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EFIGENIA CORDEIRO BARBALHO

INFLUÊNCIA DA N-ACETILCISTEÍNA (NAC) DURANTE O CULTIVO *IN VITRO* DE FOLÍCULOS ANTRAIS INICIAIS EM BOVINOS

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Dissertação apresentada ao Programa de Pós-Graduação em Biotecnologia da Universidade Federal do Ceará, como requisito à obtenção do título de Mestre em Biotecnologia. Área de concentração: Biotecnologia. Linha de Pesquisa: Análises Integrativas de Sistemas Biológicos. Área Temática: Fisiologia Reprodutiva.

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Á Deus, por todas as bençãos concedidas.
Aos meus pais, Marlene e Gonçalo por todo amor apoio.
Ao meu amado noivo Manassés por todo companherismo.
A todos os familiares e amigos.

Com amor, dedico.

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RESUMO

Este estudo investiga os efeitos de diferentes concentrações de N-acetilcisteína (NAC) no crescimento e morfologia folicular, bem como na viabilidade, níveis de espécies reativas de oxigênio (ROS) e progressão meiótica de oócitos de folículos antrais iniciais bovinos cultivados *in vitro*. Para este fim, folículos antrais isolados iniciais (~500 μm) foram cultivados em TCM-199+ sozinho ou suplementado com 1,0, 5,0 ou 25,0 mM de NAC a 38,5°C com 5% de CO2 por 8 dias. Os diâmetros dos folículos foram avaliados nos dias 0, 4 e 8 de cultivo. Ao final do cultivo, os níveis de EROs, configuração da cromatina e viabilidade (coloração de calceína-AM e homodímero-1 de etídio) foram investigados nos complexos cumulus oócitos (COCs) isolados. As comparações dos diâmetros foliculares entre os tratamentos foram realizadas pelo teste de Tukey. Dados sobre porcentagens de folículos morfologicamente normais, taxas de crescimento e configuração da cromatina foram comparados usando o teste de Fisher ($P < 0,05$). Os resultados mostraram um aumento nos diâmetros foliculares após o cultivo em todos os tratamentos em comparação ao inicio do cultivo, exceto para folículos cultivados com NAC 25,0 mM. A microscopia de fluorescência mostrou que os oócitos cultivados em todos os tratamentos coraram positivamente com calceína-AM, e que 5,0 mM de NAC reduziu a fluorescência para o homodímero-1 de etídio. Os níveis intracelulares de EROs em oócitos de folículos cultivados com NAC 1,0mM apresentaram redução significativa em relação aos demais tratamentos. A presença de NAC no meio de cultivo não influenciou as taxas de oócitos no estágio de vesícula germinativa. Em conclusão, o NAC nas concentrações de 1,0 reduz os níveis de EROs e 5,0mM reduz a coloração para o homodímero-1 de etídio, mas o NAC 25,0mM reduz o crescimento folicular e as porcentagens de folículos em crescimento contínuo.

Palavras – chaves: Antioxidante; Estresse oxidativo; EROs; Oócitos.

ABSTRACT

This study investigates the effects of different concentrations of N-acetylcysteine (NAC) on follicular growth and morphology, as well as viability, reactive oxygen species (ROS) levels and meiotic progression of oocytes from bovine early antral follicles cultured in vitro. To this end, isolated early antral follicles (~500 μm) were cultured in TCM-199+ alone or supplemented with 1.0, 5.0 or 25.0 mM NAC at 38.5°C with 5% CO2 per 8 days. Follicle diameters were evaluated on days 0, 4 and 8 of culture. At the end of cultivation, ROS levels, chromatin configuration and viability (calcein-AM and ethidium homodimer-1 staining) were investigated in isolated cumulus oocyte complexes (COCs). Comparisons of follicular diameters between treatments were performed using the Tukey test. Data on percentages of morphologically normal follicles, growth rates, and chromatin configuration were compared using Fisher's test ($P < 0.05$). The results showed an increase in follicular diameters after culture in all treatments compared to the beginning of culture, except for follicles cultured with 25.0 mM NAC. Fluorescence microscopy showed that oocytes cultured in all treatments stained positively with calcein-AM, and that 5.0 mM NAC reduced the fluorescence for ethidium homodimer-1. The intracellular levels of ROS in oocytes from follicles cultured with 1.0mM NAC showed a significant reduction in relation to the other treatments. The presence of NAC in the culture medium did not influence the rates of oocytes at the germinal vesicle stage. In conclusion, NAC at concentrations of 1.0 reduces ROS levels and 5.0mM reduces staining for ethidium homodimer-1, but NAC 25.0mM reduces follicular growth and the percentages of continuously growing follicles.

