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INFLUÊNCIA DA N-ACETILCISTEÍNA NO CRESCIMENTO E VIABILIDADE DE FOLÍCULOS PRÉ-ANTRAIS BOVINOS CULTIVADOS *IN VITRO*

DANISVÂNIA RIPARDO NASCIMENTO

SOBRAL-CE

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Dissertação de Mestrado apresentada ao Programa de Pós-Graduação em Biotecnologia, da Universidade Federal do Ceará – Campus Sobral, como requisito parcial para obtenção do título de Mestre em Biotecnologia. Área de concentração: Biotecnologia. Linha de Pesquisa: Análises Integrativas de Sistemas Biológicos. Orientador: Prof. Dr. José Roberto Viana Silva

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Aprovada em: ___/___/_____.

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A Deus.
Aos meus familiares Manoel, Danilo, Danislândia, Daniel, Danielson por serem minha base.
A minha mãe, Maria Conceição, por ser essa mulher extraordinária. Mãe você é incrível, tudo isso é por você.
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RESUMO

O estresse oxidativo durante o cultivo *in vitro* de folículos pre-antrais é um dos fatores responsáveis pela redução de viabilidade folicular *in vitro*. Desta forma, faz-se necessário a suplementação dos meios de cultivo com substâncias antioxidantes. A N-Acetilcisteína (NAC) age diretamente em radicais livres e indiretamente por meio da produção de glutationa (GSH). Desta forma, este estudo teve como objetivo investigar os efeitos de diferentes concentrações de NAC sobre crescimento, na morfologia, na formação de antro e na viabilidade de folículos pré-antrais bovinos cultivados *in vitro* por 18 dias. Para isso, os folículos foram cultivados individualmente a 38,5°C com 5% de CO_2 no ar durante 18 dias em TCM-199^{+} sozinho ou suplementado com diferentes concentrações de NAC (1.0 mM, 5.0 mM e 25.0 mM). Crescimento folicular, morfologia, formação de antro, análise de viabilidade (calceína-AM e homodímero de etídio), e histologia foram avaliados após 18 dias de cultivo. Os dados do diâmetro folicular e intensidade de coloração para viabilidade foram analisados pelo teste de Kruskal-Wallis, seguido pelo teste de comparação múltipla de Dunn. As porcentagens de sobrevivência folicular e formação de antro foram comparadas pelo teste do qui-quadrado e as porcentagens de folículos em crescimento foram comparadas pelo teste exato de Fisher ($P<0,05$). A presença de 1.0 mM de NAC no meio de cultivo aumentou a porcentagem de folículos em crescimento após 18 dias *in vitro*. A NAC (1.0 mM) também aumentou a intensidade de fluorescência para calceína-AM nos folículos pré-antrais cultivados *in vitro*. Quanto a adição de 5.0 mM de NAC não apresentou diferença significativa em nenhum parâmetro. Já a presença de 25.0 mM NAC reduziu a taxa de folículos em crescimento e aumentou a marcação para homodímero-1 de etídio. A análise histológica mostrou que os folículos morfologicamente normais no final do período de cultivo apresentavam com oócitos redondos, centralizados e células granulosas organizadas. Por outro, folículos com sinais de degeneração tinham oócitos com grande quantidade de vacúolos e células da granulosa desorganizadas. Em conclusão, 1.0 mM de NAC aumenta a viabilidade e o crescimento de folículos pré-antrais cultivados *in vitro*, enquanto 25.0 mM de NAC causam danos às membranas celulares e reduz as taxas de crescimento folicular.

Palavras chaves: Antioxidante. Bovinos. Folículos pré-antrais

ABSTRACT

The oxidative stress during *in vitro* culture of preantral follicles is one of the factors responsible for the reduction of follicular viability. Thus, it is necessary to supplement the culture media with antioxidant substances. The N-acetylcysteine (NAC) acts directly on free radicals and indirectly through the production of glutathione (GSH). This study aims to investigate the effects of different concentrations of N-acetylcysteine (NAC) on growth, morphology, antrum formation, and viability of bovine secondary follicles cultured *in vitro* for 18 days. To this end, follicles were cultured at 38.5°C with 5% CO_2 for 18 days in TCM-199^{+} alone or supplemented with 1.0, 5.0 or 25.0mM NAC. Follicular growth, morphology, antrum formation, viability (calcein-AM and ethidium homodimer-1), and histological features were evaluated at the end of culture. Follicular diameter and fluorescence intensity data for viability were analyzed by Kruskal-Wallis test, followed by Dunn's multiple comparison test. Percentages of follicle survival and antrum formation were compared by the chi-square test and percentages of growing follicles were compared by Fisher's exact test ($P<0.05$). The results showed that 1.0 mM NAC significantly increased the percentage of growing follicles when compared to other treatments. The addition of 5.0 mM of NAC did not change significantly in any parameter. On the other hand, 25.0 mM NAC significantly reduced the rate of growing follicles. Secondary follicles cultured with 1.0 mM NAC had higher fluorescence intensity for calcein-AM, while those cultured with 25.0 mM NAC were mainly stained with ethidium homodimer-1 ($P<0.05$). Histological analysis showed that oocyte vacuolization is the first sign of degeneration in cultured follicles. In conclusion, 1.0 mM NAC increases follicle viability and growth rate in bovine secondary follicles cultured *in vitro*, while 25.0 mM NAC cause damages in cellular membranes and reduces follicular growth rate.

Keyword: Antioxidant. Bovine. Preantral follicles

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LISTA DE ABREVIATURAS E SIGLAS

ANOVA	Análise de Variância
BSA	Albumina Sérica Bovina
CO^2	Gás Carbônico
Cu_2^+	Íons de cobre
CGPs	Células germinativas primordiais
DNA	Ácido desoxirribonucleico
Fe_3	Compostos contendo ferro
FSH	Hormônio Folículo Estimulante
Foxo3a	Fator de transcrição forkhead box O 3ª
Foxl2	Forkhead Box I2
H^2O^2	Peróxido de hidrogênio
IBGE	Instituto Brasileiro de Geografia e Estatística
ITS	Insulina, Transferrina, Selênio
LH	Hormônio Luteinizante
miRNAs	MicroRNAs
MOIFOPA	Manipulação de oócitos inclusos em folículos pré-antrais
NaCl	Cloreto de Sódio
O_2	Oxigênio
PBS	Tampão fosfato salino
pH	Potencial Hidrogeniônico

PTEN	Homólogo de fosfatase e tensina do cromossomo 10
P27	Inibidor de quinase dependente de ciclina 1B
RNA	Ácido ribonucleico
TCM 199	Meio de Cultivo Tecidual 199

LISTA DE SÍMBOLOS

<	Menor que
%	Porcentagem
IU	Unidades Internacionais
µg	Micrograma
µl	Microlitro
µm	Micrometros
G	Gramas
Mg	Miligrama
mL	Mililitro
Mm	Milímetros
mM	Milimolar
Ng	Nanograma
ºC	Grau Celsius

SUMÁRIO

1. INTRODUÇÃO

O Brasil contém um dos maiores rebanhos bovino do mundo, possuindo em torno de 218,2 milhões de cabeças e se classificando como um dos principais produtores e exportadores de carne (IBGE, 2020). Esse fato deve-se ao histórico de pesquisas que estimulam o desenvolvimento e aperfeiçoamento de biotécnicas reprodutivas que proporcionam o aumento na multiplicação de animais de elevado potencial econômico (VIANA; FIGUEIREDO; SIQUEIRA, 2017).

Os folículos pré-antrais correspondem a maior parte da população folicular ovariana (ALVES *et al*., 2017; FIGUEIREDO;LIMA; SANTOS, 2019). Desta forma, eles têm o potencial de fornecer um grande número de oócitos competentes para a a maturação *in vitro*. Apesar de o ovário bovino possuir uma grande quantidade de folículos pré-antrais, apenas uma pequena parcela alcança a ovulação, a maioria dos folículos irão se degenerar, através de um processo denominado atresia folicular (FIGUEIREDO *et al*., 2007). Os sistemas de cultivo disponíveis ainda não possibilitam o aproveitamento desses folículos, pois a progressão do desenvolvimento *in vitro* têm se limitado ao crescimento de folículos secundários até o estágio de folículos antrais iniciais (DA CUNHA *et al*., 2018; PAULINO *et al*., 2018; VASCONCELOS *et al*., 2021). Um dos fatores que dificulta a efetividade dos sistemas de cultivos *in vitro* de folículos pré-antrais é o excesso de espécies reativas de oxigênio (EROs), que causa estresse oxidativo. O excesso de EROs pode ocasionar danos mitocondriais, peroxidação lipídica e comprometimento da integridade de estruturas como RNA, DNA e proteínas (FILIPOVIĆ *et al.*, 2015), diminuindo assim da qualidade dos oócitos. A produção de EROs durante o cultivo *in vitro* tende a aumentar devido a ausência de mecanismos de proteção das células, manipulações e, aumento dos níveis de oxigênio no meio celular (LINS *et al*., 2017;VON MENGDEN; KLAMT; SMITZ, 2020; SOTO-HERAS; PARAMIO, 2020).

A utilização de substâncias antioxidantes no meio de cultivo folicular *in vitro* visa minimizar os danos ocasionados pelo excesso de EROs e manter as funções fisiológicas dos folículos (KASHKA; ZAVAREH; LASHKARBOLOUKI, 2016). A N-Acetilcisteina (NAC) tem alcançado destaque devido as suas atividades farmacológicas. Sua ação antioxidante pode ocorrer de forma direta e indireta: a forma direta ocorre pela ação do composto sufridila (-SH), que interage diretamente com os grupos eletrofílicos dos radicais oxidantes, e indireta, através da regulação dos níveis de GSH que é um dos principais antioxidantes endógenos (PEI *et al*., 2018). Desta forma, a NAC apresenta um elevado potencial antioxidante e citoprotetor que pode ser aproveitado como suplemento adicional em meios de

cultivo *in vitro* de folículos secundários bovinos, podendo reduzir o estresse oxidativo e manter a viabilidade folicular.

Desta forma, para um melhor esclarecimento acerca da importância desta proposta, serão explanados aspectos relacionados como: ovário dos mamíferos, processos de oogênese e foliculogenese isolamento e cultivo de folículos pré-antrais, formação das EROs e adição de antioxidantes no cultivo de folículos ovarianos, e N-Acetilcisteína.

2. REFERÊNCIAL TEÓRICO

2.1 Ovário dos mamíferos

O ovário dos mamíferos é composto por uma região cortical e uma medular. A região medular é constituída por tecido conjuntivo frouxo que possui uma grande quantidade de vasos sanguíneos e linfáticos, tais estruturas promovem a distribuição de nutrientes e sustentação do ovário. Já a região cortical é composta pela população de folículos ovarianos, além dos corpos lúteos (JUNQUEIRA; CARNEIRO, 2013; MONNIAUX *et al*., 2014). O ovário dos mamíferos e seus principais constituintes estão esquematizados na Figura 1.

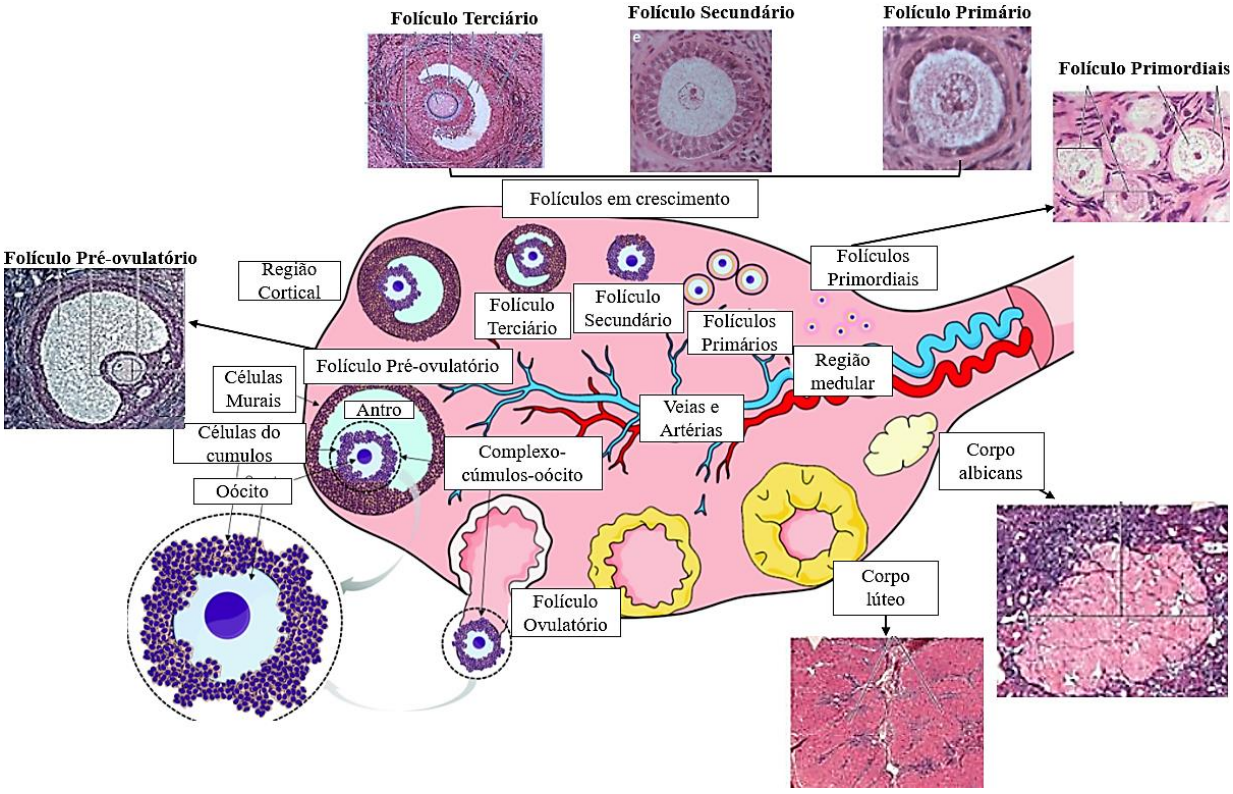

Figura 1. Esquema ilustrativo do ovário de mamíferos e seus principais constituintes. Fonte: Adaptado de Junqueira e Carneiro, (2013), Mescher, (2016) e Von mengden, Klamt e Smitz, (2020).

