of these receptors was also reported in human granulosa cells from pre-ovulatory follicles [11] and in swine cumulus cells [12]. In sheep and cows the melatonin receptors are present in cumulus oocyte complexes (COC) and their blockage with a specific inhibitor (luzindole) prevents the melatonin effects [13,14]. Luzindole (N-acetyl-2-benzyltryptamine) is a competitive melatonin receptor antagonist widely employed to study melatonin actions on cells. After blocking melatonin receptors, Wang et al. [15] revealed that, depending on the concentration, melatonin influences granulosa cells apoptosis by other pathways. In bovine ovarian follicles, however, neither is known if melatonin type 1A/B receptors are expressed in bovine preantral follicles, nor if melatonin acts via membrane receptors or other mechanisms.

In addition to its receptor-mediated actions, melatonin can pass through the cytoplasmic membrane due to its amphiphilic nature, and bind to specific DNA sequences for regulating gene expression [16]. Previous studies have reported that, after penetrating the cells, melatonin has a strong free radical scavenging property [17,18], acting as an antioxidant to protect the integrity of granulosa cells, reducing oxidative stress in nuclei, mitochondria and plasma membranes [19,20]. Oxidative stress is characterized by an imbalance between reactive oxygen species (ROS) concentrations and antioxidant activity, resulting in gradual accumulation of free radicals generated during normal metabolism [21]. The ROS affect multiple physiological processes from oocyte maturation to fertilization, embryo development and pregnancy [22]. The defense mechanisms of cells against ROS accumulation can be enzymatic or non-enzymatic. Enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPX), peroxiredoxin 6 (PRDX6) and catalase (CAT) are related to antioxidant defense. In bovine

oocytes, melatonin up-regulates the expression of CAT, SOD and GPX [23]. The oxidative stress during *in vitro* follicular development negatively affects oocyte quality [24,25]. In this way, the presence of antioxidants in the culture medium can reduces the oxidative stress and maintains oocyte quality *in vitro* [26].

Melatonin exerts a variety of beneficial effects on the follicle and oocyte in several mammalian species [27-31]. In mice, the rate of fertilization and embryo development *in vitro* was improved by melatonin in a dose-dependent pattern [27]. Melatonin also reduced apoptosis, ROS production and mitochondrial damage in mice treated with cisplatin. This hormone helped to preserve follicular morphology and cell proliferation, and increased glutathione levels [28]. In pig, melatonin has beneficial effects on oocyte meiotic maturation through tubulin formation factors [29]. In sheep, melatonin reduces the negative effects caused by thermal stress and decreased apoptotic rate in granulosa cells [30]. In goats, the development of preantral follicles was improved by melatonin in combination with FSH [31]. However, there is still little evidence reporting the effects of melatonin on bovine preantral follicle development, as well as on the expression of antioxidant enzymes.

The aims of this study were to evaluate the expression of melatonin receptors 1A/B in bovine ovarian follicles by immunohistochemistry and to investigate the effect of different concentrations of melatonin on growth and viability of bovine secondary follicles after 18 days of culture. Melatonin receptor antagonist, luzindole, was used to further evaluate the mechanisms of actions of melatonin on growth, viability, and levels of mRNA for *SOD*, *CAT*, *GPX1* and *PRDX6* in cultured secondary follicles.

2. Materials and methods

The culture media and other chemicals used in the present study were purchased from Sigma Chemical Co. (St. Louis, USA) unless otherwise indicated in the text.

2.1. Experiment 1: Immunolocalization of melatonin receptors and effects of melatonin in cultured bovine secondary follicles

Bovine ovaries (n=82) were collected from 41 adult cows in a local slaughterhouse. After collecting, the ovaries were washed in 70% ethanol for about 10 sec, followed by two rinses in TCM-199 buffered with HEPES and supplemented with penicillin (100 µg/mL) and streptomycin (100 µg/mL). The ovaries were transported within 1h to the laboratory in TCM-199 at 4°C. Ovaries from five cows were destined to immunohistochemistry, while the secondary follicles intended to *in vitro* culture were isolated from ovaries from 36 cows.

In order to identify the presence of melatonin receptors 1 A/B in bovine ovarian follicles, the immunohistochemistry was performed according to previous studies [30]. Ovaries were fixed in 10% buffered formalin (Dinâmica, São Paulo, Brazil) for 24 h, dehydrated with increasing concentrations of ethanol (Dinâmica), clarified in xylene (Dinâmica), and embedded in paraffin (Dinâmica). Tissue sections (5 mm thick) were mounted in Starfrost glass slides (Knittel, Braunschweig, Germany) and incubated in citrate buffer (Dinâmica) at 95^0C in a deckloaking chamber (Biocare, Concord, USA) for 40 min for antigen recover. Endogenous peroxidase activity was blocked by incubation with 3% H_2O_2 (Dinâmica) and methyl ethanol (QEEL, São Paulo, Brazil) for 10 min. Nonspecific binding sites were blocked using 1% normal goat serum (Biocare) diluted in PBS (Sigma Aldrich Chemical Co., St. Louis, MO, USA). Subsequently, the sections were incubated in a humidified chamber for 60 min at room temperature with monoclonal rabbit anti MEL-1A/B-R (1:50; reference: (B-08) sc 9013, Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibody. Thereafter, the sections were incubated for 20min with MACH4 Universal HRP-polymer (Biocare). Protein localization was demonstrated with diaminobenzidine (DAB; Biocare), and the sections were counterstained with hematoxylin (Vetec, São Paulo, Brazil) for 1min. Negative controls underwent all steps except the primary antibody incubation. Follicles were classified as primordial, primary,

secondary and antral follicles according to characteristics described previously [32] In the different follicular compartments, i.e., oocyte, granulosa and theca cells, the immunostaining was classified as absent, weak, moderate or strong. The slides were examined using a microscope (Nikon, Tokyo, Japan) under 400 magnification.