Keywords: Antioxidant; Oxidative stress; ROS; Oocytes.

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LISTA DE ABREVIATURA E SIGLAS

AI	Anáfase I
AC	Adenilato cyclase
ANOVA	Análise de Variância
AMPc	Adenosina Monofosfato Cíclica
BMP -4	Proteína Morfogenética Óssea 4
BMP - 15	Proteína Morfogenética Óssea 15
BSA	Albumina Sérica Bovina
bFGF	Fator Básico de Crescimento de Fibroblastos
CAT	Catalase
CC	Células do Cumulus
CG	Células da granulosa
CT	Células da teca
CCNB1	Ciclina B1
COC	Complexo Cumulus-oócito
CNP	Peptídeo Natiurético do tipo C
CGP	Células Germinativas Primordiais
DNA	Ácido Desoxiribonucleico
eIF4E	fator de iniciação da tradução
EROS	Espécies Reativas de Oxigênio
EREs	Espécies Reativas de Enxofre
ERNs	Espécies Reativas de Nitrogênio
EO	Estresse Oxidativo
EGF	Fator de Crescimento Epidérmico
IGF	Insulina Fator de Crescimento
et al	Colaboradores
FIV	Fertilização *In vitro*
FSH	Hormônio Folículo-estimulante
FSK	Forskolina
GDF9	Fator de Crescimento e diferenciação – 9
GnRH	Hormônio liberador de gonadotrofina
GMPc	Guanosina Monofosfato Cíclica
GSH	Glutationa

GPX	Glutationa redutase/peroxidase
GVBD	Vesícula Germinative Rompida
H1FOO	Histona específica do oócito 1
IBMX	3-isobutil-1 metilxantina
LPS	Lipopolysaccharide
LH	Hormônio Luteinizante
MAPKs	Proteínas quinases ativadas por mitógenos
MI	Metáfase I
MII	Metáfase II
MIV	Maturação *In vitro*
MET	Microscopia Eletrônica de Transmissão
c-MOS	Fator de maturação de oócitos MOS
MPF	Fator Promotor de Maturação
mRNA	Ácido Ribonucleico mensageiro
NAC	N-Acetilcisteína
PARN	Ribonuclease específica poli (a)
PBS	Phosphate Bufferid Saline
PDE3	Fosfodiesterase tipo 3
PIV	Produção *In vitro*
PPGB	Programa de Pós-Graduação em Biotecnologia
RENORBIO	Rede Nordeste de Biotecnologia
RNA	Ácido Ribonucleico
GPX	Glutationa Peroxidase
SH	Grupo Sulfridila
SNK	Student Newman-Keuls
SOD	Superóxido Desmutase
SOP	Síndrome do Ovário Policístico
TCM-199	Meio de cultivo tecidual
TCM-199^{+}	Meio de cultivo tecidual Suplementado
TGFβ	Fator Transformador de Crescimento β

TI	Telófase I
TZPs	transzonal projections
UFC	Universidade Federal do Ceará
VG	Vesícula Germinativa