O ovário apresenta duas funções principais, a primeira está relacionada à função endócrina, correspondendo à liberação de estrogênio e progesterona, o controle do eixo hipotálamo-hipófise-ovário, o controle do ciclo estral/menstrual e o funcionamento do ovário (EDSON; NAGARAJA; MATZUK, 2009). A segunda função está relacionada ao papel

gametogênico, que corresponde ao desenvolvimento e maturação de oócitos inclusos nos folículos ovarianos tornando-os aptos para a fecundação. Os folículos ovarianos contém um oócito circundado por células somáticas (células da granulosa e tecais) e são responsáveis por determinar o ciclo reprodutivo (SPITSCHAK; HOEFLICH, 2018).

A função gametogênica do ovário envolve dois eventos; a foliculogênese e a oogênese. São processos complexos que levam as mudanças morfológicas e funcionais nos folículos, assim como o desenvolvimento e maturação dos oócitos (PORRAS-GÓMEZ; MORENO-MENDOZA, 2017; SÁNCHEZ; SMITZ, 2012). Em adição, o entendimento desses eventos permite elucidar a dinâmica folicular, assim como compreender os mecanismos e fatores envolvidos nesses processos biológicos.

2.2 Processos de oogênese e foliculogênese

A foliculogênese e a oogênese são processos cruciais para liberação do oócito maduro e viável durante o processo de ovulação (FIGUEIREDO *et al*., 2007). A oogênese é o processo de formação dos oócitos que se inicia durante o desenvolvimento embrionário. No primeiro mês de gestação em mamíferos, as células germinativas primordiais (CGPs) se deslocam do saco vitelino até as gônadas primitivas. Nas gônadas, essas células se dividem, formando as oogônias. No terceiro mês, as oogônias entram no estágio de prófase da primeira divisão meiótica e param na fase de diplóteno da meiose, formando assim os oócitos. Os oócitos na fase de diplóteno são envolvidos por uma camada de células da pré-granulosa de formato pavimentos e formam os folículos primordiais (GROSSMAN; SHALGI, 2016).

Na puberdade, por conta da liberação do pico pré-ovulatório de FSH e LH, ocorre uma progressão da divisão meiótica e, com isso, são formados os oócitos secundários, que se encontram na fase de metáfase II. Após a fecundação do oócito pelo espermatozoide, a meiose é retomada novamente e a partir disso, origina-se o oócito haploide fecundado, encerrando assim a oogênese (HURK; ZHAO, 2005).

A foliculogênese é o processo que envolve a formação, o desenvolvimento e a maturação dos folículos. Inicia-se com os folículos primordiais quiescentes, e finaliza com a formação dos folículos maduros e com oócitos aptos a serem fertilizados (folículos de *Graff* ou pré-ovulatórios) (JONES; SHIKANOV, 2019). Os folículos são as unidades morfofuncionais

do ovário, que são responsáveis pelo desenvolvimento e maturação do oócito (FIGUEIREDO *et al*., 2007; FIGUEIREDO *et al*., 2019).

Os folículos ovarianos são caracterizados pelo tamanho e quantidade de células somáticas que os circundam, sendo divididos de acordo com as características morfológicas em folículos pré-antrais ou não cavitários (primordiais, primários e secundários) e folículos antrais ou cavitários (terciários e pré-ovulatórios) (FIGUEIREDO *et al*., 2007). Os processos de oogênese e foliculogênese estão esquematizados na Figura 2.

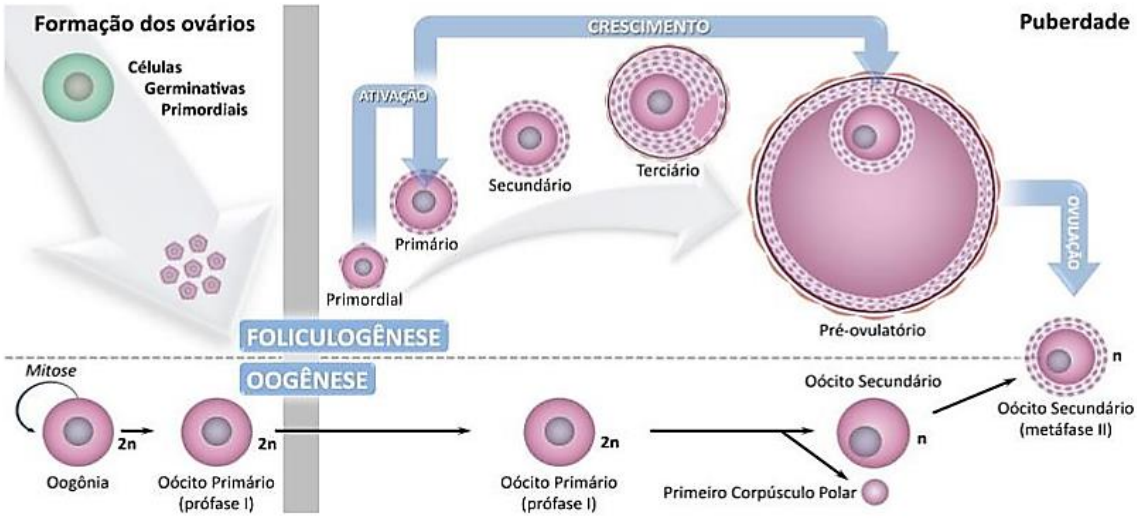

Figura 2. Esquema representando o processo de foliculogênese em paralelo com a oogênese. Fonte: Rossetto, (2013b)

2.2.1 Folículos pré-antrais

Na espécie bovina, os folículos primordiais são formados por volta do dia 110 de gestação (ERICKSON, 1966; DINIZ *et al*., 2005). Estes folículos são constituídos por uma única camada de células somáticas achatadas (pré-granulosa) (Figura 3). Os oócitos presentes nesses folículos permanecem em repouso até serem ativados e dar origem aos folículos primários e secundários (GOUGEON; BUSSO, 2000; GROSSMAN; SHALGI, 2016).

Após a ativação dos folículos primordiais, ocorrem mudanças morfológicas como um aumento do tamanho oócitário, as células da granulosa se multiplicam e dão origem aos folículos em transição que possuem células da granulosa tanto em formato cúbico como

achatadas. Estes folículos posteriormente darão origem aos folículos primários (GOUGEON; BUSSO, 2000; ARAÚJO *et al.*, 2014).

A ativação dos folículos primordiais ocorre por influência de fatores de crescimento, hormônios e peptídeos. Dentre estas substâncias, destaca-se o fator de crescimento e diferenciação 9 (GDF9), os fatores de crescimento semelhantes à insulina 1 e 2 (IGFs 1 e 2), o kit ligante (KL), o fator de crescimento epidermal (EGF), as proteínas morfogenéticas ósseas 4, 7 e 15 (BMPs 4, 7 e 15) (MONNIAUX *et al*., 2014;CHANG; QIAO; LEUNG, 2016; DA CUNHA *et al*., 2018; REINERI *et al*., 2018;PAULINO *et al*., 2020, 2018). Os mecanismos relacionados à ativação e crescimento folicular ainda permanecem em constante investigação (ARAÚJO *et al*., 2014; GOUGEON; BUSSO, 2000; MARTINS *et al*., 2008). No entanto, pesquisas indicam que a ausência da atividade das moléculas inibidoras da ativação folicular, como PTEN, Foxo3a, p27 e Foxl2, leva à ativação prematura do *pool* de folículos primordiais (CASTRILLON *et al*., 2003; SCHMIDT *et al*., 2004; RAJAREDDY *et al*., 2007; REDDY *et al*., 2008;ADHIKARI; LIU, 2009; MAIDARTI *et al*., 2019).

Os folículos primários são constituídos por uma camada de células da granulosa no formato cúbico (Figura 3). Além disso, ocorre o aumento no volume do citoplasma e núcleo do oócito (LUCCI *et al*., 2001; RODGERS; IRVING-RODGERS, 2010; ARAÚJO *et al*., 2014). Em adição, nota-se uma maior quantidade de mitocôndrias alongadas e sua dispersão por todo o ooplasma, além de um início de formação da zona pelúcida (BASSO; ESPER, 2002).

Para a formação dos folículos secundários, as células da granulosa sofrem intensa multiplicação dando origem a mais de uma camada de células, também ocorre um aumento do diâmetro folicular e oocitário. Nos folículos secundários, o oócito encontra-se cercado por duas ou mais camadas de células da granulosa cuboides (Figura 3), nota-se um espessamento da zona pelúcida, mitocôndrias alongadas e a presença de grânulos corticais no oócito (BASSO; ESPER, 2002). Em bovinos tem-se o aparecimento de folículos secundários em torno de 210 dias de gestação (RÜSSE, 1983). Vale destacar que nesta fase os folículos são responsivos ao FSH(BARROSO *et al*., 2020; BEZERRA *et al*., 2019; PAULINO *et al*., 2020). Os folículos pré-antrais são ilustrados na Figura 3.

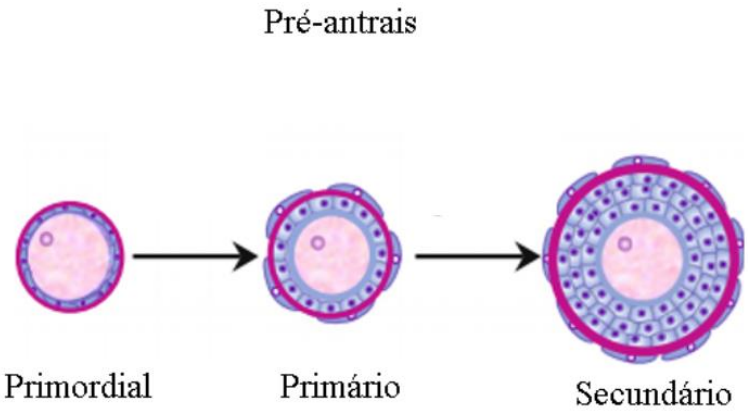

Figura 3. Ilustração representando os folículos ovarianos pré-antrais**.** Fonte: Adaptado de Araújo *et al.*(2014).

Com a progressão do crescimento folicular, as células das granulosas se multiplicam e se diferenciam. Estudos indicam que fatores como GDF-9 e BMP-15 estão envolvidos na proliferação, diferenciação das células das células da granulosa e formação do antro (ALAM; LEE; MIYANO, 2018)**.**

2.3 Manipulação de oócitos inclusos em folículos pré-antrais (MOIFOPA)

A biotécnica de MOIFOPA, também conhecida como técnica do ovário artificial, propõe a recuperação/preservação de um grande número de oócitos inclusos em folículos pré-antrais para o uso em outras biotécnicas bem estabelecidas como, a fertilização *in vitro* (FIGUEIREDO *et al*., 2007).

O apropriado método de isolamento garante a sobrevivência folicular durante o cultivo *in vitro* de folículos pré-antrais (ROSSETTO *et al*., 2011). O isolamento de folículos consiste na retirada dos folículos inclusos no tecido cortical ovariano, o que pode ocorrer de forma mecânica ou com método químico com auxílio de enzimas que digerem o tecido cortical ovariano (FIGUEIREDO *et al*., 2007; ROSSETTO *et al*., 2011). Para o método químico, as substâncias amplamente utilizadas são as enzimas colagenase, tripsina, e DNAses, já que o tecido cortical ovariano é repleto de fibroblastos e fibras colágenas. No entanto tais substâncias podem também danificar a membrana basal e as células da teca dos folículos (ARAÚJO *et al*., 2014; DEMEESTERE *et al*., 2005).

A forma mecânica de isolamento folicular conta com auxílio instrumentos como tesouras cirúrgicas, tissue chopper, micro fórceps ou agulhas para isolamento dos folículos inclusos nos fragmentos ovarianos, no entanto o número de folículos alcançados é menor quando equiparado ao método enzimático (KATSKA; RYŃSKA, 1998). Apesar deste entrave, é o mais recomendado em relação ao método químico, tanto economicamente, como pela facilidade de execução. Além disso, não danifica as células da membrana basal (KATSKA; RYŃSKA, 1998; ROSSETTO *et al*., 2011). A presença de uma membrana basal melhora os efeitos de suplementos acrescentado ao meio de cultura, já que possui proteo-heparans sulfato que se liga a diversos fatores de crescimentos (FIGUEIREDO *et al*., 1995; GOSPODAROWICZ; GREENBURG; BIRDWELL, 1978). Uma união dos dois métodos de isolamento também pode ser utilizada. Tal metodologia envolve inicialmente o uso de enzimas que irá facilitar o posterior processo de remoção dos folículos com o uso do método mecânico (ARAÚJO *et al*., 2014; ROSSETTO *et al*., 2011).

Em bovinos o método mecânico com auxílio de agulhas de insulinas (25G) para isolamento dos folículos pré-antrais, em especial os secundários é amplamente utilizado com sucesso no cultivo *in vitro* (BARROSO *et al*., 2020; DA CUNHA *et al*., 2018; GOMES *et al*., 2019; PAULINO *et al*., 2020; VASCONCELOS *et al*., 2021)

Sabe-se que avanços significativos já foram alcançados em camundongos, ou seja, a produção de embriões e nascimento de animais após o cultivo *in vitro* de folículos primordiais. No entanto, para animais de médio e grande porte, esta técnica ainda está em processo de aperfeiçoamento. Atualmente, um dos desafios está na recuperação de oócitos competentes inclusos em folículos pré-antrais cultivados *in vitro* (PAULINO *et al*., 2022). Durante o cultivo *in vitro* é necessário um aumento substancial do diâmetro oócitário, assim como a transcrição de uma grande quantidade proteínas. Em bovinos, por exemplo, até atingir a competência oócitaria, os oócitos oriundos de folículos secundários deve crescer de 69 μm até 125 μm, além disso a transcrição de diversos genes é necessária, ao qual já foram detectados em torno de 39.014 transcritos (CANDELARIA; DENICOL, 2020; CHOWDHURY *et al*., 2021; PAULINO *et al*., 2022).