For *in vitro* culture, secondary follicles with approximately 200 μm in diameter were manually dissected from the strips of the ovarian cortex (1-2 mm) using 26-gauge needles under a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan). After isolation, follicles with a visible oocyte surrounded by granulosa cells, an intact basement membrane, and no antral cavity were selected for culture. Then, follicles were individually cultured in 100 μL of culture medium under mineral oil in Petri dishes (60 x 15 mm, Corning, USA). The control culture medium, called TCM-199^{+}, consisted of TCM-199 (pH 7.2-7.4) supplemented with 10 μg/mL insulin, 5.5 μg/mL transferrin and 5 ng/mL selenium (ITS), 3.0 mg/mL bovine serum albumin (BSA), 2mM glutamine, 2mM hypoxanthine, 50 μg/mL of ascorbic acid and 100 ng/mL FSH. The secondary follicles were randomly cultured in TCM-199^{+} alone or supplemented with melatonin (10^{-11}, 10^{-9}, 10^{-7} or 10^{-5} M). Concentrations of melatonin were chosen according to results of previous studies [13]. This experiment was repeated six times, with six cows in each repetition.

In each treatment, from 74 to 79 follicles were cultured. The follicles were cultured at 38.5°C, with 5% CO_2 in air, for 18 days to evaluate follicle development. Every two days of culture, 60 μL of medium was replaced by fresh medium. At days 0, 6, 12 and 18, follicular diameters and morphology were assessed. Follicles with opaque oocyte and granulosa cells were considered degenerated. In addition, two perpendicular measurements were performed in the normal follicles using an inverted microscope with NIS Elements 2.4 software (Nikon, Nikon Instruments Inc., Japan).

After culture, follicles (n=20/treatment) were incubated in 100 μL droplets of TCM-199 containing 4mM calcein-AM and 2mM ethidium homodimer-1 (Molecular Probes, Invitrogen, Karlsruhe, Germany) at 37°C for 15 min. Calcein-AM is a substrate for esterase enzyme, while ethidium homodimer-1 penetrated in dead cells with damaged plasma membranes and binds to their DNA. Afterwards, the follicles were washed three times in TCM-199 and examined under fluorescence microscope (Nikon, Eclipse, TS 100., Japan). Oocytes and granulosa cells were considered viable if the cytoplasm was stained positively with calcein (green) and if the chromatin was not labeled with ethidium homodimer-1 (red).

2.2. Experiment 2: Effects of melatonin and melatonin receptor inhibitor (luzindole) on follicular growth and gene expression

Bovine ovaries (n = 80) were collected from 40 adult cows in a local slaughterhouse and the secondary follicles were isolated as described in experiment 1. After isolation, the secondary follicles were randomly cultured in TCM-199^{+} alone or supplemented with 10^{-7} M melatonin, 10 μM luzindole or both 10^{-7} M melatonin and 10μM luzindole. Concentration of melatonin was chosen according to results of experiment 1, while the concentration of luzindole was chosen according to Cao et al. [33]. At days 0, 6, 12 and 18, follicular diameters, morphology and antrum formation were assessed. Evaluation of follicular viability by fluorescence microscopy was performed according to experiment 1. This experiment was repeated six times, with six or seven cows in each repetition.

Morphologically normal follicles that were cultured in each treatment were then stored at -80°C until extraction of the total RNA for further analysis of the levels of mRNAs for *SOD, CAT, PRDX1 and GPX1*. Total RNA extraction was performed using a Trizol® purification kit (Invitrogen, São Paulo, Brazil) in accordance with the manufacturer's instructions. Quantification of mRNA was performed using SYBR Green. PCR reactions were composed of

1 µl cDNA as a template in 9.4 µl of SYBR Green Master Mix (PE Applied Biosystems, Foster City, CA, USA), 9.4 µL of ultra-pure water, and 0.5 µM of each primer. The primers were designed to perform amplification of *SOD, CAT, PRDX6, GPX1* and *GAPDH* (Table 5). The specificity of each primer pair was confirmed by melting curve analysis of PCR products. The thermal cycling profile for the first round of PCR was initial denaturation and activation of the polymerase for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30s at 58°C, and 30 s at 72°C. The final extension was for 10 min at 72°C. All reactions were performed in a Step One Plus instrument (Applied Biosystems, Foster City, CA, USA). The ΔΔCt method was used to transform the Ct values into normalized relative expression levels [34].

2.3 Statistical analysis

Statistical analyzes were performed using the GraphPad Prism software. The data of follicular diameters were initially subjected to analysis of normal distribution using the Kolmogorov-Smirnov test. Then, these data were evaluated by ANOVA. Comparisons of follicular diameters among treatments were performed using the Student-Newman-Keuls (SNK) test. Data concerning follicular survival and antrum formation after in-vitro culture in each treatment were compared by Chi-square test, and the results were expressed as percentages. The levels of mRNA were analyzed by Kruskal-Wallis test, followed by the post hoc Dunn's multiple comparison test. Differences were considered significant when $P<0.05$.

3. Results

3.1. *Experiment 1: Immunolocalization of melatonin receptors and in vitro effects of melatonin on bovine secondary follicles*

The immunohistochemistry showed weak staining for melatonin receptor type 1A/B in granulosa cells from primordial, primary, and secondary follicles, while antral follicles had

moderate staining in these cells. Oocytes from all follicular categories had a strong staining for melatonin receptor type 1A/B. There was no positive reaction in theca cells for melatonin receptor type 1A/B (Figure 17).

Secondary follicles cultured with 10^{-11}, 10^{-9} or 10^{-7} M melatonin showed a progressive and significant increase in follicle diameter with the increase of culture period from days 0 to 6, 12 and 18. After 6 days, follicles cultured in medium containing 10^{-7} or 10^{-5} M melatonin had larger diameters than those of cultured in control medium alone or supplemented with 10^{-11} or 10^{-9} M melatonin. In addition, after 12 days of culture, follicles cultured with 10^{-7} M melatonin had larger diameters than those cultured in other treatments. At day 18, follicles cultured with 10^{-7} M melatonin remained with significantly larger diameters than those cultured in other treatments (Table 6).

After 18 days, follicles cultured in control medium alone or supplemented with 10^{-11}, 10^{-9}, 10^{-7} or 10^{-5} M melatonin had 80% (33/41), 80% (33/41), 86% (38/44), 95% (42/44), and 90% (40/44) of morphologically normal follicles, respectively. No significant differences were observed among treatments. Follicles cultured in all treatments were positively stained with calcein, but those cultured in control medium alone or with 10^{-5} M melatonin had low staining in the central region of the follicles (Figure 18).