LISTA DE SÍMBOLOS

%	Porcentagem
A	Alfa
B	Beta
C	Carbono
Cm	Centímetro
D	Dia
µg	Microgrma
µL	Microlitro
µM	Micromolar
µm	Micrometro
Ml	Mililitro
Mm	Milímetro
mM	Milimolar
M	Molar
O_2	Oxigênio
CO_2	Dióxido de carbono
°C	Grau Celsios
H	Hora
IU	Unidades Internacionais
G	Gravidade
G	Grama
UV	Ultravioleta
<	Menor que
>	Maior que
≤	Menor ou igual

SUMÁRIO

1 INTRODUÇÃO

A bovinocultura representa uma das áreas que mais contribuem com a economia brasileira, e vem ganhando destaque no mercado nacional e internacional, sendo responsável por fornecer produtos de alta qualidade, proporcionando geração de emprego e aumento da renda no país (SOARES *et al.*, 2019). Desta forma, a busca por estratégias que possibilitem aumentar a eficiência reprodutiva de animais que apresentem características genéticas superiores, torna-se bastante relevante (MOREIRA *et al*., 2019). Neste sentido, várias biotécnicas reprodutivas vêm sendo aplicadas nos rebanhos, as quais exercem grande impacto no setor do agronegócio no Brasil, sendo responsáveis pela inovação na pecuária. Essas biotécnicas contribuem para o melhoramento da produtividade e permitem uma maior eficácia na seleção de animais geneticamente superiores (FIGUEIREDO e LIMA, 2017).

Dentre essas biotécnicas aplicadas a reprodução animal, a maturação *in vitro* (MIV) de complexos cumulus oócito (COC) vem sendo extensivamente utilizada para a produção *in vitro* de embriões. No entanto, o tamanho dos folículos os quais os COCs são coletados podem comprometer a eficiência dessa técnica, tendo em vista que a competência oócitaria é adquirida gradativamente durante o crescimento folicular (DODE *et al*., 2000). Neste aspecto, estudos demonstraram que oócitos coletados a partir de folículos antrais iniciais com cerca de 1 e 2 mm possuem competência significativamente reduzida, consequentemente, resultam em menor taxa de maturação, enquanto oócitos recuperados de folículos maiores (>3 mm) são capazes de completarem a maturação nuclear *in vitro* (PAVLOK *et al*., 1992; NEMCOVÁ *et al*., 2019). Desta forma, estudos envolvendo o cultivo *in vitro* de foliculos antrais iniciais pode favorecer a elucidação dos fatores que regulam o desenvolvimento folicular *in vitro* e a ultilização dos oócitos desses foliculos em protocolos de MIV, bem como, pode contribuir com o aperfeiçoamento dos sistemas de cultivo *in vitro*. No entanto, o aumento das espécies reativas de oxigênio (EROs) durante o cultivo é um dos principais fatores associados à baixa qualidade dos folículos ovarianos cultivados *in vitro* (PAULINO *et al*., 2022; SÁ *et al*., 2018).

A N- Acetilcisteína (NAC) é uma substância precursora de cisteína e glutationa reduzida, que apresenta grande atividade antioxidante (MAHMOODI *et al*., 2015). Tuncer *et al*. (2018) mostraram que a NAC é capaz de prevenir a atresia de folículos ovarianos em camundongos. Outro estudo também revelou que a NAC melhora a qualidade de oócitos murinos após a vitrificação (MATILLA *et al.*, 2019).