Durante o cultivo de folículos pré-antrais, eles são expostos a elevadas concentrações de oxigênio, o que promove o aumento de EROS. Também vale destacar o tempo de cultivo prolongado, manipulações, exposição a luz ultravioleta, composição do meio

de cultura, temperatura e osmolaridade (LINS *et al*., 2017; SÁ *et al*., 2018; SOTO-HERAS; PARAMIO, 2020).

Em bovinos, os avanços limitam-se ao cultivo de folículos pré-antrais, especificamente, folículos secundários que possuem diâmetro em torno de 200 µm á 250 µm até o crescimento com diâmetro em torno de 300 µm á 400 µm, com a respectiva formação da cavidade antral, além disso, tem-se a recuperação de oócitos, que por sua vez não são competentes para suportar as fases iniciais da embriogênese (BEZERRA *et al*., 2019; GOMES *et al*., 2019; BARROSO *et al*., 2020; PAULINO *et al*., 2020, 2022; VASCONCELOS *et al*., 2021). Em bovinos, assim como em caprinos e ovinos, há um baixo percentual de embriões produzidos após o cultivo *in vitro* de folículos secundários (SILVA; VAN DEN HURK; FIGUEIREDO, 2016). Desta forma, pesquisas ainda são necessárias para que a MOIFOPA se torne uma biotécnica de uso imediato. Atualmente, as pesquisas relacionadas a MOIFOPA estão mais voltadas para elucidação dos mecanismos envolvidos na foliculogênese (SILVA, 2017).

As constantes pesquisas sobre o aperfeiçoamento dos sistemas de cultivos podem inicialmente contribuir para uma maior compreensão da folículogênese, e futuramente permitir a recuperação de um grande número oócitos oriundos de folículos pré-antrais, assim como multiplicação de animais de alto valor zootécnico e até em risco de extinção (ROSSETTO *et al*., 2011). Um do grande desafio a ser enfrentado durante o cultivo *in vitro* é a formação de grandes quantidades de EROS (KHAZAEI; AGHAZ, 2017; SOHEL *et al*., 2019; SOTO-HERAS; PARAMIO, 2020).

2.4 Formação das EROs e adição de antioxidantes durante o cultivo de folículos ovarianos

Um dos fatores que interferem na efetividade dos sistemas de cultivos *in vitro* de folículos pré-antrais bovinos é a produção excessiva de EROs, o que desencadeia o estresse oxidativo. O acúmulo de EROs causa peroxidação lipídica, comprometimento da integridade celular e estruturas como RNA, DNA e proteínas (FILIPOVIĆ *et al.,* 2015) e diminui a qualidade dos oócitos para próximas etapas, como maturação e fertilização.

Em condições de equilíbrio, as EROs possuem papeis essenciais, como por exemplo, no processo de ovulação, facilitando a liberação do oócito (SIES, 2017). Entretanto, quando ocorre a produção excessiva de EROS associado a falta de defesa antioxidante pode ocorrer danos oxidativos (FILIPOVIĆ *et al*., 2015). Na espécie bovina, pesquisas demonstram

que o estresse oxidativo durante o cultivo afeta diretamente o desenvolvimento folicular e a maturação oocitária *in vitro* (AGARWAL; SENGUPTA; DURAIRAJANAYAGAM, 2018). Além disso, durante a maturação *in vitro* (MIV), as EROs podem provocar efeitos como apoptose e bloqueio no desenvolvimento embrionário (CHEN *et al*., 2017). O aumento de EROs durante o desenvolvimento folicular pode causar crescimento anormal das células da granulosa e perda da função, levando à apoptose dessas células (LI *et al*., 2016; ZHANG *et al*., 2016). Em adição, estudos demonstram que a produção excessiva de EROs aumenta a expressão de miRNAs associados a apoptose como miR-365b-3p, miR-26b-5p, miR-128a e miR-181 (SOHEL *et al*., 2019). Em sistema de cultivo *in vitro,* o excesso de EROs tende a aumentar devido a superexposição ao oxigênio, ineficiência de mecanismos de defesa antioxidantes das células, manipulações, e aumento de oxigênio no meio celular (LINS *et al*., 2017;SOTO-HERAS; PARAMIO, 2020).

Os mecanismos de defesa das células contra a acumulação de EROs podem ser enzimáticos ou não enzimáticos. Os enzimáticos contam com a ação de enzimas relacionadas à defesa antioxidante, como a superóxido dismutase (SOD), a glutationa peroxidase (GPX), a Catalase (CAT) e a peroxiredoxinas (PRXs) (KHAZAEI; AGHAZ, 2017; SOHEL *et al*., 2017; WANG *et al*., 2017).

Os mecanismos de defesa não enzimáticos contam com substâncias antioxidantes que podem ser adquiridos de fontes fora do organismo. Entre as principais classes de compostos com atividade antioxidante estão: vitaminas (vitamina C e vitamina E), carotenoides e polifenois que são amplamente utilizados em meios de cultivo, pois possui ação antioxidante comprovada (SOUSA *et al*., 2007; PAULINO *et al*., 2020;VASCONCELOS *et al*., 2021).

Os antioxidantes agem na estabilização de radicais livres por meio da doação de um elétron, desta forma, neutralizam a ação dos radicais livres e impedem a reação em cadeia causada pelas espécies reativas (SOUSA *et al*., 2007). A utilização de substâncias antioxidantes no meio de cultivo *in vitro* de folículos pré-antrias busca eliminar a formação das EROs e manter as funções fisiológicas dos folículos (KASHKA; ZAVAREH; LASHKARBOLOUKI, 2016). Desta forma, diferentes antioxidantes com diferentes mecanismos de ação são amplamente utilizados em meios de cultivo para inibir os danos causados pelo estresse, tais como a transferrina, selênio, ácido ascórbico (DA CUNHA *et al.*, 2018; GOUVEIA *et al.*, 2016;

PAULINO *et al.*, 2018; SANTOS ; SCHOEVERS; ROELEN, 2014). No entanto, ainda há a busca por substâncias com um grande potencial antioxidante para serem aproveitadas em meios de cultivo para folículos secundários, tal como a NAC.

2.5 NAC

A NAC é um aminoácido também conhecido como N-acetil-L-cisteína, é amplamente utilizado como fármaco, pois além de possuir eficiência comprovada também é de fácil acesso e possui um baixo custo comercial (ELBINI DHOUIB *et al*., 2016; MOKHTARI *et al*., 2017; SAMUNI *et al*., 2013).

Devido a sua estrutura (Figura 4), a NAC interage diretamente com radicais livres através de suas cadeias laterais, reagindo rapidamente com os radicais hidroxila (OH^-), dióxido de nitrogênio (NO^2) e trióxido de carbono íon (CO^{3-}), eliminando assim as EROs produzidas pelas células (AKCA *et al*., 2005). Além disso, a NAC atua como quelante de íons metálicos, como Cu^{2+} e Fe^3, o que facilita a remoção desses íons (KOH *et al*., 2002).

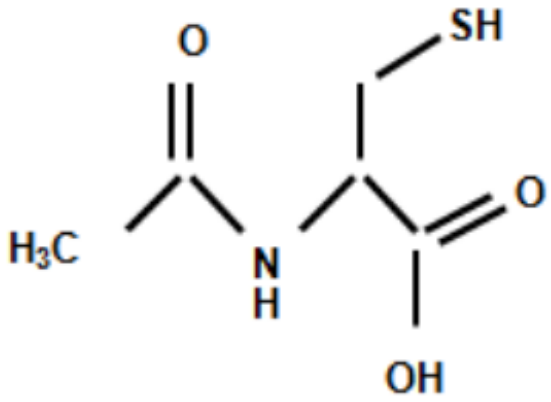

Figura 4. Fórmula da N-acetilcisteína. Fonte: Caz, (2018) Adaptado de Rushworth e Megson, (2014).

A ação da NAC também é atualmente explorada contra o vírus Sars-CoV-2(COVID-19), já que devido ao seu grupo tiol livre (SH) reduz as ligações dissulfeto do muco, diminuindo a sua viscosidade (JORGE-AARÓN; ROSA-ESTER, 2020). Além disso, também é precursor da cisteína, que é necessária para a síntese de GSH (ALDINI *et al*., 2018; JORGE-AARÓN; ROSA-ESTER, 2020). Quanto a estudos relacionados a biologia reprodutiva,

Cheraghi *et al*. (2018) demonstrou que a administração de NAC em mulheres portadoras da síndrome dos ovários policísticos (SOP) melhorou a qualidade dos oócitos, a maturação oocitária e a qualidade do embrião, tais benefícios estão relacionados a modulação da expressão do GDF-9 e do receptor de tirosina quinase (c-Kit).

A suplementação do meio de maturação oocitária com NAC aumentou as taxas de desenvolvimento de blastocistos em suínos (WHITAKER; CASEY; TAUPIER, 2012). Outros estudos mostraram que a NAC reduz o estresse oxidativo causado pelo encurtamento do telômeros e a instabilidade cromossômica em oócitos, e melhora as taxas de maturação oocitária e de desenvolvimento embrionário inicial (LIU *et al*., 2012). A NAC também demonstra proteger o DNA das células da granulosa, assim como também evidencia sua ação anti-apoptótica e anti-inflamatória ao regular positivamente a defesa antioxidante das células (BHARDWAJ; SARAF, 2020; OLESEN *et al*., 2021).

Além de seu papel como antioxidante direto, a NAC funciona como um antioxidante indireto. Os efeitos indiretos da NAC dependem da reposição de GSH intracelular, que é o principal antioxidante das células (PEI *et al*., 2018). A GPX age na redução do peróxido de hidrogênio, transformando-o em sua forma oxidada ($2GSH + H^2O^2 \rightarrow GSSG + 2\ H_2O$). Por sua vez, a forma oxidada da glutationa é reduzida, pela enzima glutationa redutase na presença de nicotinamida adenina dinucleotídeo fosfato (NADPH), como mostra a seguinte equação:

$GSSH + NADPH + H^+ \rightarrow 2GSH + NADP^+$ (ADEOYE *et al*., 2018).

Os níveis mais elevados de GSH produzem embriões de melhor qualidade, pois, protegem os oócitos de caprinos dos danos causados pelo estresse oxidativo (MUKHERJEE *et al*., 2014). Tem sido também relatado que a deficiência de glutationa está relacionada ao envelhecimento ovariano prematuro (LIM *et al*., 2013).

A NAC adentra na célula sem a necessidade de um transportador, em meio intracelular. A NAC é convertida em Acetil e L-cisteína pela enzina N-desatilcilase, posteriormente a enzima glutamato cisteína ligase (GCL) agrupa o L-glutamato à L-cisteína e, posteriormente a L-glicina é incorporada via GSH sintetase (GS) (RUSHWORTH; MEGSON, 2014).A conversão da NAC para Glutationa é esquematizada na Figura 5.

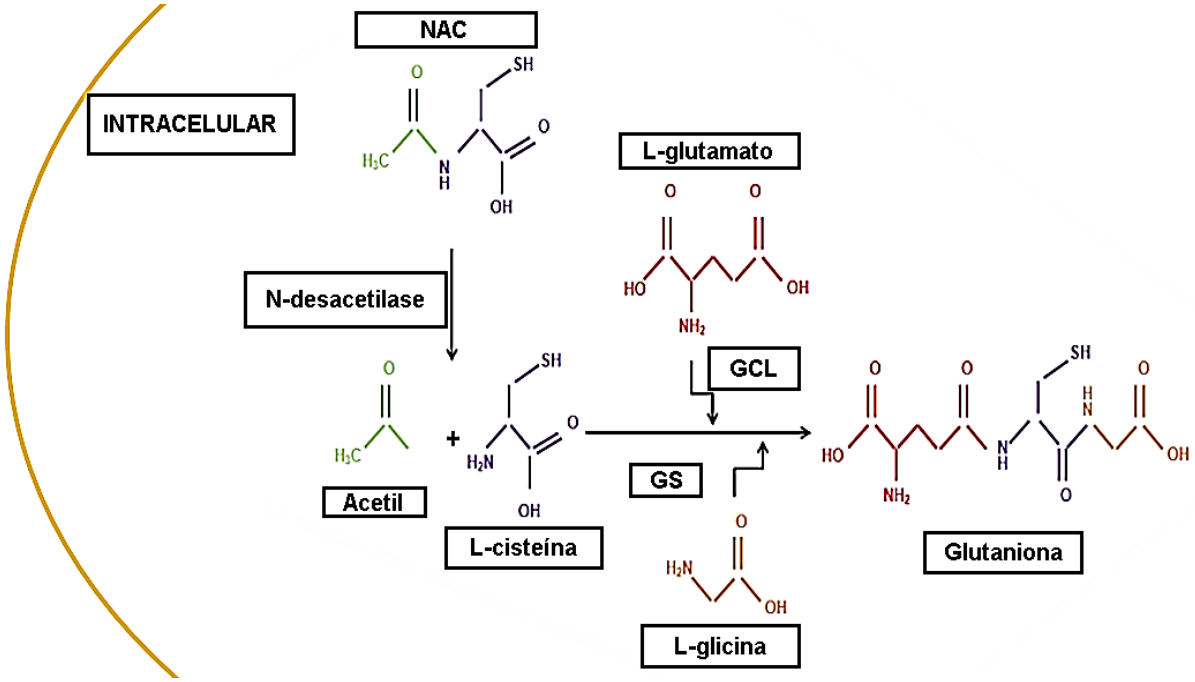

Figura 5. Esquema ilustrando a conversão da N-Acetilcisteína em Glutationa no meio intracelular. Fonte: Adaptado de Caz, (2018); Rushworth e Megson, (2014).