3.2. *Experiment 2: Effects of melatonin and melatonin receptor inhibitor (luzindole) on follicular growth and gene expression*

A progressive increase in follicular diameters was observed with the increase of culture period from days 0 to 6, 12 and 18 in all treatments. After 6, 12 and 18 days, 10^{-7} M melatonin significantly increased follicular diameters, but luzindole blocked these positive effects (Table 7), emphasizing that melatonin is acting via receptors to promote growth.

The percentage of normal follicles after 18 days of culture in medium supplemented with 10^{-7} M melatonin, 10mM luzindole and both melatonin and luzindole did not differ from those follicles cultured in control medium. The follicles cultured with luzindole had, however, lower percentage of normal follicles than those cultured with melatonin (Figure 19).

Fluorescence microscopy showed that follicles cultured for 18 days in control medium alone or supplemented with melatonin, luzindole and both melatonin and luzindole stained positively for calcein, but not for ethidium-1 homodimer. However, low calcein staining was observed in the central region of follicles cultured in control medium alone or supplemented with luzindole or both melatonin and luzindole (Figure 20).

Figure 21 shows that follicles cultured in presence of melatonin had significantly higher rates of antrum formation than those cultured in control medium, while those culture in presence of luzindole or both melatonin and luzindole did not differ from control group.

Figure 22 shows that melatonin, luzindole or both did not influence the levels of mRNA for CAT and SOD when compared to control group. Follicles cultured in medium with only melatonin had, however, higher levels of mRNA for CAT and SOD than to those cultured with luzindole or both melatonin and luzindole. Follicles cultured with both melatonin and luzindole had lower levels of mRNA for PRDX6 than that cultured control medium. In addition, luzindole or both melatonin and luzindole reduced the levels of mRNA for GPX1 when compared to control group.

4. Discussion

This study shows that type 1A/B melatonin receptors are expressed in oocytes and granulosa cells from bovine follicles and that 10^{-7} M melatonin promotes in vitro growth of secondary follicles after binding to these membrane receptors. Consistent with our work, a similar concentration of melatonin increased the survival and rate of development of preantral

follicles in mice and goats [31,35]. Melatonin can stimulate follicular development by promoting increased production of insulin-like growth factor 1, an important mitogen growth factor in granulosa cells [36]. Xiao et al (2019) also reported that melatonin suppressed apoptosis of bovine granulosa cells and up-regulated the expression of genes that antagonize apoptosis and down-regulated genes that normally increase apoptosis [37]. In our study, the positive effects of melatonin on follicle growth were blocked by its receptor antagonist luzindole, emphasizing that melatonin acts through receptors to promote follicle growth. Depending on the tissue, organ and species, melatonin activates different second messenger cascades by interacting with its receptors [38]. Activation of melatonin receptors promotes changes in intracellular cAMP, cGMP and calcium levels [39].

This study showed that melatonin increased antrum in cultured bovine preantral follicles. During follicular development, both granulosa cell proliferation and antrum formation are essential to increase follicular diameter. Previous studies have also reported that melatonin increases human follicular fluid formation during folliculogenesis [40]. The production of hyaluronan and proteoglycan by granulosa cells generates an osmotic gradient that attracts fluid from thecal blood vessels [41]. Proteoglycans and their glycosaminoglycan side chains are osmotic solutes that act to increase the osmotic pressure within the follicle, resulting in fluid accumulation [42-45]. Versican and versican proteoglycans have been identified in the follicular fluid of several species, including bovine [45,46] and humans [47], while perlecan (also known as heparan sulfate proteoglycan 2, HSPG2) protein and HSPG2 mRNA have been identified in granulosa cells of bovine antral follicles [46,48,49].

Melatonin also acts as free radical scavenging and its antioxidant activity can improve preantral follicle growth through the protection of follicular cells against damage caused by ROS formed during in vitro culture [50, 51]. The antioxidant activity of melatonin is related to its ability to donate electrons and, therefore, neutralize ROS [52,53]. Furthermore, due to its

small size and highly amphiphilic properties [54,55], melatonin can act in aqueous and lipid media, accumulating in the most diverse cellular structures, preventing damage to the DNA and lipid peroxidation and preserving the function of organelles, especially mitochondria [19, 56, 57]. In our study, luzindole reduced the levels of mRNA for SOD, CAT and Prdx6 in comparison to follicles cultured with only melatonin. In accordance with our results, Mayo et al. [58] showed that melatonin increases the expression of mRNA for SOD and GPx, and that regulation of expression of these enzymes is receptor mediated. By blocking melatonin receptors, luzindole also reduced mRNA expression for Prdx6 and GPx1 in cultured secondary follicles. It is important to emphasize that oocyte [21] and granulosa cells [37] produce melatonin, which can act in an autocrine manner in the follicles. Thus, luzindole can also inhibit endogenous effects of melatonin, which explain the reduction of mRNA for Prdx6 and GPX1 in follicles cultured in medium with only luzindole. Ceko et al. [59] showed that the high expression of GPx1 in bovine granulosa cells is associated with follicle survival, as well as estradiol and progesterone secretion. SOD catalyzes the dismutation of superoxide radicals into H_2O_2 which is later converted to water and oxygen by CAT or GPx1 [60]. CAT is known to play a crucial role in antioxidant defense pathways, effectively catalyzing the conversion of H_2O_2 to water and oxygen. Due to its greater catalytic effectiveness, CAT has been considered an important antioxidant enzyme in reproductive processes. Khan et al. [61] demonstrated that CAT gene silencing compromised the physiological functions of bovine granulosa cells, in addition to increasing intracellular levels of ROS, inducing apoptosis and impairing matrix metalloproteinase homeostasis, estradiol and progesterone production. A reduction in the activity of these antioxidant enzymes can result in in vitro growth regression of preantral follicles [62].

In conclusion, melatonin receptors type 1A/B are expressed in oocytes and granulosa cells of primordial, secondary and antral bovine follicles and melatonin stimulates development

of preantral follicles through its membrane-coupled receptors. Furthermore, luzindole blocks the effects of melatonin on expression of antioxidant enzymes (SOD, CAT and Prdx6) in cultured follicles.