Dessa forma, a adição de NAC ao meio de cultivo de foliculos antrais iniciais (~500µM) bovinos pode contribuir com o controle do estresse oxidativo *in vitro,* consequentemente,

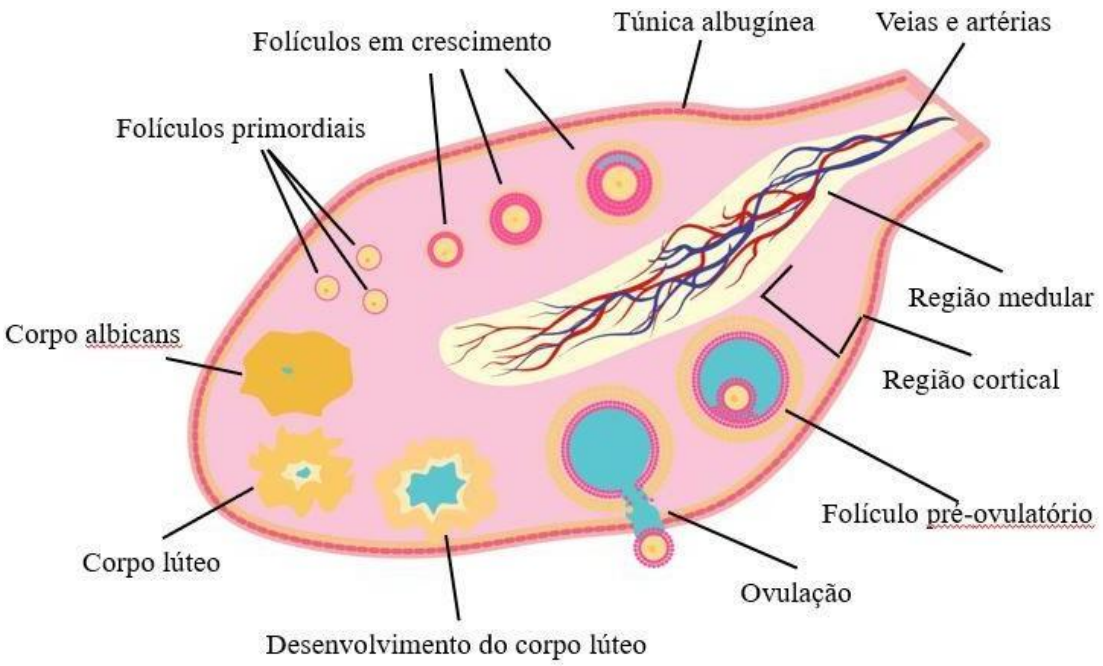
propiciando um aumento na eficiência dos sistemas de cultivo folicular *in vitro*. Além disso, a viabilização do cultivo de foliculos antrais iniciais fornece perspectivas para melhorar a compreensão da foliculogênese.

2 REVISÃO DE LITERATURA

2.1 Ovário bovino

Os ovários bovinos (Figura 1) são geralmente ovais e aplanados lateralmente, medindo cerca de 3,0 a 4,5 cm de comprimento, 1,5 a 2,0 cm de largura e 2,0 a 2,8 cm de profundidade, podendo haver grande variação na sua forma e tamanho ao longo do ciclo estral (NASCIMENTO *et al*., 2003). A superfície ovariana é coberta por um epitélio pavimentoso, denominado epitélio germinativo, em baixo do qual está presente a túnica albugínea responsável pela cor esbranquiçada do ovário, e sob ela fica a região cortical, onde estão localizados os folículos contendo os oócitos (JUNQUEIRA e CARNEIRO, 2013).

Figura 1 - Esquema ilustrativo do ovário mamífero e suas principais estruturas.



Fonte: Autor, 2022.

Os ovários são responsáveis pela realização de dois processos fundamentais, sendo eles; a função gametogênica, que envolve a formação, maturação e liberação de oócitos competentes para fertilização; e a função endócrina que envolve a síntese e secreção de hormônio que são essenciais para o desenvolvimento folicular (ARAÚJO *et al*., 2014; PORRAS-GOMÉZ e MENDOZA, 2017). A função gametogênica envolve a interação entre dois fenômenos complexos que tem início ainda na fase fetal. O primeiro é a oogênese que se refere a uma sequênciade eventos em

que as células germinativas primordiais diferenciam-se inicialmente em oogônias, seguindo para ovócitos primários e posteriormente secundários, quando ocorre a extrusão do primeiro corpúsculo polar (ADONA *et al*., 2013). O segundo é a foliculogênese, que compreende uma sequência de eventos que inclui a formação, crescimento e maturação folicular,até a ovulação (VAN DEN HURK e ZHAO, 2005).