3. JUSTIFICATIVA

O interesse na espécie bovina justifica-se pela importância socioeconômica esses animais para o Brasil. Nesse cenário, a realização de pesquisas que impulsionem a multiplicação de espécies de alto valor genético pode contribuir para a economia do país e para a evolução de pesquisas na área de reprodução animal, assim como sua aplicabilidade em humanos e em espécies em risco de extinção (VIANA; FIGUEIREDO; SIQUEIRA, 2017). O cultivo *in vitro* de folículos pré-antrais possibilita o desenvolvimento e crescimento isolado dessas estruturas, assim como o controle de fatores ambientais tais como pH, temperatura, luminosidade, substâncias (RAMESH; ARCHIBONG; NIAZ, 2010; ROSSETTO *et al.*, 2013b; ROSSETTO *et al*., 2016). Além disso, espécie bovina é indicada como o modelo experimental comparativo a espécie humana, por apresentar características semelhantes em aspectos reprodutivos, como a duração da foliculogênese, o tamanho e a dominância dos folículos (BAERWALD, 2009; SANTOS; SCHOEVERS; ROELEN, 2014). Assim, avanços biotecnológicos no cultivo *in vitro* de folículos bovinos podem ser aplicáveis a espécie humana, assim como contribui para o entendimento do complexo mecanismo da foliculogênese (ARAÚJO *et al*., 2014; BAERWALD, 2009).

Sabe-se que um dos fatores que interferem negativamente no cultivo *in vitro* de folículos pré-antrais é a produção excessiva de EROs (AGARWAL *et al*., 2012; LINS *et al*., 2017). O acúmulo de EROs causa diversos danos na membrana, nas mitocondriais, no DNA e RNAs das células cultivadas *in vitro* (FILIPOVIĆ *et al*., 2015). Desta forma, a produção excessiva de EROs pode afetar a viabilidade e morfologia de folículos secundários. Com a finalidade de evitar o acúmulo de EROs, existe a necessidade da adição de substâncias com ação antioxidante aos meios de cultivo *in vitro* (KASHKA; ZAVAREH; LASHKARBOLOUKI, 2016; LINS *et al*., 2017). Neste cenário, a NAC é um fármaco amplamente conhecido que possui ação antioxidante comprovada, com a possibilidade de agir diretamente contra os radicais livres devido ao seu grupo tiol livre (SH) , e indiretamente na síntese de GSH (BHARDWAJ; SARAF, 2020; LIU *et al*., 2012; OLESEN *et al*., 2021). Estudos demonstram que a NAC protege o DNA das células, como também protege contra os danos causados pela a produção excessiva de EROs (BHARDWAJ; SARAF, 2020; LIU *et al*., 2012; OLESEN *et al*., 2021). As propriedades positivas da NAC estimulam a investigação desta substância e de seus efeitos em meios de cultivo *in vitro* para folículos pré-antrais. Além disso,

dentre os antioxidantes rotineiramente adicionados nos meios de cultivo *in vitro*, nenhum promove a síntese de glutationa.

A adição de NAC ao meio de cultivo pode trazer novas perspectivas ao prevenir e minimizar o estresse oxidativo e, consequentemente, e contribuir para o desenvolvimento de meios de cultivo mais eficientes para promover o desenvolvimento de folículos pré-antrais *in vitro*. Isto pode facilitar o aproveitamento dos milhares de folículos pré-antrais inclusos nos ovários de fêmeas da espécie bovina.

4. HIPÓTESES

- A NAC influencia positivamente o crescimento e a manutenção da morfologia e viabilidade de folículos secundários bovinos cultivados *in vitro* por 18 dias.
- A NAC induz a formação de antro durante o cultivo *in vitro* de folículos secundários bovinos por 18 dias.

5. OBJETIVOS

5.1 Objetivo Geral

- Avaliar a influência de diferentes concentrações de NAC (1.0 mM, 5.0 mM e 25.0 mM) sobre a sobrevivência e o desenvolvimento de folículos secundários bovinos cultivados *in vitro.*

5.2 Objetivos específicos

- Avaliar o efeito de diferentes concentrações de NAC (1.0 mM, 5.0 mM e 25.0 mM) sobre a morfologia, crescimento e a taxa de formação de antro de folículos secundários bovinos cultivados *in vitro* por 18 dias;
- Avaliar o efeito de diferentes concentrações de NAC (1.0 mM, 5.0 mM e 25.0 mM) sobre a viabilidade de folículos secundários bovinos após 18 dias de cultivo *in vitro*;

CAPÍTULO 1

ARTIGO DE REVISÃO

The mechanisms that control the preantral to early antral follicle transition and the strategies to have efficient culture systems to promote their growth *in vitro*

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Abstract:

The interaction among oocyte, granulosa and theca cells regulates the preantral to antral follicles transition. Understanding the mechanisms that regulates this step of folliculogenesis is important to improve *in vitro* culture system, and to open new perspectives to use oocytes from preantral follicles for assisted reproductive technologies. Thus, this review aims to discuss the endocrine and paracrine mechanisms that control granulosa cells proliferation and differentiation, formation of the antral cavity, estradiol production, atresia, and follicular fluid formation during transition from preantral to early antral follicles. The strategies that promote *in vitro* growth of preantral follicles are also discussed.

Key-words: Oocyte. Granulosa cells. Preantral. Antral. Transition.

Introduction

The transition from preantral (multilaminar follicles) to early antral follicles requires the action of local factors, hormones, and an efficient bidirectional communication between granulosa cells and oocytes. Several attempts to promote *in vitro* growth of preantral follicle up to maturation have been reported in various domestic species (bovine: Paulino *et al*, 2018; Antonino *et al*, 2019; Bezerra *et al*, 2019; Paulino *et al*, 2020; Vasconcelos *et al*, 2021, caprine: Silva *et al*., 2017; Ferreira *et al*., 2018; Soares- Costa *et al*, 2018; Pontes *et al*., 2019, ovine: Jobard *et al*., 2019; Mbemya *et al*., 2019; Barros *et al*, 2020; Gomes *et al*, 2021; Silva *et al*, 2021, swine:de Lima *et al*., 2017;Kere *et al*., 2020). However, different from mice (O'Brien *et al*., 2003), embryo production from *in vitro* cultured preantral follicles is not yet reported in the literature (Wu and Tian, 2007; Arunakumari *et al*., 2010;Figueiredo *et al*., 2018; Figueiredo *et al*., 2019, Paulino *et al*., 2022). The maintenance of bidirectional communication between the oocyte and the granulosa cells in cultured follicles, as well as the large quantity of messenger RNA (mRNA) and proteins that the oocyte need to synthesize during its growth make it difficult the complete their development *in vitro*, especially for human and domestic animals, in which follicular development can length up to 180 days (Figueiredo *et al*., 2019; Alam and Miyano, 2020; Paulino *et al*., 2022).

The endocrine and paracrine control that regulates the transition from preantral to antral follicles is complex and involves a precise interaction of several factors (Araújo *et al*., 2014; Figueiredo *et al*., 2018; Figueiredo *et al*., 2019). Any interference with this control can lead to ovarian disorders, such as polycystic ovary syndrome (Abdel Aziz *et al*., 2021; Asghari *et al*., 2021). The present review highlights the mechanisms involved in endocrine and paracrine control during the transition from preantral to early antral follicles, as well as the importance of granulosa cell proliferation, antral cavity formation, and estradiol production for proper follicle development. The strategies to promote *in vitro* growth of preantral follicles are also discussed.

1. Endocrine and paracrine control during the transition from preantral to early antral follicles

The transition from preantral to early antral follicles is a step in which follicle development is regulated by intraovarian factors, but the follicles are responsive to gonadotrophins. The slow growth of preantral and early antral follicles is gonadotropin-independent, but progression to late antral follicular state requires follicle stimulating hormone (FSH) (Iber and Geyter, 2013). The FSH is the main regulator of follicle development and its receptors are detectable in granulosa cells of preantral follicles (Ferreira *et al*., 2018; Paulino *et al*., 2018; 2019; Vasconcelos *et al*., 2021). This hormone stimulates granulosa cell proliferation and promotes follicular growth and antrum formation (Ferreirra *et al*., 2018;Figueiredo *et al*., 2019; Fushii *et al*., 2020). Other hormones, such as melatonin have a functional role in preantral follicles by influencing their development, increasing production of active mitochondria in oocytes and steroidogenesis in granulosa cells (Riaz *et al*., 2019; Barros *et al*., 2020). It is well established that FSH stimulates the production of aromatase (Cyp19a1), which synthesizes 17β-estradiol, an important hormone for granulosa cell proliferation (Fitzpatrick and Richards, 1994; Bishonga *et al*., 2001). Anti-mullerian hormone (AMH) is also produced by granulosa cells of preantral and antral follicles (Umer *et al*., 2019; Gautam *et al*., 2021). It is already well established that this hormone prevents early depletion of follicles, but there is still much to elucidate about the role of this hormone during folliculogenesis. Rocha *et al*. (2021) has shown that there is an interaction between AMH and FSH, in which AMH reduces FSH-induced estradiol and progesterone production. Tanimoto *et al*. (2021) showed that for the development of a viable follicle, blockage of AMH production by estrogen is needed.

Oocyte-derived factors, such as growth differentiation factor-9 (GDF-9), bone morphogenetic protein 15 (BMP-15), and fibroblast growth factor- 2 (FGF-2) are important regulators of preantral follicle growth, by inducing granulosa cell proliferation and differentiation, and antral cavity formation (Reineri *et al*., 2018; Monte *et al*., 2019a). Other factors, such as epidermal growth factor (EGF) and insulin like growth factor 1 (IGF-1) influence the development of preantral follicles by expanding oocyte diameter and inducing granulosa cell proliferation (Monte *et al*., 2019b; Paulino *et al*., 2020). Vascular endothelial growth factor (VEGF) is an important angiogenic factor that induces granulosa cell proliferation, an essential characteristic for the transition from preantral to antral follicles, and

improves oocyte maturation (Araújo *et al*., 2011; Da Silva *et al*., 2015; Cadenas *et al*., 2017). Activin is another intra-ovarian factor that accelerate the growth of preantral follicles, estradiol synthesis, and mRNA expression for FSH receptor in rat granulosa cells (Tanaka *et al*., 2019). On the other hand, in bovine species, activin decreases the FSH stimulating action in preantral follicles cultured *in vitro*, which was associated with decreased levels of transcripts for hyaluronan synthases (HAS-1,HAS-2) and proliferating cell nuclear antigen (PCNA)(Silva *et al*., 2014).

2. Granulosa cell proliferation and oocyte-granulosa cell interaction during the transition from preantral to early antral follicles

Granulosa cells form a favorable metabolic and hormonal environment for oocyte growth and maturation (Baumgarten and Stocco, 2018). Likewise, the oocyte can influence the proliferation of granulosa cells, providing follicular development through the production of growth factors, such as BMP-15 and GDF- 9 (de Figuereido and Lima, 2017; Baumgarten and Stocco, 2018). Orisaka *et al*. (2006) showed that GDF-9 controls follicular fate by promoting its survival and growth during the preantral to early antral transition, suppressing granulosa cell apoptosis and follicular atresia through PI3K/Akt activation.

During the development of preantral follicle, GDF-9 and BMP-15 continue to stimulate the proliferation of granulosa cells; however, they decrease the production of progesterone and increase the expansion and reorganization of the cumulus to form the antral cavity. In addition, GDF-9 stimulates the expression of FSH receptors in granulosa cells, which become responsive to gonadotropins (de Conto; Matte; Cunha-Filho, 2021). Bone morphogenetic protein 4 (BMP-4) and FSH also play an important regulatory role in growth and steroidogenesis of preantral follicles. According to Sakaguchi *et al*. (2017), BMP-4 inhibits the luteinization of granulosa cells, while FSH increases their proliferation and the viability of the oocyte-cumulus-granulosa complex.

Granulosa cells produce several autocrine and paracrine factors that may be involved in the initiation of antrum formation; such as kit ligand, activins, inhibins, hyaluronan, versican, AMH and transforming growth factor α (TGF-α) that synchronize oocyte growth, granulosa cell proliferation and theca cell differentiation (Vasconcelos *et al*.,2013; Dumesic *et al*., 2015). In this sense, the interaction between oocyte and granulosa cells, as well as the

differentiation and proliferation of theca cells can be determinant for the progress of preantral follicle growth, steroidogenesis and oocyte maturation (Chu *et al*., 2018; Alam and Miyanno, 2020).

After antrum formation, granulosa cells are physically separated into mural granulosa cells, which organize along the follicle wall, and cumulus granulosa cells, which surround the oocyte (Zhang, 2018; Baumgarten and Stocco, 2018). Cumulus granulosa cells nourish the oocyte by providing specific amino acids, glycolysis products and cholesterol biosynthesis substrates through gap junctions (Baumgarten and Stocco, 2018). In addition, they prevent premature oocyte maturation and resumption of meiosis in the oocyte, by maintaining high levels of cAMP in the oocyte (Zhang, 2018; Russell and Robker, 2018). The mechanisms that control the transition from preantral to early antral follicles are shown in Fig. 1.