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Conflict of interest

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

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Figure 20. Viability of bovine secondary follicles cultured for 18 days after staining with calcein-AM (green) and ethidium homodimer-1 (red). Secondary follicles cultured in TCM-199^{+} alone (a, b) or supplemented with melatonin (c, d), luzindole (e, f) or melatonin associated with luzindole (g, h). The scale bars represent 100 μm.

Figure 21. Rate of antrum formation in secondary follicles after 18 days of in vitro culture in TCM-199^{+} alone supplemented with melatonin, luzindole and both melatonin and luzindole.

Figure 22. mRNA levels (mean ± standard deviation) for (A) CAT, (B) SOD, (C) PRDX6 or (D) GPX1 in secondary follicles cultured in vitro for 18 days in TCM-199 + alone supplemented with melatonin, luzindole and melatonin associated with luzindole. a, b, c Lowercase letters represent statistically significant differences between treatments ($P < 0.05$).

Table 5.

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

Table 6.

	Day 0	Day 6	Day 12	Day 18
TCM-199^{+}	192.3±4.3^{aA}	241.0±6.0^{aB}	267.0±9.2^{aC}	282.0±12.2^{abC}
MEL 10^{-11}M	203.4±4.2^{aA}	249.5±5.4^{aB}	283.2±6^{aC}	297.5±5.2^{bD}
MEL 10^{-9}M	202.0±5.1^{aA}	251.4±9,5^{aB}	289.2±10^{aC}	313.7±9.6^{bD}
MEL 10^{-7}M	204.0±4.3^{aA}	275.6±7.7^{bB}	314.7±8.2^{bC}	342.1±7.6^{cD}
MEL 10^{-5}M	199.7±6,0^{aA}	277.4±6.6^{bB}	290.4±8.6^{aB}	275.0±9.2^{aC}

A, B, C, D Capital letters represent statistically significant differences between days of culture for each treatment (between columns) ($p < 0.05$)
a, b, c, d Lower case represent statistically significant differences between treatments at the same day of culture (same column) ($p < 0.05$)

Table 7.

	Day 0	Day 6	Day 12	Day 18
TCM-199^{+}	201.8±3.3^{aA}	238.9±5.8^{aB}	274.2±5.8^{aC}	313.7±7.3^{aD}
Melatonin	213.3±3.1^{aA}	277.4±5.1^{bB}	336.3±5.9^{bC}	418.1±9.2^{bD}
Luzindole	205.4±4.1^{aA}	232.6±4.1^{aB}	262.3±5.1^{aC}	347.2±7.7^{aD}
Mel+Luzindole	205.6±3.4^{aA}	248.2±5.3^{aB}	285.7±6.5^{aC}	326.5±8.5^{aD}

A, B, C, D Capital letters represent statistically significant differences between days of culture for each treatment (between columns) ($p < 0.05$)
a, b, c, d Lower case represent statistically significant differences between treatments at the same day of culture (same column) ($p < 0.05$)

Figure 17.

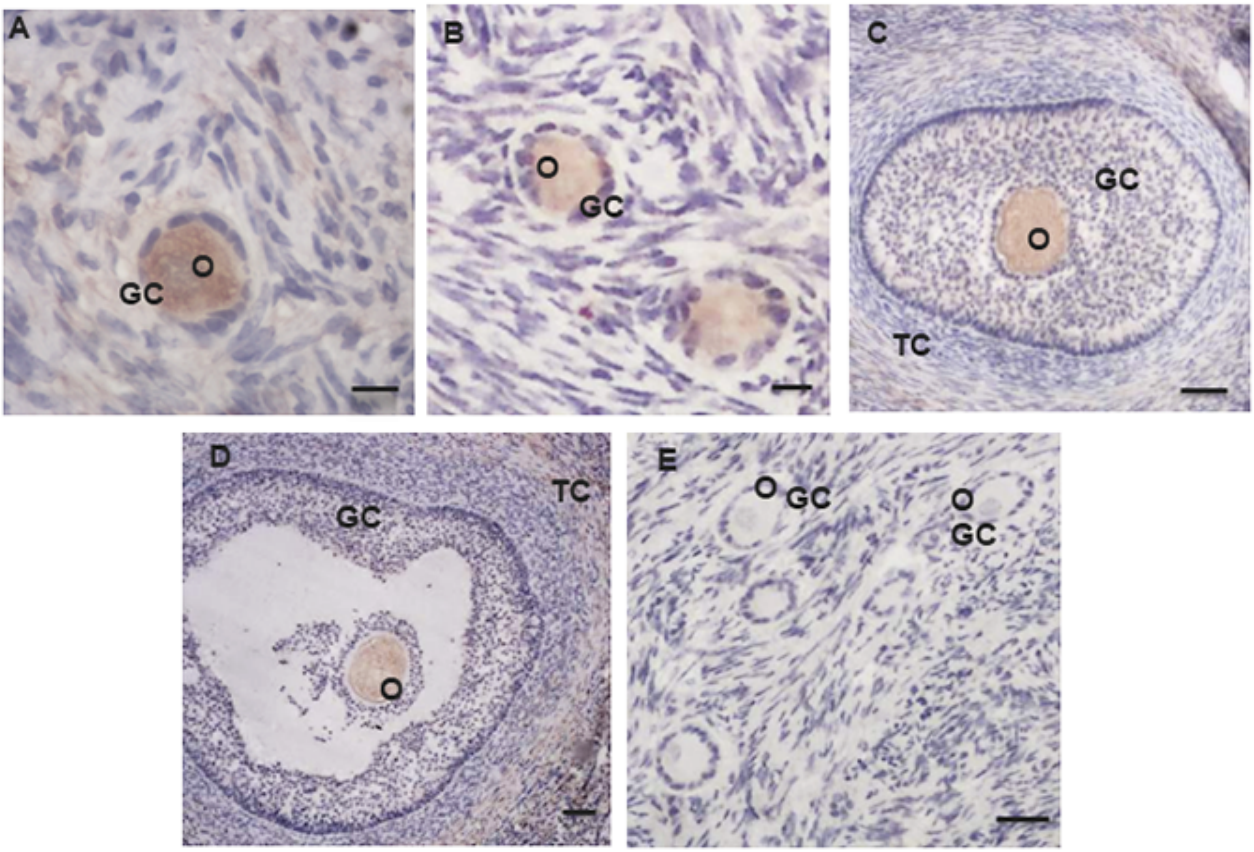


Figure 18.