2.2 Oogênese

A oogênese inicia-se, com a colonização da gônoda primitiva pelas células germinativas primordiais (CGPs) ou gonócitos, que são originadas do mesoderma extraembrionário (ARAÚJO *et al.,* 2015). Essa colonização das gônodas pelas CGPs, é controlada por diversos fatores, dentre eles estão os membros da família do fator transformador de crescimento β (TGFβ), como a proteína morfogenética óssea 4 (BMP-4), a BMP-8b e a BMP-2 (SÁNCHEZ e SMITZ, 2012). As CGPs, sofrem mitoses, diferenciam-se em oócitos primários e dão início a primeira divisão meiótica (CROCOMO *et al*., 2019). No entanto, a meiose é interrompida ainda no estágio inicial, na fase diplóteno da prófase I (vesícula germinativa). A retomada da meiose ocorre com a liberação de gonadotrofinas na puberdade (LONERGAN e FAIR, 2016;), quando os oócitos imaturos progridem para a metáfase II (MII), fase em que permanecerão até a fecundação ou ativação da partenogênese (LONERGAN *et al*., 2000).

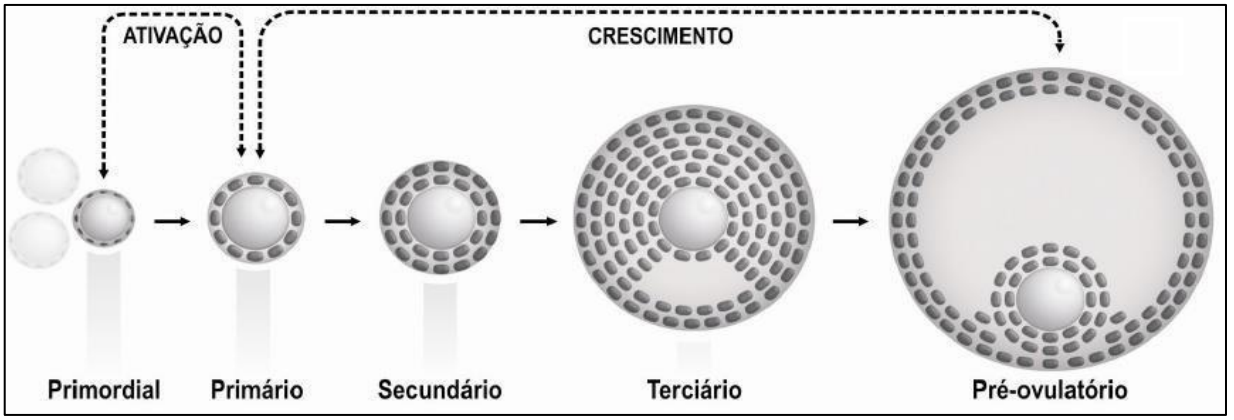
2.3 Foliculogênese ovariana

Na maioria das espécies, a foliculogênese (Figura 2) tem início na vida pré-natal, e pode ser definida como o processo de formação, crescimento e maturação folicular (VAN DEN HURK e ZHAO, 2005). Ela tem início com a formação dos folículos primordiais que iniciam seu crescimento para formar sequencialmente os folículos primários (oócito circundado por uma única camada de células da granulosa cuboides), secundários (oócito circundado por duas ou mais camadas de células da granulosa); terciários (com presença de cavidade antral). Por fim, formam-se os folículos pré- ovulatórios que terminam com a ovulação do oócito maduro (ADONA *et al*., 2013; MATSUDA *et al.,* 2012).