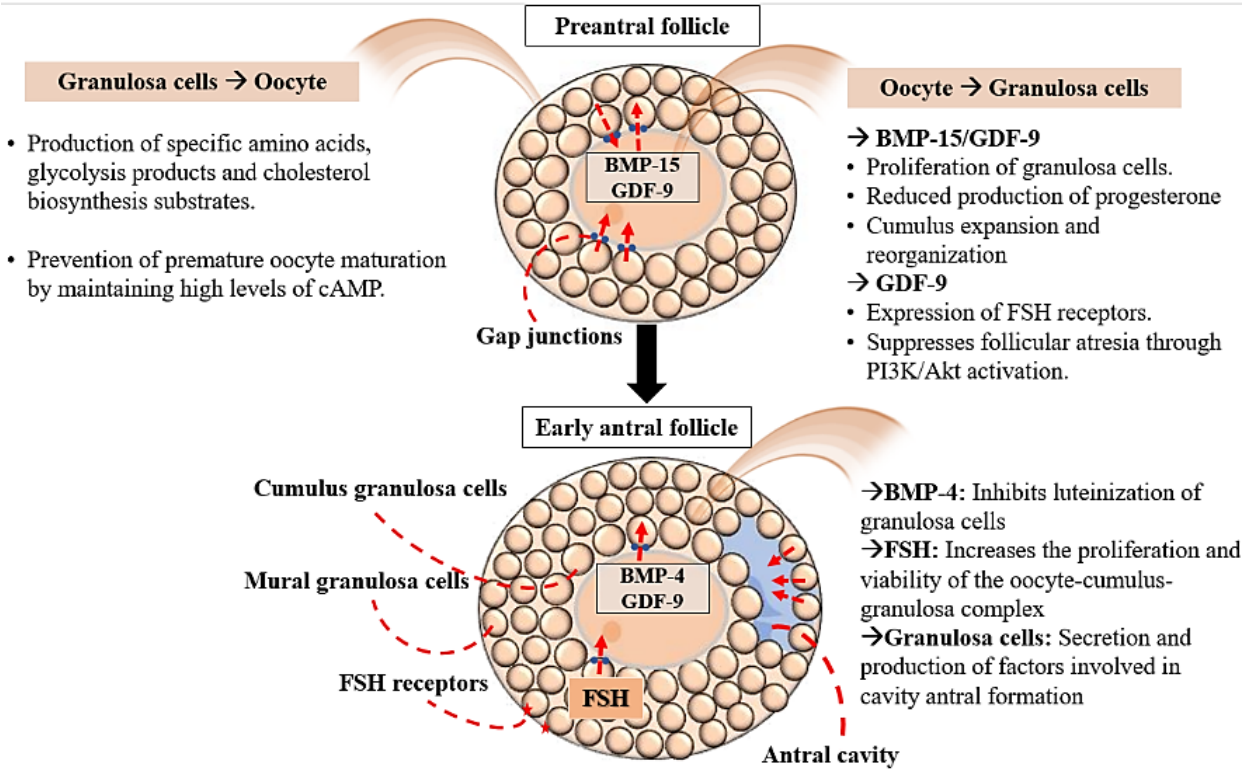

Figure 1. Factors that control the transition from preantral to early antral follicles.

3. **Mechanisms of antrum formation in preantral follicles**

When the distance between the mural granulosa and the cumulus cells increases, the formation of the antral cavity occurs, marking the preantral to early antral follicles transition (Chu *et al*., 2018). The antral cavity is formed between the granulosa cells and requires a fluid ingress from the vascularization of theca cells via membrane proteins, such as aquaporins (AQPs)(Kawashima and Kawamura, 2018; Paz *et al*., 2018). Antrum formation depends on the stimulation of local factors for the production and secretion of polysaccharides and proteins by granulosa cells, which accumulate and generate an osmotic gradient that attracts fluid from the thecal vasculature (Baumgarten; Stocco, 2018). It is recognized that granulosa cells express the enzymes that synthesize hyaluronic acid, versican and perlecan, which are responsible for the formation of follicular fluid (Schoenfelder and Einspanier, 2003; Clarke *et al*., 2006;Vasconcelos *et al*., 2013; Nagyova *et al*., 2020). Several AQPs are related to the influx of water through the follicle wall, such as AQP5, AQP7, AQP8, and AQP9. These membrane proteins are detected in granulosa cells of different species, including pig (Skowronski *et al*., 2009), sheep (Sales *et al*., 2015) and cattle (Ishibashi *et al*., 2009). Paz *et al*. (2018) also demonstrated the presence of AQP3 in granulosa cells, which is also involved in the expansion of the antral cavity in the transition from preantral to the antral follicle.

The development of the antral cavity is intensified by granulosa cell activity. Alam *et al*. (2018) showed that even without the presence of an oocyte, GDF-9 and BMP-15 influence the production of antrum-like structures. GDF-9 is known to stimulate versican and perlecan expression and interacts favorably with FSH to increase hyaluronan synthetase 2 expression (Vasconcelos *et al*., 2013; Silva *et al*., 2014). Some studies demonstrate epidermal growth factor (EGF) increases mRNA levels for GDF-9, such factor is essential for antral cavity formation in *in vitro* cultured preantral follicles (Alam *et al*., 2018; Paulino *et al*., 2019). The mechanisms involved in the formation of antral cavity formation are shown in Fig. 2.

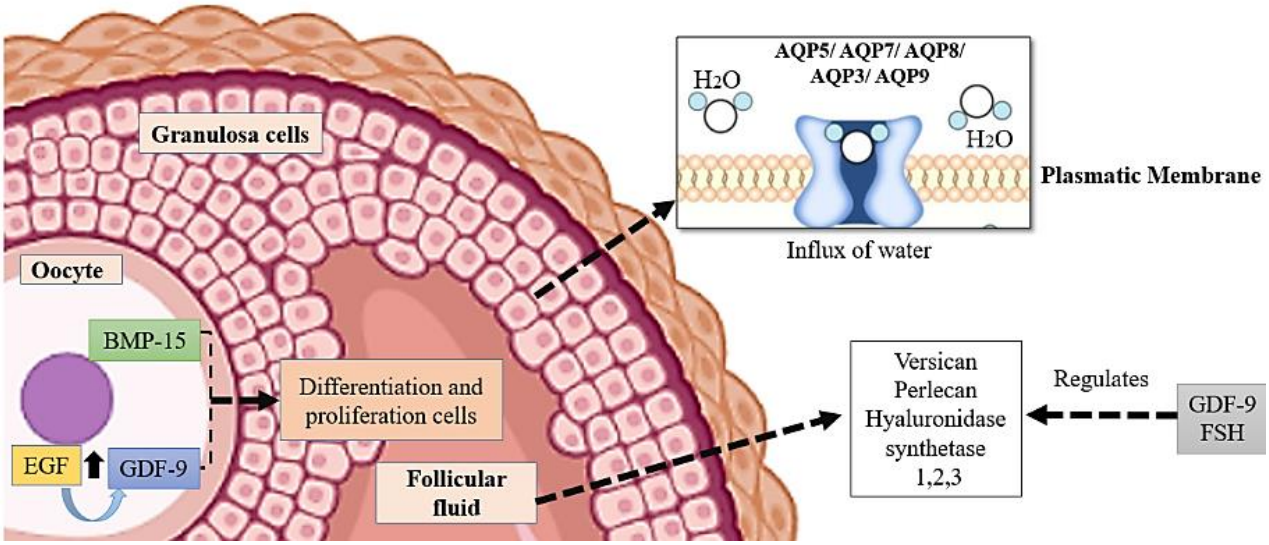

Figure 2. Hormones and growth factors that control the formation of antral cavity during the transition from preantral to early antral follicles.

4. **Production of estradiol during the transition from preantral to early antral follicles**

When preantral follicles reach six to seven layers of granulosa cells, the inner layer of the theca becomes active and the formation of the antral cavity begins. Increased 17β-estradiol in follicular fluid is associated with increased mRNA expression for CYP19A1 in granulosa cells (García-Guerra *et al*., 2018).

As a growing follicle acquires sufficient aromatase activity as a result of FSH stimulation, estradiol production suppresses FSH secretion below what is necessary to support the development of less mature follicles, which consequently suffer atresia (Zeleznik, 2004). Thus, 17β-estradiol biosynthesis is a tightly regulated molecular process, dependent on the expression of key steroidogenic enzymes influenced by FSH and intra-ovarian signaling molecules, including beta-catenin, an essential co-transcription factor for maximal induction of FSH from the aromatase mRNA transcript and subsequent estradiol production (Forrest *et al*., 2022). Furthermore, LH promotes preantral to antral follicle transition by up regulating follicular androgen biosynthesis (Orisaka *et al*., 2013).

Expression of luteinizing hormone/choriogonadotropin receptor (LHCGR) and cytochrome P450 family 17 subfamily A member 1 (CYP17A1) mRNAs appear in large preantral follicles, concomitantly with theca differentiation. Followed by the expression of

steroidogenic acute regulatory protein (StAR) in 1 mm antral follicles, granulosa cells from preantral and early antral follicles do not express StAR. Thus, steroidogenesis in bovine follicles potentially begins in follicles ≧ 1 mm. Furthermore, the mRNA for CYP17A1 was located exclusively in theca internal cells, which indicates that the conversion of progestogens to androgens occurs only in theca interna (Braw-Tal and Roth, 2005). Furthermore, the neonatal rat ovary is completely devoid of antral follicles at birth. By day 12 of age, small to medium-sized antral follicles are present, in addition to follicles at all preceding stages of development. During the intervening period the ovary becomes steroidogenically active, and responsive to gonadotrophins on days 7-9 of age, suggesting that granulosa and theca cells become active at that time (Carson and Smith, 1986).

The expression of key enzymes involved in steroidogenesis is crucial for the proper development of the follicle. It has been observed that mRNA encoding P450arom was not detectable until early antral cavity formation, in addition to being expressed only in granulosa cells (Yuan *et al*., 2008).The mechanisms involved in the production of estradiol during the transition from preantral to early antral follicles is shown Fig. 3.

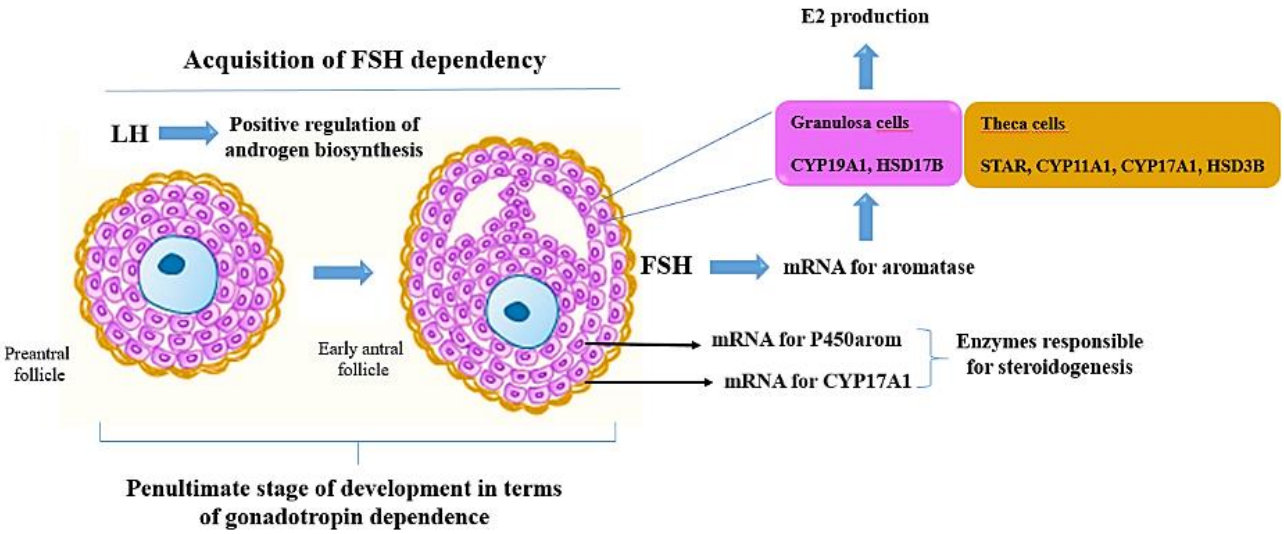

Figure 3. Mechanisms that control production of estradiol during the transition from preantral to early antral follicles.

5. Follicle atresia during the transition from preantral to early antral follicles

Follicular atresia during preantral to early antral follicles is mainly controlled by intrafollicular regulators, such as growth factors, cytokines, and steroids (Orisaka *et al*., 2009). Atresia is characterized by degenerative morphological changes in oocyte, granulosa and theca cells (Makarevich *et al*., 2018).

In preantral follicles, degeneration initially occurs in the oocyte and, subsequently, in granulosa cells (Meng *et al*., 2018). The first signs of atresia in the oocyte are retraction of nuclear chromatin and oocyte fragmentation, which triggers the process of irreversible elimination of ovarian follicles at this stage of development. In the granulosa cells of preantral follicles, changes are rarely observed.

Zoheir (2021) showed that follicular atresia is related to cellular deoxyribonucleic acid (DNA) fragmentation, an important biochemical marker of apoptosis. Apoptosis has morphological and biochemical characteristics and is one type of programmed cell death. Recent studies demonstrate that in granulosa cells from cultured preantral follicles of buffaloes, transmembrane protein AQP8 is involved in the regulation of cycle progression and apoptosis (Cao *et al*., 2021). Additional evidence shows that apoptosis is not the only death pathway active in the ovary. Gannom *et al*. (2012) reported a decrease in preantral follicle numbers without a concomitant increase in apoptosis, and no change in apoptosis markers caspase 3 and TUNEL. Furthermore, miRNAs have been shown to control several fundamental biological processes, including follicular atresia through their target genes and signaling pathways, and play a central role in the regulation of autophagy (Ma *et al*., 2020). Gannom *et al*. (2012) showed that degeneration of preantral follicles is associated with of activation the autophagy cascade. Meng *et al*. (2018) reported that the standard pathway of degeneration in preantral follicles is through autophagy, and that the activation of this pathway occurs under normal physiological conditions.

Meng *et al*. (2018) reported that atresia in antral follicles is initiated by granulosa cell apoptosis by insufficient FSH level or reduced numbers of FSH receptors. Although apoptosis can occur at all stages of follicular development, the antral follicles (~2.0 mm in diameter – human species) are susceptible to apoptotic signals (Orisaka *et al*., 2009). In these follicles, with the progression of atresia, there is a reduction in the number of layers of granulosa cells, and invasion of fibroblasts and macrophages (Seneda, 2021). However, the early antral

follicles smaller than 2mm in human and domestic animal species are still not dependent of gonadotropins. Probably atresia in these follicles can be associated with of activation the autophagy cascade, as described for preantral follicles (Meng *et al*., 2018). Factors that control follicle atresia during the transition from preantral to early antral follicles are represented in Fig. 4.