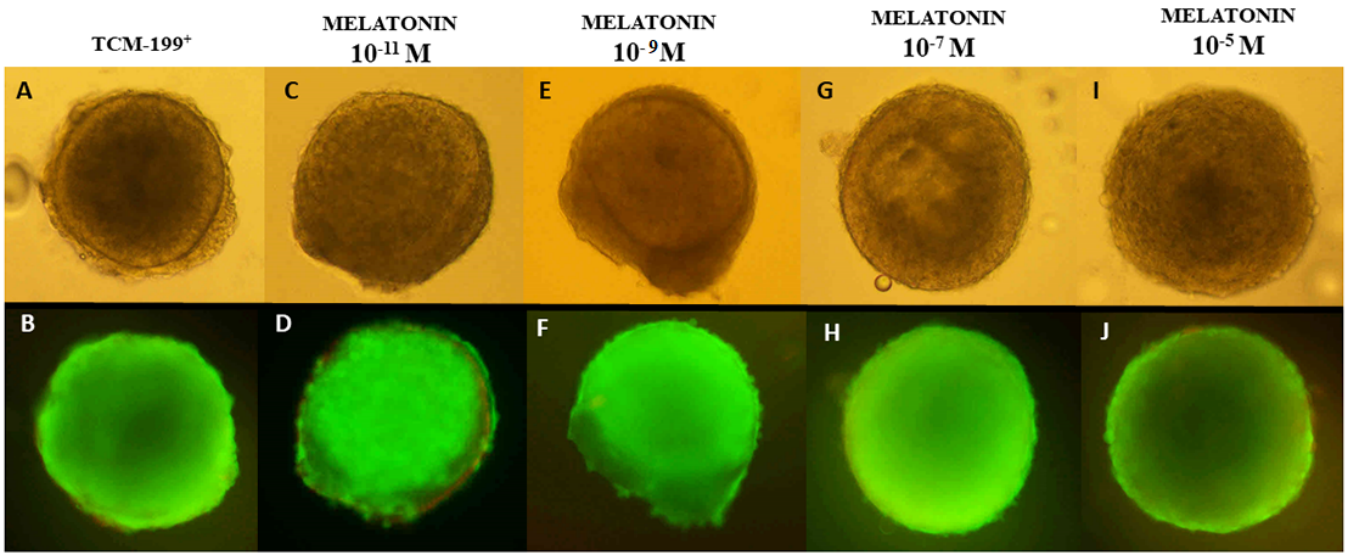

TCM-199+
MELATONIN 10-11 M
MELATONIN 10-9M
MELATONIN 10-7 M
MELATONIN 10-5 M
A
C
E
G
I
B
D
F
H
J

Figure 19.

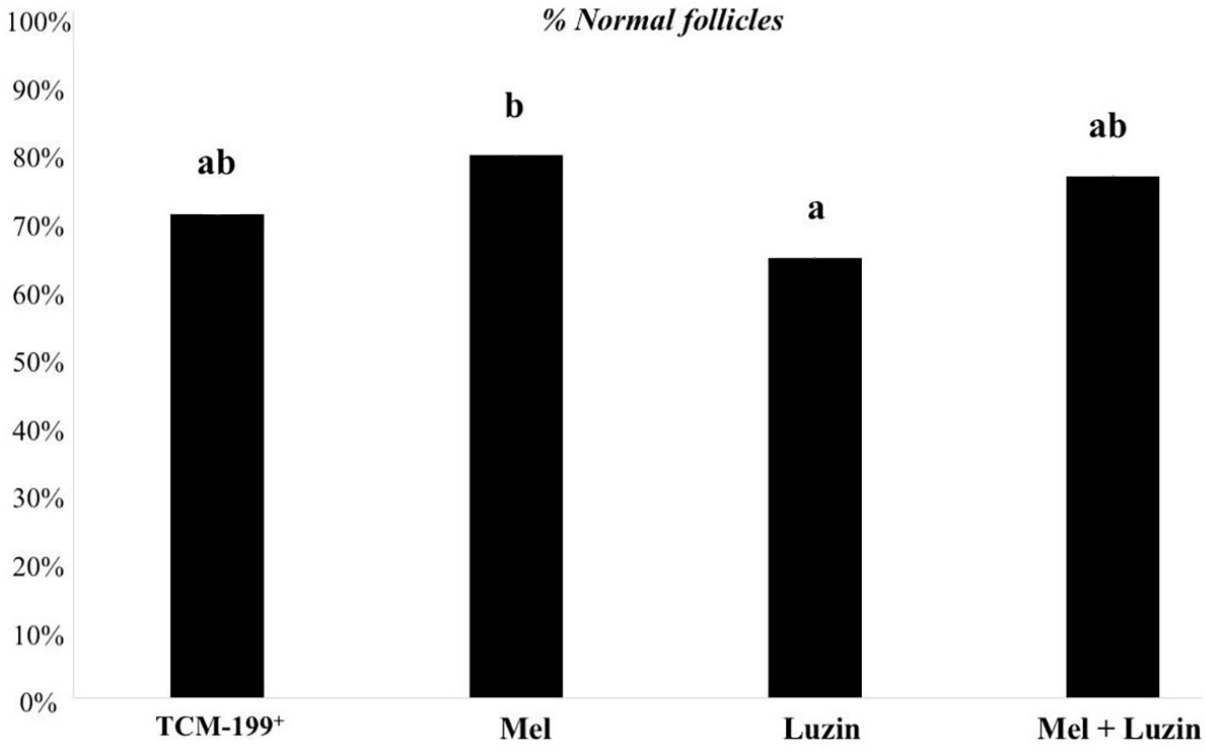

% Normal follicles
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
ab
b
a
ab
TCM-199+
Mel
Luzin
Mel + Luzin

Figure 20.