O desenvolvimento folicular pode ser dividido em duas etapas; ou seja, a fase pré- antral, que abrange o crescimento dos folículos primordiais até os folículos secundários e a fase antral, quando os foliculos crescemm até o estágio pré-ovulatório. O desenvolvimento de folículos antrais é caracterizado pela organização das células da granulosa em várias camadas e pela formação de uma cavidade antral repleta de líquido folicular, composto por água,

eletrólitos, proteínas séricas e hormônios esteroides (SOUSA *et al*., 2008; BARNETT *et al.*, 2006; VASCONCELOS *et al.*, 2013). As células da granulosa são fisicamente separadas em células da granulosa murais, que se organizam ao longo da parede do folículo, e células do cumulus granulosa, que circundam o oócito (ZHANG, 2018; BAUMGARTEN e STOCCO, 2018). A formação da cavidade antral está relacionada a competência meiótica, que se deve à expressão reduzida de genes que ativam vias de sinalização para aumentar a capacidade do oócito de responder ao aumento das gonadotrofinas (SÁNCHEZ e SMITZ, 2012; ROUHOLLAHI, *et al*., 2020). Tendo em vista que nessa fase os foliculos tornam-se responsivos e dependentes das gonadotrofinas. Essa responsividade às gonadotrofinas possibilita que os folículos cresçam até a seleção e dominância (OLIVEIRA *et al*., 2011; MAGALHÃES-PADILHA *et al*, 2013).

Figura 2: Visão geral da foliculogênese. A representação esquemática ilustra as diferentes classes do desenvolvimento folicular, incluindo folículos pré-antrais: primordiais, primários e secundários, e folículos antrais: terciários e pré-ovulatórios.



Fonte: Lima-Verde; Rossetto; Figueiredo (2011).

2.4 Mecanismos de controle do desenvolvimento de folículos antrais iniciais e estratégias para promover o crescimento destes folículos in vitro

ARTIGO 1

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

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

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Abstract

This review aims to discuss the main factors involved in the development of early antral follicle up to gonadotropin dependence. This follicular phase is characterized by intense proliferation of granulosa cells, formation of a fluid-filled cavity, morphological differentiation of cumulus and mural granulosa cells, and recruitment of theca cells. The interaction between oocyte, granulosa and theca cells is determinant for follicle growth and hormone production. The oocyte influences follicle development through the production of growth factors, which stimulates the production of FSH receptors in granulosa cells. Expression of these receptors enables the follicles to become responsive and later dependent on gonadotropins. FSH induces proliferation and viability of the cumulus-granulosa complex, but many other local factors are involved in the regulation of follicular development. Therefore, this review is of great relevance for a better understanding of the mechanisms involved in the control of ovarian follicular growth and steroidogenesis, as well as for the establishment of efficient culture systems for in vitro growth of early antral follicles.

Keywords: In vitro culture; early antral follicles; folliculogenesis.

1. Introduction

Oocyte developmental competence refers to the ability of a female gamete to reach maturation, to be fertilized, and to support embryonic development until the blastocyst stage [1]. According to Dode *et al* [2], this competence is acquired gradually during preantral and early antral follicular growth.

To reinforce this information, oocytes from 3 mm antral follicles are able to complete nuclear

maturation in vitro, while those from smaller follicles (1 and 2 mm) have reduced competence [3,4]. This non-competence of oocytes from small antral follicles is due to the reduced expression of genes that activate signaling pathways to increase the oocyte ability to respond to the increase in gonadotropins [5]. Responsiveness to gonadotropins enable the follicles to grow until selection and dominance [6,7].