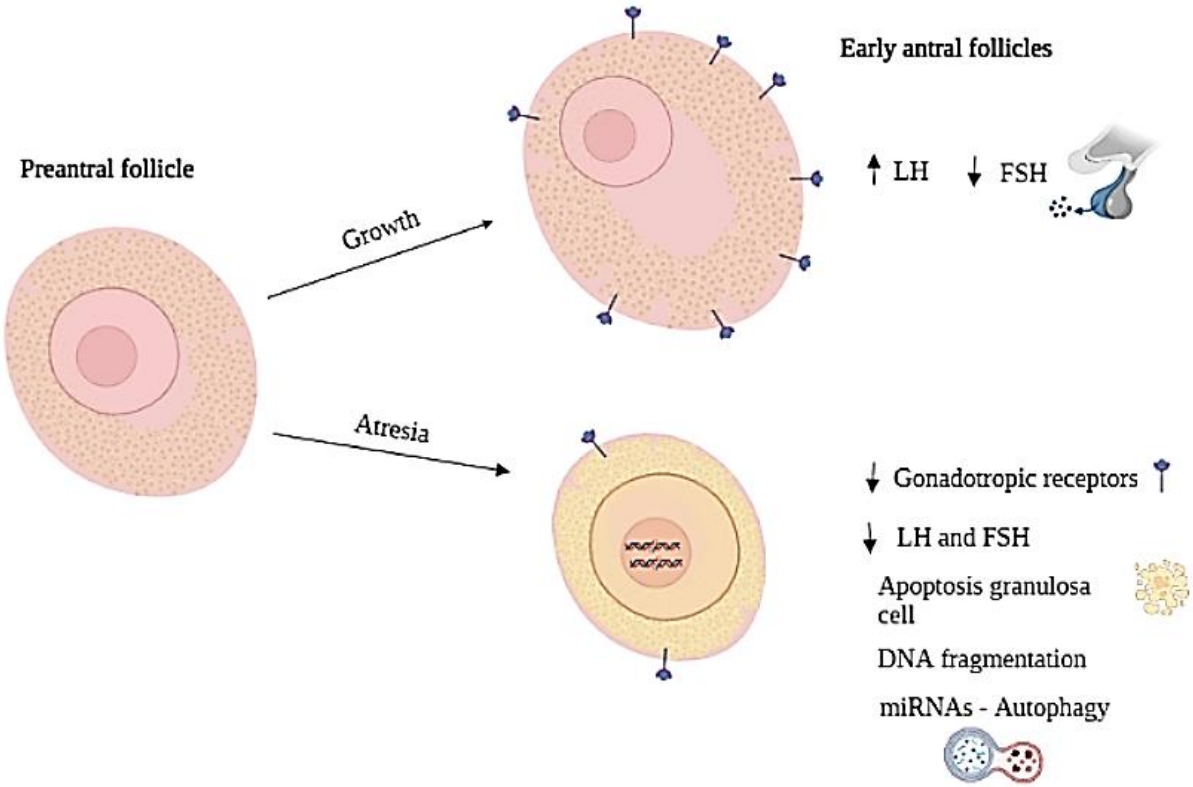

Figure 4. Factors that control follicle atresia during the transition from preantral to early antral follicles.

6. Strategies to promote *in vitro* growth of preantral follicles

The limitations of *in vitro* culture systems for preantral follicles come from the difficulty of maintaining the three-dimensional structure of the follicles, and the many signals necessary to coordinate stimulation of follicular growth (Paulino *et al*., 2022). Although bi-dimensional (2D) follicle culture has been successful performed in many studies (da Cunha *et al*., 2018; Paulino *et al*., 2018; Paulino *et al*., 2020; Vasconcelos *et al*., 2021), a major limitation of these systems is their inability to maintain follicle architecture, with the oocyte surrounded by granulosa cells. This is particularly problematic with follicles from large mammalian

species, which require longer-term culture (Simon *et al*., 2020). Given the importance of maintaining the follicle complex architecture, three-dimensional (3D) culture systems can help maintaining the follicle complex architecture (Simon *et al*., 2020). In this system, follicles are encapsulated in biomaterials. The various types of matrices used to encapsulate follicles allow the somatic cells to proliferate and helps to maintain follicle architecture and cell–cell interactions, thereby creating a microenvironment very similar to that of the *in vivo* ovary (Belli *et al*., 2012). In general, matrices have been engineered from natural components such as collagen and alginate (Healy *et al*., 2021), or matrigel (Hao *et al*., 2020), or from synthetic components such as polyethylene glycol (Green and Shikanov, 2016; Tomaszewski *et al*., 2021).

Preantral follicles cultured in the 3D system had greater homogeneity of daily growth, higher rates of viability and antrum formation, as well as low rates of degeneration (Antonino *et al*., 2019; Panta *et al*., 2019). Preantral follicles from other species have been successfully cultured in a 3D culture system using alginate and fibrin (monkey:Xu *et al*., 2013; Bulgarelli *et al*., 2018; cat: Chansaenroj *et al*., 2019; human:Chiti *et al*., 2017). Besides, multistep culture systems have been developed to further mimic the physiologic environment of developing follicles (Green and Shikanov, 2016; Simon *et al*., 2020). These systems have been used for culturing early preantral follicles. The multistep method starts with culture of ovarian cortical tissue during 3 weeks to initiate primordial follicle activation and to support follicle growth to the preantral stage. At the end of ovarian tissue culture, preantral follicles are isolated mechanically and cultured individually or in the group for 6 weeks (Xu *et al*., 2021). In this system, Xu *et al*. (2021) demonstrated that ~50% of human follicles survived for 6 weeks. Most surviving follicles grew to the antral stage and produced ovarian steroid hormones estradiol and progesterone, in addition to paracrine factors such as activin A, insulin like growth factor 2 (IGF-2), and VEGF. In addition, the cultured preantral follicles exhibited morphology similar to that of human follicles developed *in vivo*.

Many efforts have been made to elucidate the mechanisms involved in the growth of preantral follicles, as recently reviewed by Paulino *et al*. (2022). Although comprehension of the molecular regulation and composition of the microenvironment coordinating the events in preantral follicles remains incomplete, over time, many studies have been conducted to optimize culture systems to support follicular growth (Healy *et al*., 2021).

Preantral follicles from human and animal species require the development of a tightly regulated culture system with adequate nutrients, cytokines, growth factors, and developmental stage-dependent hormones to support cell survival and proliferation, as well as cellular function, which changes as follicles grow and oocytes mature (Simon *et al*., 2020; Paulino *et al*., 2022). Therefore, understanding the influence of several compounds for supplementation of culture medium for preantral follicles is fundamental. In this context, melatonin increases follicular and oocyte diameters, formation of antral cavity and preserves high rates of morphologically intact sheep preantral follicles for up to 18 days of culture (Barros *et al*., 2020).

Another alternative to improve follicular development *in vitro* is to supplement the culture medium with EGF, whose signaling regulates many cellular processes associated with survival (Sabbah *et al*., 2020). EGF has an important role in folliculogenesis, by promoting several processes, like granulosa and theca cell proliferation (Jachter *et al*., 2022). The effects of adding EGF to the culture medium are directly associated with improved survival of bovine preantral follicles. In general, EGF-treated follicles reach a larger diameter at the end of the culture period (Paulino *et al*., 2020; Jachter *et al*., 2022). Hormones such as progesterone and cytokines such as tumor necrosis factor alpha (TNF-α) are able to maintain healthy follicle morphology and positively influence follicular growth and antrum formation in cattle (Paulino *et al*., 2018; Paulino *et al*., 2020). Also in cattle, preantral follicles cultured in the presence of bone morphogenetic protein 2 (BMP-2) or BMP-4 show a significant increase in follicular diameter and greater average daily growth (da Cunha *et al*., 2018).

Understanding the mRNA transcription of preantral follicles can provide important insights to detect follicular genes expression at several critical stages of its development under different culture conditions. Thus, the discovery of genes differentially expressed at each of growth and follicular stage development can be used to elucidate the processes involved in follicular growth (Paulino *et al*., 2022). In bovine species, an over expression of mRNA for oocyte maturation factor Mos (cMOS) and GDF-9 was observed when oocytes from preantral follicles cultured in the presence of EGF. Furthermore, higher levels of cyclin B1 and GDF9 mRNA were observed in oocytes from follicles cultured with progesterone (Paulino *et al*., 2020). In addition, bovine preantral follicles cultured in the presence of alpha-lipoic acid express higher levels of transcripts for FSH receptor, LHCGR, IGF-1, bone morphogenetic protein receptor 1A (BMPR1a), transforming growth factor beta-receptor 1

(TGFβR1), transforming growth factor beta 1 (TGFβ1), activin receptor type-2B (ActRIIB), GDF-9, and activin. The expression of pro-apoptotic genes, BCL2 associated XBAX) and C-Myc were also down regulated (Zoheir *et al*., 2017).

To mitigate the effects of oxidative stress induced by culture conditions, such as lower quality oocytes, follicular cell death, inactivation of antioxidant enzymes, and mitochondrial damage, there has been an increasing interest in the antioxidant potential of the natural compounds. Sheep preantral follicles cultured in medium supplemented with kaempferol showed high percentages of follicular survival, antrum formation, and greater follicular diameter. In addition, kaempferol preserves higher levels of metabolically active mitochondria (Santos *et al*., 2019). In cattle, the presence of eugenol in culture medium promotes higher daily growth rates of bovine preantral follicles, in addition to stimulating the expression of mRNA for glutathione peroxidase 1 (GPX1) (Vasconcelos *et al*., 2021). The strategies to promote *in vitro* growth of preantral follicles are represents in Fig. 5.

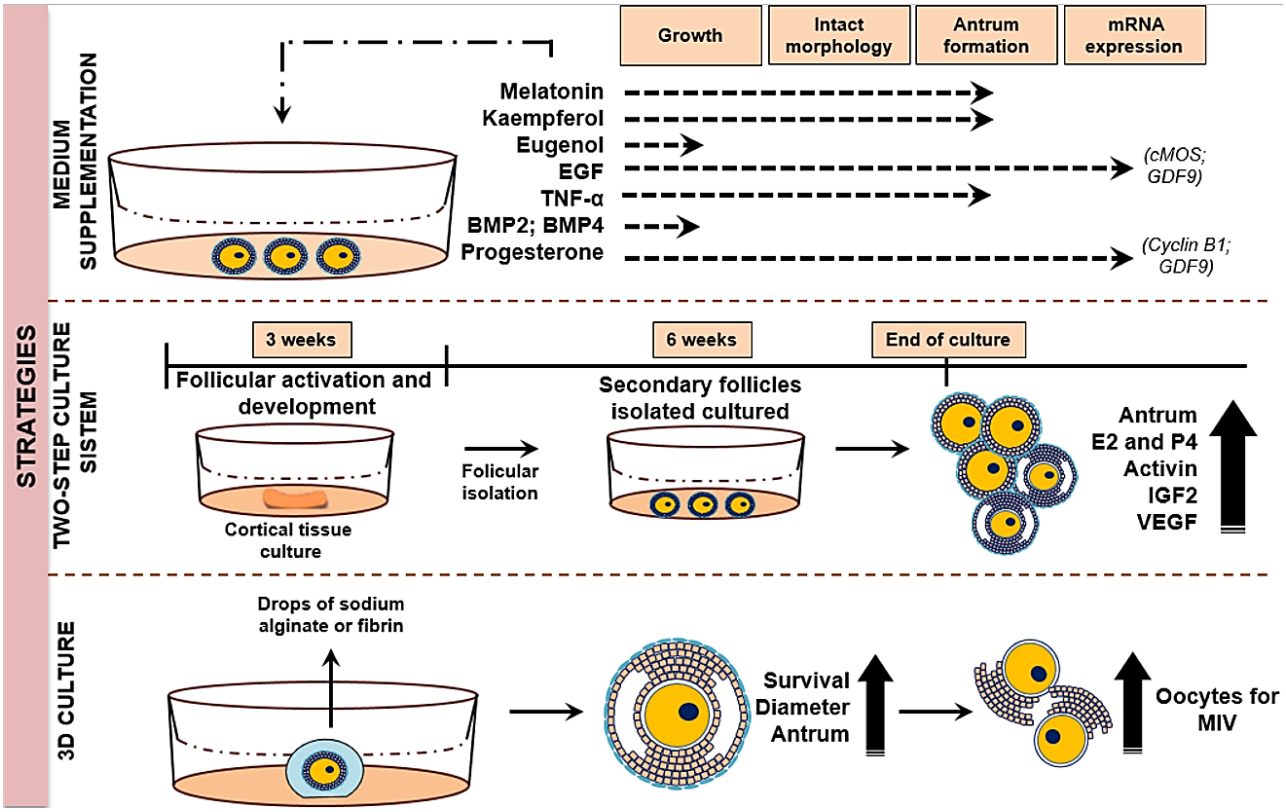

Figure 5. Strategies to promote *in vitro* growth of preantral follicles.

Final considerations

The transition from preantral to antral requires intense and precise granulosa-oocyte interaction, any dysregulation can interfere with acquisition of oocyte competence. Endocrine, paracrine and autocrine factors are essential to follicular growth, antral cavity formation, granulosa cell proliferation, differentiation, and future gonadotropin dependence.

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Conflict of interest

The authors declare that they have no competing interests.