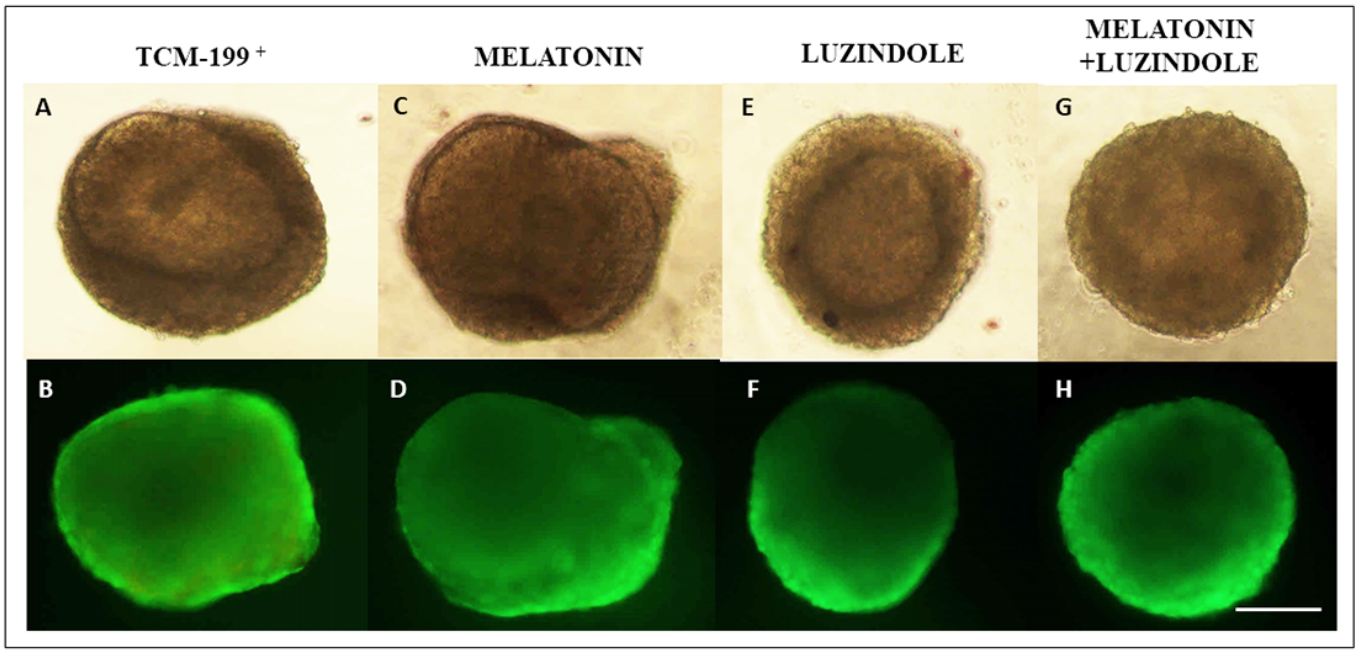

TCM-199 +
MELATONIN
LUZINDOLE
MELATONIN
+LUZINDOLE
A
B
C
D
E
F
G
H

Figure 21.

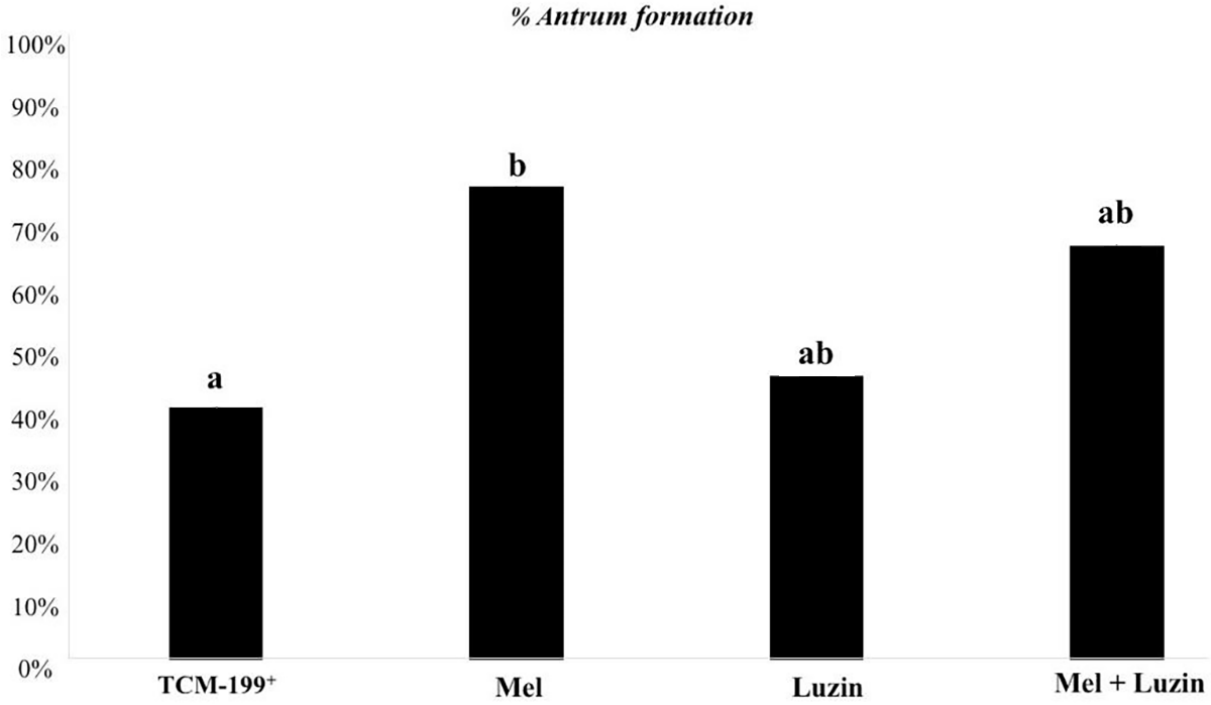
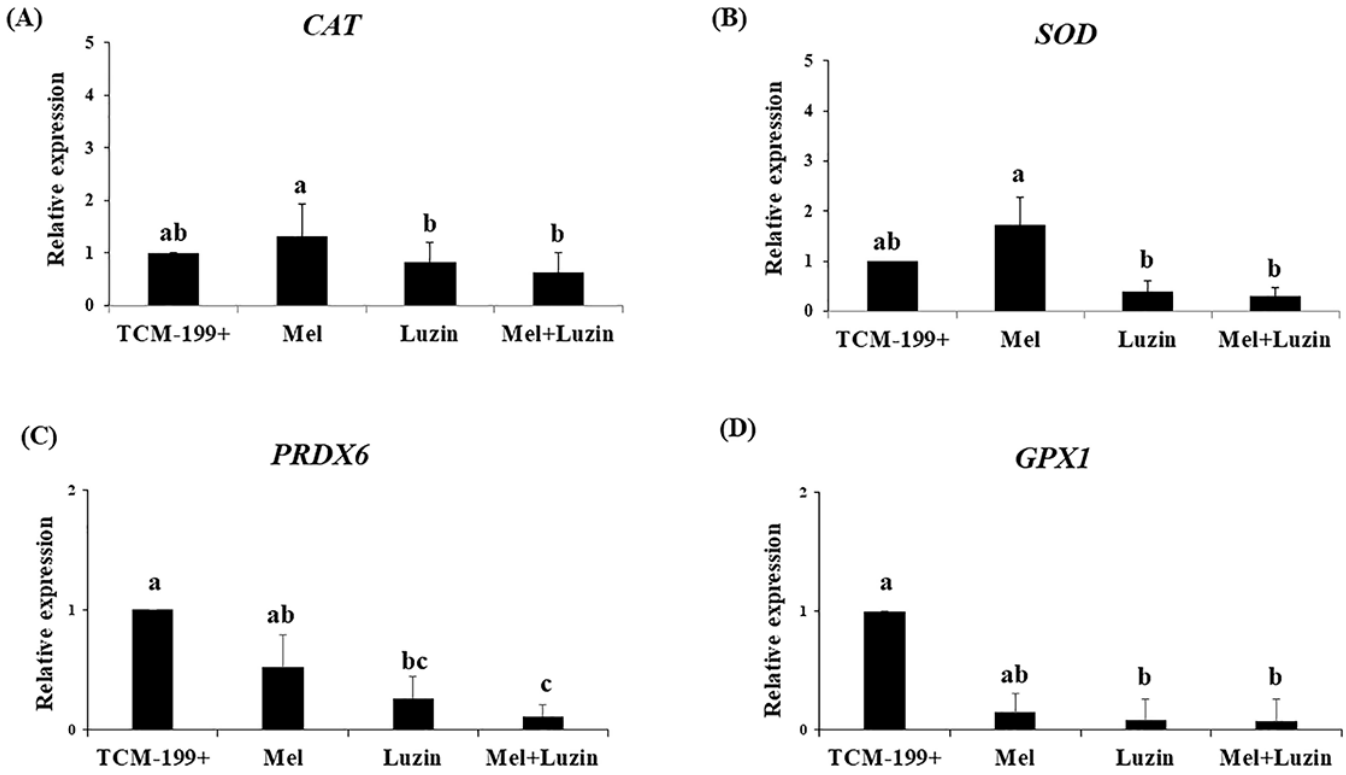