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CAPÍTULO 2

ARTIGO TÉCNICO

Effects of N-acetylcysteine on growth, morphology and viability of *in vitro* cultured bovine secondary follicles

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Abstract

This study aims to investigate the effects of different concentrations of N-acetylcysteine (NAC) on growth, morphology, antrum formation, and viability of bovine secondary follicles cultured *in vitro* for 18 days. To this end, follicles were cultured at 38.5°C with 5% CO_2 for 18 days in TCM-199^{+} alone or supplemented with 1.0, 5.0 or 25.0mM NAC. Follicular growth, morphology, antrum formation, viability (calcein-AM and ethidium homodimer-1), and histological features were evaluated at the end of culture. Follicular diameter and fluorescence intensity data for viability were analyzed by Kruskal-Wallis test, followed by Dunn's multiple comparison test. Percentages of follicle survival and antrum formation were compared by the chi-square test and percentages of growing follicles were compared by Fisher's exact test ($P<0.05$). The results showed that 1.0 mM NAC significantly increased the percentage of growing follicles when compared to other treatments. On the other hand, 25.0 mM NAC significantly reduced the rate of growing follicles. Secondary follicles cultured with 1.0 mM NAC had higher fluorescence intensity for calcein-AM, while those cultured with 25.0 mM

NAC were mainly stained with ethidium homodimer-1 (P<0.05). Histological analysis showed that oocyte vacuolization is the first sign of degeneration in cultured follicles. In conclusion, 1mM NAC increases follicle viability and growth rate in bovine secondary follicles cultured *in vitro*, while 25mM NAC cause damages in cellular membranes and reduces follicular growth rate.

Key-words: Viability. Growth. NAC. Secondary. Follicle.

1. Introduction

The development of *in vitro* culture systems to explore the population of immature oocytes enclosed in preantral follicles has a great potential to provide a large number of oocytes for *in vitro* fertilization (Araújo *et al*., 2014; Ferreira *et al*., 2018; De Figueiredo *et al*., 2019). The *in vitro* culture of preantral follicles is a technique that, besides allowing their development *in vitro*, helps to understand the mechanisms that control follicular growth and maturation (Figueiredo *et al*., 2018, 2019; Ferreira *et al*., 2018; Paulino *et al*., 2018). The complex mechanisms controlling follicular development are, however, obstacles to have competent oocytes *in vitro* (Paulino *et al*., 2022). The development of *in vitro* conditions to reproduce follicular development is the main challenge for researchers in the field. 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. In bovine species, various studies have shown that secondary follicles cultured during 18 days develop up to early antral follicles, but an increase in degeneration rate is frequently reported at this stage (da Cunha *et al*., 2018; Paulino *et al*., 2018; Vasconcelos *et al*., 2021)

During *in vitro* culture of preantral follicles, various factors, like variation in medium osmolality, exposure to light, large volumes of culture media, variation in temperature, and static nature of culture systems, can favor oxidative stress (Lins *et al*., 2017; Sies, 2017). High concentration of oxygen *in vitro* (up to 20%) can also result in accumulation of reactive oxygen species (Sá *et al*., 2018). Oxidative stress can cause damages in DNA, proteins and mitochondria of oocyte and granulosa cells, and increase apoptosis (Yang *et al*., 2017; Sá *et al*., 2018). Shi *et al*. (2016) reported that long-term moderate oxidative stress causes ultrastrucutral changes in ovarian follicles and reduces fertility in mice. These authors showed that the oocytes had swollen Golgi apparatus, large number of lipid droplets, and mitochondria with

vacuolization and degeneration of the cristae and matrix. To minimize the adverse effects of oxidative stress, various studies have reported the importance of adding antioxidant substances to *in vitro* culture media (Lins *et al*., 2017; Paulino *et al*., 2018; Vasconcelos *et al*., 2021). N-acetyl-cysteine (NAC) is a substance that can be used to optimize *in vitro* conditions during follicle growth (Barrozo *et al*., 2021). The antioxidant effects of NAC occur directly by donating electrons to reactive species, and indirectly by being a precursor of glutathione that integrates the intracellular antioxidant system (Pei *et al*., 2018). Several studies emphasize the potential of NAC to improve the quality of oocytes, granulosa cells, ovarian tissues, and embryos during *in vitro* culture (Whitaker *et al*., 2012; Cheraghi *et al*., 2018; Bhardwaj and Saraf, 2020; Olesen *et al*., 2021). Fabbri *et al*. (2007) reported a synergic action between NAC and FSH to promote preantral follicle growth and viability in cultured human ovarian tissues. However, it is not known if NAC improves the growth, morphology and viability of bovine secondary follicles cultured *in vitro*.

This study aims to investigate the effects of different concentrations of NAC on growth, antrum formation and viability of bovine secondary follicles culture *in vitro*.

2. **Material and methods**

2.1. Ovarian collection

Bovine (*Bos taurus*) ovaries (n=60) were collected in a local slaughterhouse (Sobral, Ceará, Brazil) after official administration permission to use the ovaries for research purposes. Immediately after death, the ovaries were washed in 70% ethanol followed by two washes in TCM-199 supplemented with penicillin (100IU) and streptomycin (0.1 mg/ml) and buffered with HEPES. Subsequently, the ovaries were transported within 1 h to the laboratory in TCM-199 at 4°C.

2.2 Isolation and culture of secondary follicles

For follicular isolation, the methodology of Vasconcelos *et al*. (2021) was followed. Fragments (1-2mm) of the ovarian cortex were removed with the aid of a sterilized scalpel blade and placed in TCM-199 medium supplemented with HEPES (0.05 mM / mL) and penicillin (100IU) /streptomycin (0.1 mg/mL). Subsequently, the fragments were analyzed in a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan) to identify the secondary follicles (150-250 μm). Follicles that did not have antral cavity were manually isolated using 25G needles. After

isolation, follicles with intact basement membrane, spherical oocyte, surrounded by granulosa cells, and intact theca cell layers were selected for culture.

Secondary follicles were cultured individually in microdrops of TCM-199^{+}, i.e., TCM-199 medium (pH 7.2-7.4) supplemented with FSH (100ng/ml), ITS (insulin (10µg/mL), transferrin (5.5µg/mL) and selenium (5ng/mL) mL)), ascorbic acid (50µg/mL), BSA (3.0mg/mL), glutamine (2mM), hypoxanthine (2mM), penicillin (100IU) and streptomycin (0.1 mg/mL) and HEPES (0.05mM). The follicles were randomly cultured in TCM-199^{+} alone or supplemented with 1.0, 5.0, 25.0 mM NAC. The concentrations of NAC were based on previous studies (Bhardwaj and Saraf, 2020; Olesen *et al*., 2021). Culture was carried out in an incubator with 5% CO_2 in the air, at 38.5 °C, for 18 days. Every two days, 60 µL of culture medium was replaced. At the end of the culture, follicular diameters and antrum formation were analyzed under a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan). This experiment was repeated eight times.

2.3 Assessment of secondary follicle morphology, growth and antrum formation

The follicles were evaluated at days 0 and 18 of culture using an inverted microscope (Nikon, Eclipse, TS 100, Japan) and an image capture system (NIS Elements 2.4 Software, Nikon Instruments Inc., Japan). Follicle diameters were measured only in morphologically normal follicles. The average of two perpendicular measurements of the outer layer of the thecal cells was reported as follicular diameter (µm). Antrum formation was characterized by the presence of a translucent cavity visible within the granulosa cell layers. All evaluations and measurements were made by the same operator.

2.4 Assessment of secondary follicles viability by fluorescence microscopy

At the end of culture period, the follicles (n = *20* / treatment) were incubated in 100 µL of drops of TCM-199 containing 4 mM of calcein-AM and 2 mM of ethidium homodimer-1 (Molecular Probes, Invitrogen, Karlsruhe, Germany) at 37° C for 15 min. Then, the follicles were washed in TCM-199 and evaluated under a fluorescence microscope (Nikon, Eclipse, TS 100m Japan). Oocytes and granulosa cells were classified as viable if the cytoplasm was positively stained with calcein AM (green) and not viable if the chromatin was positively labeled with ethidium homodimer-1 (red) (Schotanus *et al*., 1997; Van der Huck *et al*., 1998).

The fluorescence intensity was analyzed using Image J software (National Institute of Health, Bethesda, MD, USA). The staining intensity of cultured follicles was determined by measuring the average pixels intensity of the follicular area after background subtraction.

2.5 Histological characteristics of morphologically normal or degenerated follicles

After the culture period, the follicles (n = 16, per treatment) were fixed for 1 hour in 4% paraformaldehyde, then transferred to a maintenance solution (PBS, 0.1% BSA, and Tween20 0, 1%), where they remained until processing. After fixation, the follicles were embedded in a 10µl drop of histogel (HistoGel™) for 3 to 5 minutes and stained with Alcian blue for 2 minutes. Soon after, the follicles were subjected to dehydration in a graded series of ethanol concentrations, clarified with xylene and embedded in paraffin. For each group of follicles, sections of 5 µm were cut and mounted on slides, and later stained with hematoxylin-eosin. The slides were examined under an optical microscope (Nikon, Tokyo, Japan). Follicles were classified according to morphology as normal and degenerated.

2.6 Statistical analysis

Statistical analyses were performed using GraphPad Prism software, version 9.0. Data of follicular diameter and growth rate between culture days were initially subjected to normal distribution analysis using the Kolmogorov-Smirnov test. Data of follicular diameters and staining intensity for calcein-AM and ethidium homodimer-1 were analyzed by Kruskal-Wallis test, followed by Dunn's multiple comparison test. Percentages of follicle survival and antrum formation were compared by Chi-square test, while the percentages of growing follicles were compared by Fisher's exact test. Differences were considered significant when $P<0.05$.

3. Results

3.1. Effects of NAC on follicle growth, morphology and antrum formation

Table 1 shows the presence of 1.0 mM NAC in cultured medium significantly increased the percentage of growing follicles after 18 days of culture, when compared to other treatments. NAC, however, did not influence the percentage of morphologically normal follicles or antrum formation. On the other hand, 25.0 mM NAC significantly reduced the rate of growing follicles during culture.

Table 1. Percentages of morphologically normal secondary follicles, antrum formation and of growing follicles after 18 days of *in vitro* culture in TCM-199^{+} alone or supplemented with different concentrations of NAC.

Treatments	Morphologically normal follicles	Antrum Formation	Growing follicles
TCM-199^{+}	97.97% (97/99)	20.20% (20/99)	62.63% (62/99)^{a}
NAC 1.0 mM	100.00%(101/101)	29.70% (30/101)	76.24% (77/101)^{b}
NAC 5.0 mM	100.00% (99/99)	24.24% (24/99)	54.55% (54/99)^{ac}
NAC 25.0 mM	98.98% (98/99)	29.29% (29/99)	47.47% (47/99)^{c}

a, b,c Lowercase letters represent statistically significant differences between Day 0 and Day 18 ($P < 0.05$).

Table 2 show that follicles cultured with TCM-199^{+} alone or supplemented with different concentration of NAC had a significant increase in their diameters after 18 days, when compared to day 0 ($P<0.05$).

Table 2. Diameters (mean ± S.E.M) of bovine secondary follicles after 0 and 18 days of *in vitro* culture in TCM-199^{+} alone or supplemented with different concentrations of NAC.

Treatments	Day 0	Day 18	Day 0-18	Daily growth average (µm)
TCM-199^{+}	203.69±3.52 ^{a}	279.46±8.21^{b}	75.77±7.46	4.21±0.41
NAC 1.0 mM	201.76±3.04 ^{a}	280.77±8.76 ^{b}	82.80±8.51	4.39±0.43
NAC 5.0 mM	203.40±3.41 ^{a}	265.92±8.17 ^{b}	66.42± 8.41	3.46±0.41
NAC 25.0 mM	210.95±4.15 ^{a}	271.18±8.81 ^{b}	60.24±7.63	3.34±0.42

a, b Lower case letters represent statistically significant differences between days 0 and 18 ($P < 0.05$).

3.2. Effects of NAC on follicular viability

Fig.1 shows secondary follicles stained with calcein-AM and ethidium homodimer-1 after 18 days of *in vitro* culture. Secondary follicles cultured in a medium

supplemented with 1.0 mM NAC had higher fluorescence intensity for calcein-AM than those cultured in control medium ($P<0.05$) (Fig. 2A). On the other hand, follicles cultured in a medium with 5.0 and 25.0 mM NAC did not differ significantly from each other or with those cultured in control medium ($P>0.05$). The fluorescence intensity for ethidium homodimer-1 was higher in follicles cultured in medium supplemented with 25.0 mM NAC when compared to follicles from control group ($P<0.05$) (Fig. 2B). It can be observed that follicles cultured in the presence of 25.0 mM NAC and TCM-199+ had stromal cells surrounding the follicles stained positively with ethidium homodimer-1 (Fig. 1).

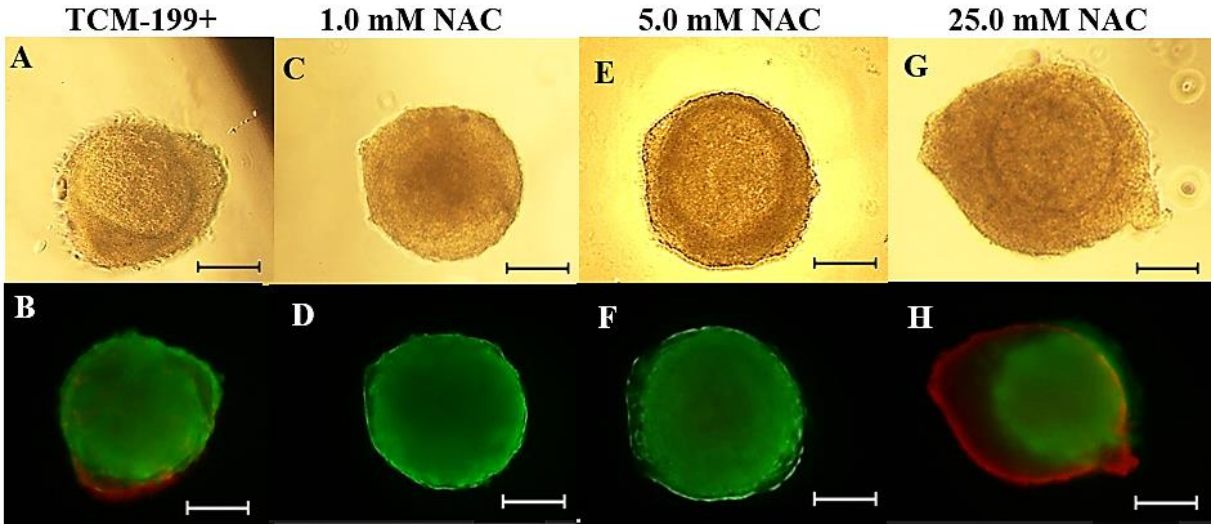

Figure 1. Fluorescence microscopy of bovine secondary follicles after 18 days of culture after staining with calcein-AM (green) and ethidium homodimer (red). Secondary follicles were cultured in TCM-199^{+} alone (A, B) or supplemented with 1.0 mM (C, D), 5.0 mM (E, F) or 25.0, mM NAC (G, H). Scale bars represent 100µm.

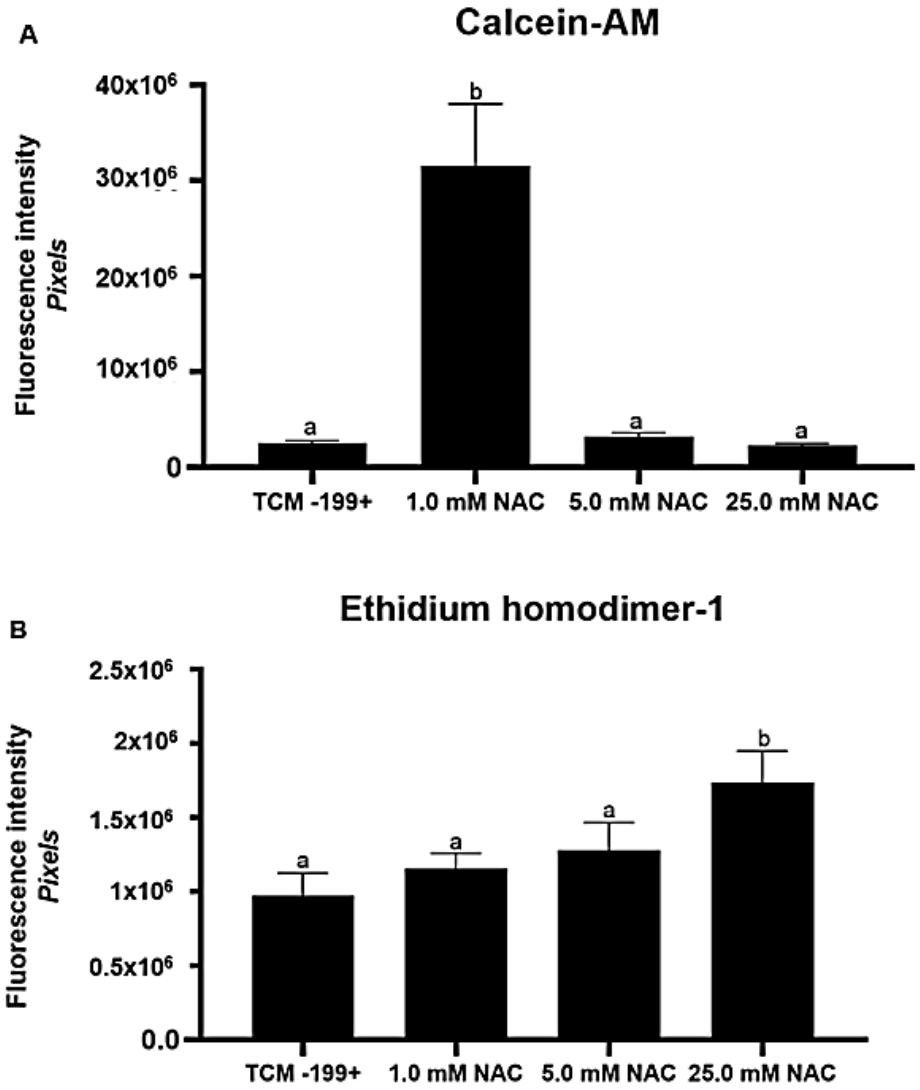

Figure 2. Fluorescence intensity levels (mean ± S.E.M) of calcein-AM (A) ethidium homodimer-1 (B) in bovine secondary follicle after 18 days of culture in TCM-199^{+} alone or supplemented with different concentrations of NAC (1.0 mM, 5.0 mM and 25.0 mM). a,b capital letters represent statistically significant differences between treatments ($P<0.05$).

3.3. Histological characteristics of morphologically normal or degenerated follicles after culture

Fig. 3A shows a morphologically normal follicle with organized granulosa cell layers, intact basement membrane, small spacing between granulosa cells (beginning of antral cavity formation), a centralized oocyte and no indication of degeneration. Fig. 3B shows a degenerated type 1 follicle, with organized granulosa cell layers, but the oocyte had large

number of vacuoles in the ooplasm. Fig. 3C shows a degenerated type 2 follicles, with disorganized granulosa cells, and a vacuolizeted retracted oocyte.

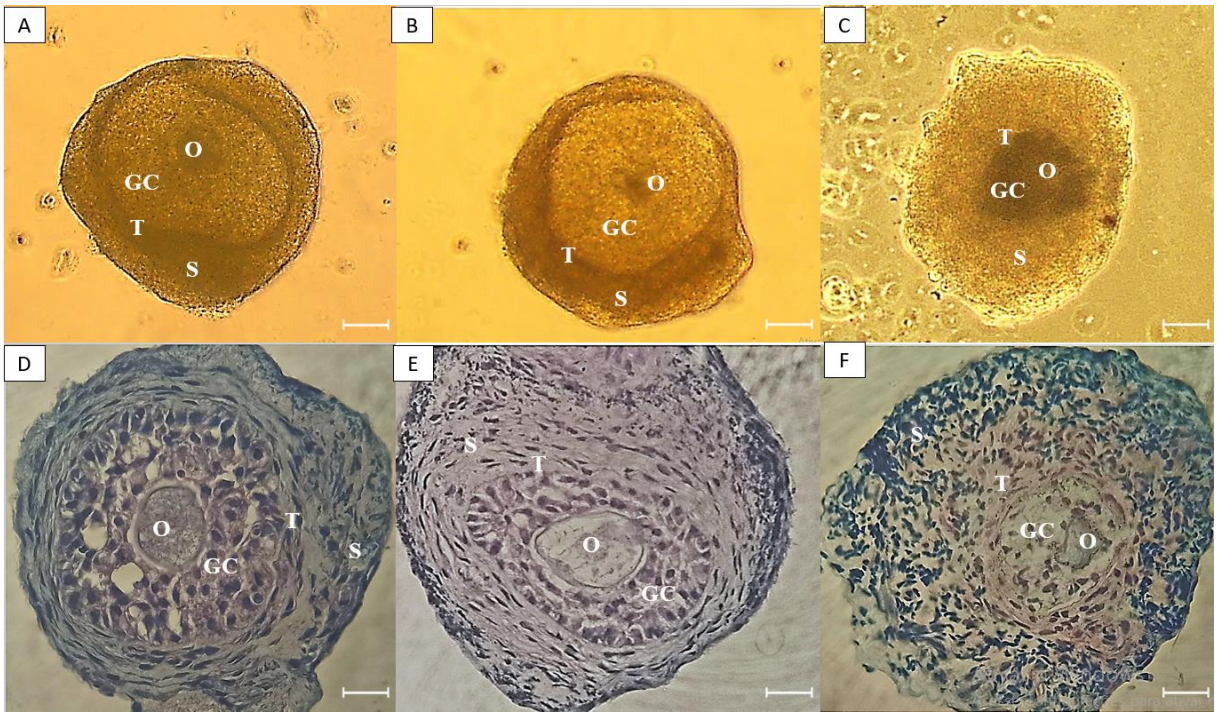

Figure 3. Morphological characteristics of secondary follicles after 18 days of *in vitro* culture. (A, D) Normal follicle, (B, E) follicle with initial signs of degeneration in the oocyte, (C, F) follicle with degeneration of both oocyte and granulosa cells. Theca cells (T), oocyte (O), stromal tissue (S) and granulosa cells (GC). Scale bar: 100 µm.

4. Discussion

The present study demonstrates for the first time that 1 mM NAC increases the viability and growth rate of bovine secondary follicles after 18 days of *in vitro* culture. Previous reports also showed that 1 mM NAC improves the quality of vitrified murine oocytes and the development of mouse embryos (Silva *et al*., 2015, Matilla *et al*., 2019). The thiol group of NAC provides electrons to free radicals (Yi *et al*., 2016; Pei *et al*., 2018) acting as a direct antioxidant. NAC is also a precursor of glutathione (GSH) by liberating a cysteine group and increasing the concentration of GSH in cells (Yi *et al*., 2016; Pei *et al*., 2018). The GSH is a major component of intracellular antioxidant control that prevents injuries caused by oxidative stress in oocytes (Mukherjee *et al*., 2014). It is also interesting to note that several studies indicate that NAC promotes survive and proliferation of various cell types (fibroblasts: Kunnavatana *et al*., 2005; adipose tissue-derived stem cells: Xiong *et al*., 2012; corneal

endothelial cell: Kim *et al*., 2014; intestinal epithelial cells: Yi *et al*., 2016; human retinal pigment epithelial cells: Terluk *et al*., 2019). Recently, Ding *et al*. (2021) has showed that NAC increases the production of transcripts for cyclin D1, which is related to cell proliferation and may have influenced the growth of bovine secondary follicles *in vitro*. In contrast, follicles cultured with 25 mM NAC had reduced growth rate. Ding *et al*. (2021) demonstrated that NAC in concentrations higher than 10 mM suppressed cell proliferation of porcine placental trophoblastic cells cultured *in vitro*, while Sun *et al*. (2021) showed that NAC in concentrations higher than 10.0 mM causes a reduction in the pH of culture medium and is harmful to oocyte growth during *in vitro* maturation.

Regarding viability, 1mM NAC increased calcein staining in cultured follicles. Calcein-acetoxymethyl ester (calcein AM) is non-fluorescent and cell membrane permeable, which is converted to calcein (fluorescent form) when its AM ester group is cleaved by intracellular non-specific esterases (De Clerck *et al*., 1994; Van Der Hoek *et al*., 1998). Esterases are an important group of enzymes for animal cells, with a catalytic function in the reaction of ester hydrolysis, in particular, the acetylesterases that hydrolyze acetyl esters (Oesch-Bartlomowicz and Oesch, 2008). In secondary and tertiary ovarian follicles of buffaloes, activity of non-specific esterases in granulosa cells, corona radiata, and theca cells have been reported to be important for cell growth and viability (Pathak and Bansal, 2019). Other studies showed that NAC protects granulosa cells against apoptosis, and improves cell viability in humans (Cheraghi *et al*., 2018; Olesen *et al*., 2021) and murines (Bhardwaj and Saraf, 2020). The 1 mM concentration of NAC has also been shown to improve cell viability in human osteochondral tissue chondrocytes (Calvo *et al*., 2020).

The presence of 25 mM NAC in culture medium, however, increased the percentages of cells with damaged membranes, as indicated by ethidium homodimer-1 staining. This marker also indicates an extravasation of cytoplasmic contents (Sanfilippo *et al*., 2011). This NAC concentration may be toxic for bovine secondary follicles; such effect can be associated with a pro-oxidant action (Finn and Kemp, 2012). The same molecules that scavenge active free radicals can also cause oxidative stress, prompting the induction of cellular signaling to increase antioxidant defense (Halliwell, 2009; Xanthis *et al*., 2021). NAC acts as a pro-oxidant molecule when, for example, it comes into contact with transition metals and thus can react with H_2O_2 to generate hydroxyl radical, which can cause lipid peroxidation and,

consequently, causes damage to cell membranes. (Sagristá *et al*., 2002; Ates *et al*., 2008). Such a factor may have contributed to reduce the percentage of developing follicles after culture in medium supplemented with 25 mM NAC.

Data from histological analysis show that the first signs of degeneration were observed in the oocyte. Such degeneration patterns in preantral follicles have also been reported in ovaries of various species (ovine: Jorio *et al*., 1991, caprine: Silva *et al*., 2001, canine: Spanel-Borowski, 2012). Previous *in vitro* studies have also shown that the oocyte of preantral follicles degenerates while granulosa cells appear healthy and continue to proliferate, showing that the oocyte is much more sensitive to degenerative events than granulosa cells (Figueiredo *et al*., 1994; Braw-Tal and Yossefi, 1997). In preantral follicles, autophagy is involved in the degeneration of oocyte and later in the granulosa cells (Rajakoski, 1960; Rodgers and Irving-Rodgers, 2010; Kassouri-Maouche *et al*., 2018; Meng *et al*., 2018). The overexposure to oxygen, light, temperature variations, osmolarity are involved in the increase of ROS levels and, consequently, oocyte degeneration (Sá *et al*., 2018; Soto-Heras and Paramio, 2020; Paulino *et al*., 2022).

In conclusion, 1mM NAC increases the viability and growth rate of bovine secondary follicles cultured *in vitro*, while 25mM NAC damages cell membranes and reduce the percentage of growing follicles.

5. Ethics standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals. This study was registered in Ethics and Animal Welfare Committee of the Federal University of Ceará (N° 02/2021).

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7. Author Contribution Statement

All authors contributed equally to write the manuscript. J.R.V. Silva critically revised the manuscript.

8. Conflict of interest

The authors declare that they have no competing interests.

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CONCLUSÕES GERAIS

- A NAC (1.0 mM) aumenta a porcentagem de folículos em crescimento e a viabilidade de folículos secundários bovinos cultivados *in vitro* por 18 dias.
- Adição de 25.0 mM de NAC ao meio de cultivo promove a diminuição da taxa de folículos em crescimento e aumento da fluorescência para homodímero-1 de etídio.

PERSPECTIVAS

A N-acetilcisteína é um fármaco que possui um grande potencial antioxidante que pode ser explorado durante o cultivo *in vitro* de folículos secundários. A adição de NAC aos meios de cultivo *in vitro* de folículos secundários bovinos pode contribuir para o desenvolvimento de sistemas de cultivo *in vitro* mais eficientes para promover o crescimento de folículos secundários e a melhoria das taxas de viabilidade. Todavia ainda há a necessidade de mais investigações para uma compreensão de maneira mais detalhada os mecanismos de ação da NAC durante o crescimento folicular *in vitro.*

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