% Antrum formation
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
a
b
ab
ab
TCM-199+
Mel
Luzin
Mel + Luzin

Figure 22.


(A)
CAT
Relative expression
5
4
3
2
1
0
ab
a
b
b
TCM-199+
Mel
Luzin
Mel+Luzin
(B)
SOD
Relative expression
5
4
3
2
1
0
ab
a
b
b
TCM-199+
Mel
Luzin
Mel+Luzin
(C)
PRDX6
Relative expression
2
1
0
a
ab
bc
c
TCM-199+
Mel
Luzin
Mel+Luzin
(D)
GPX1
Relative expression
2
1
0
a
ab
b
b
TCM-199+
Mel
Luzin
Mel+Luzin

8 CONCLUSÕES GERAIS

O EGF promove o crescimento folicular e aumenta a expressão de RNAm para GDF9 e cMOS em folículos bovinos cultivados *in vitro*, enquanto a progesterona não influencia o crescimento folicular *in vitro*, mas aumenta a expressão de GDF9 e ciclina B1 em oócitos. No entanto, o EGF e a progesterona não têm efeito sinérgico durante o crescimento de folículos pré-antrais bovinos.

Os receptores de melatonina dos tipos 1A/B são expressos em oócitos e células da granulosa de folículos primordiais, secundários e antrais de bovinos e a melatonina estimula o desenvolvimento de folículos pré-antrais por meio de seus receptores acoplados à membrana. Além disso, o luzindole bloqueia os efeitos de crescimento e expressão de enzimas antioxidantes (SOD, CAT e Prdx6) promovidos pela a melatonina, mostrando que este hormônio atua por meio da ligação com seus receptores de membrana.

9 PERSPECTIVAS

O desenvolvimento de um sistema *in vitro* adequado para apoiar o cultivo a longo prazo de folículos pré-antrais de espécies humanas e animais domésticos para proporcionar um ambiente folicular adequado capaz de recriar uma estrutura tridimensional pode ser uma alternativa para melhorar o desenvolvimento folicular *in vitro*. E ainda, a adição de substâncias como EGF, P4 e melatonina podem contribuir para o aumento da taxa de competência oocitária após o cultivo *in vitro* de folículos secundários aptos a assegurar o desenvolvimento embrionário inicial, bem como podem apoiar o conhecimento dos níveis de expressão de RNAm que são estocados nos oócitos.

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ANEXOS

ANEXO A- PUBLICAÇÃO – ARTIGO TÉCNICO (CAPÍTULO I)

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Effects of epidermal growth factor and progesterone on development, ultrastructure and gene expression of bovine secondary follicles cultured in vitro

L.R.F.M. Paulino, P.A.A. Barroso, A.W.B. Silva, A.L.P. Souza, F.T.G. Bezerra, B.R. Silva, M.M.A. Donato, C.A. Peixoto, J.R.V. Silva *

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ABSTRACT

The aims of this study were to investigate the effects of epidermal growth factor (EGF) and progesterone on the development, viability and the gene expression of bovine secondary follicle culture in vitro for 18 days. Secondary follicles (~0.2 mm) were isolated from ovarian cortex and individually cultured at 38.5 °C, with 5% CO_2 in air, for 18 days, in TCM-199® (n = 63) alone (control medium) or supplemented with 10 ng/mL progesterone (n = 64), 10 ng/mL EGF (n = 61) or both EGF and progesterone (n = 66). The effects of these treatments on growth, antrum formation, viability, ultrastructure and mRNA levels for *GDF-9*, *c-MOS*, *H1foo* and *cyclin B1* were evaluated, significantly different ($p < 0.05$). The results showed that there was a progressive increase in follicular diameter in all treatments, but only follicles cultured in medium supplemented with EGF had increased significantly in diameter when compared to follicles cultured in the control medium at the end of the culture period, significantly different ($p < 0.05$). A positive interaction between EGF and progesterone was not observed. In addition, the presence of EGF, progesterone or both in culture medium did not influence the rate of follicle survival and antrum formation. However, the presence of only progesterone in cultured medium increased the expression of mRNAs for *GDF9* and *cyclin B1* in oocytes. EGF also significantly increased the levels of mRNAs for *cMOS* and *GDF9* when compared to follicles cultured in control medium. Ultrastructural analyzes showed that cultured follicles in all treatments maintained the integrity of granulosa cells. In conclusion, the EGF promotes the development of secondary follicles cultured in vitro for 18 days and increases the expression of *cMOS* and *GDF9*, while progesterone alone or in association with EGF have not a positive effect on follicular growth. However, progesterone increases the expression of *GDF9* and *cyclin B1* in oocytes.

1. Introduction

Preantral follicles are the majority of the ovarian follicle population and their use as a source of homogeneous oocytes for bovine reproductive biotechnologies could result in a substantial advance in this field [1]. *In vitro* culture of secondary follicles of ruminants have already been performed in an attempt to obtain oocytes suitable for fertilization (caprine [2], bovine [3]) and production of embryos have been reported for caprine [4] and ovine species [5]. However, in the bovine species, the results are limited to the formation of early antral follicles during secondary follicle culture in vitro [3,6–9]. In this way, in vitro culture of secondary follicles is necessary to provide information on the follicular needs during development, which can help to make possible the use of their oocytes in assisted reproduction techniques. Thus, it is important to improve culture systems by adding growth factors and hormones to understand its mechanisms and to optimize the in vitro development of bovine secondary follicles. Among these substances, epidermal growth factor (EGF) and progesterone (P4) are candidates to improve follicular survival and growth in vitro.

Previous studies have shown that EGF has an important role in ovarian folliculogenesis, by regulating several processes, like proliferation of granulosa cells and atresia (caprine [10], equine [11]). In cattle, the addition of EGF increases cumulus cells expansion and improved maturation and oocyte fertilization rate [12]. More recently, Choi et al. [13] showed that the effects of EGF on goat oocyte maturation can be potentiated by progesterone, since this hormone increases the expression of EGF-like factors that influences oocyte maturation. However, it is still not yet known if EGF and progesterone interacts positively to promote in vitro development of bovine preantral follicles.

Progesterone regulates various reproductive processes in females, including ovulation, implantation and sexual differentiation. Signaling downstream of P4 has been studied primarily through the activation of nuclear receptors that act as transcription factors to stimulate P4-dependent gene expression [14]. In addition, P4 has been shown to play an important role in regulating bovine oocyte maturation both in vivo and in vitro by signaling through both nuclear (PGRs) and membrane P4 receptors (mPR). Inhibition of P4 synthesis and blockage of the

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ANEXO B- PUBLICAÇÃO – ARTIGO DE REVISÃO

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REVIEW

Why Is It So Difficult To Have Competent Oocytes from In vitro Cultured Preantral Follicles?

Laís R. F. M. Paulino[1] · Ernando I. T. de Assis[1] · Venâncio A. N. Azevedo[1] · Bianca R. Silva[1] · Ellen V. da Cunha[1] · José R. V. Silva[1]

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Abstract
The developmental competence of oocytes is acquired gradually during follicular development, mainly through oocyte accumulation of RNA molecules and proteins that will be used during fertilization and early embryonic development. Several attempts to develop in vitro culture systems to support preantral follicle development up to maturation are reported in the literature, but oocyte competence has not yet been achieved in human and domestic animals. The difficulties to have fertilizable oocytes are related to thousands of mRNAs and proteins that need to be synthesized, long-term duration of follicular development, size of preovulatory follicles, composition of in vitro culture medium, and the need of multi-step culture systems. The development of a culture system that maintains bidirectional communication between the oocyte and granulosa cells and that meets the metabolic demands of each stage of follicle growth is the key to sustain an extended culture period. This review discusses the physiological and molecular mechanisms that determine acquisition of oocyte competence in vitro, like oocyte transcriptional activity, follicle and oocyte sizes, and length and regulation of follicular development in murine, human, and domestic animal species. The state of art of in vitro follicular development and the challenges to have complete follicular development in vitro are also highlighted.

Keywords Follicle growth · Oocyte maturation · Culture systems · Preantral follicles

Introduction

For most mammalian species, formation of primordial follicles takes place pre-natally and constitutes the basic reserve of gametes, which are recruited for growth throughout the reproductive age. Follicular development involves growth of preantral follicles, i.e., activation of primordial follicle and development to primary and secondary follicle stages, as well as formation of antral and Graafian follicles to release a cumulus-oocyte complex (COC) at ovulation [1, 2]. During follicle growth, oocytes have great transcriptional activity and store a large amount of RNA to support the processes of fertilization, embryonic development, and activation of embryonic genome [3]. Additionally, changes in carbohydrate and lipid metabolism, mitochondrial function and location, reduction of oxygen radicals, epigenetic reprogramming, and bidirectional communication between cumulus cells and oocytes are observed during oocyte cytoplasmic maturation [4]. All these events are crucial for a proper acquisition of oocyte competence.

Current advances in in vitro embryo production are still dependent on a supply of oocytes from large antral follicles, which are present in the ovary in relatively small numbers [5]. Thus, the development of in vitro culture systems to explore immature oocytes enclosed in preantral follicles has a great potential to provide a large number of oocytes for in vitro fertilization in human [6] and domestic animal species [7–10]. It is important to consider that preantral follicles represent 90 to 95% of the entire follicular population and store the majority of oocytes present in mammalian ovaries [11]. The complex mechanisms controlling follicular development are, however, obstacles to have competent oocytes in vitro. Over time, many studies have been conducted to optimize culture systems to support three crucial stages, i.e., primordial follicles

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ANEXO C- COMPROVANTE DE SUBMISSÃO – ARTIGO TÉCNICO (CAPÍTILO II)

Please verify your contribution to Immunolocalization of melatonin receptors in bovine ovarian follicles and in vitro effects of melatonin on growth, viability and gene expression in secondary follicles

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