In the course of follicular development, proliferation and morphological differentiation of granulosa cells, are of great importance to prepare the follicle to respond to gonadotropins and to create a favorable environment for oocyte development [8]. Granulosa cells produce several autocrine and paracrine factors that may be involved in oocyte growth and antrum formation [9]. Additionally, oocyte derived factors stimulate the expression of FSH receptors in granulosa cells, to enable them to become responsive to gonadotropins [10]. FSH induces proliferation and viability of the oocyte-cumulus-granulosa complex, and may also induce granulosa cell differentiation [11]. In addition, oocyte-derived factors also stimulate antral cavity formation by increasing the expression proteoglycans, as a result of interaction with FSH [9]. Thus, understanding the endocrine, paracrine and autocrine mechanisms that control the interaction between follicular cells and the oocyte during early antral follicles is very important to develop strategies to promote their development in vitro [12].

The present review provides an overview of the main factors that control the development of early antral follicles up to gonadotropin dependence, i.e., regulation of granulosa cell proliferation, steroidogenesis, atresia, interaction between oocyte and granulosa cells, as well as the strategies to promote the development of early antral follicles in vitro.

2. Endocrine control of early antral follicle development

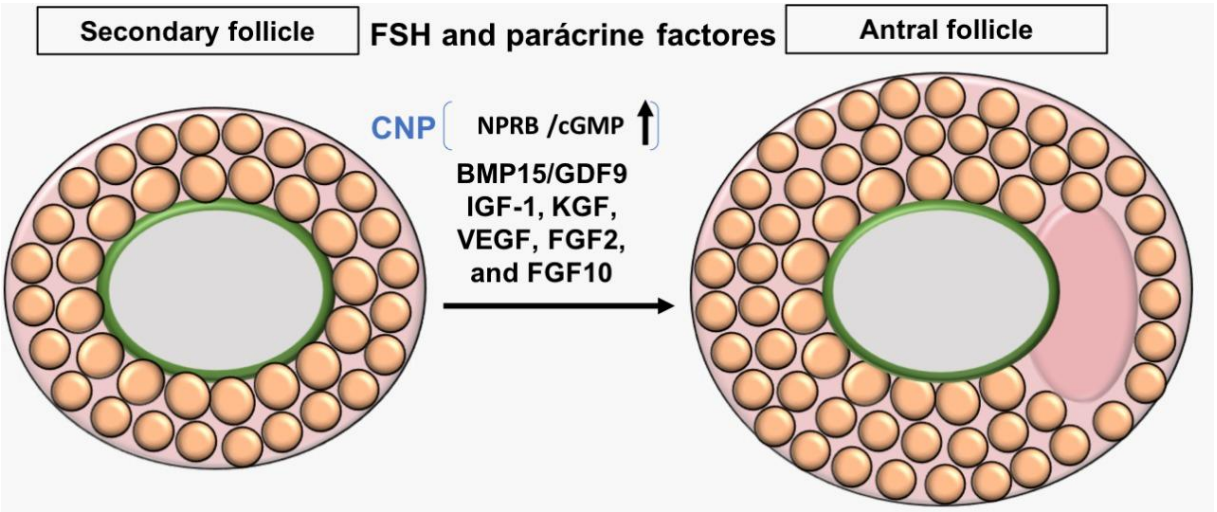
Follicle development from the preantral to the early antral stage is primarily controlled by intraovarian regulators, but it can be stimulated by FSH. The specific receptors for FSH are expressed in granulosa cells of secondary and early antral follicles [13]. When secondary follicles are formed, granulosa cells express FSHR and theca cells express LH receptor (LHR) [14]. In domestic and human species, antrum formation is observed when the follicles have around 0.2mm [15] and become dependent of gonadotrophin when they reach 3.0mm in cow [16], 4.0mm in sheep [17], 3.0mm in goat [18] and 5.0mm in human [19]. Follicle growth and maturation beyond this stage, which includes follicle recruitment, selection, dominance and ovulation is gonadotropin-dependent [20,21]. Acquisition of FSH dependence during this interval of growth is crucial to

determine follicular fate, i.e growth or atresia, in addition to stimulating the importance of C-type natriuretic peptide (CNP) which is a preantral and antral follicular growth factor (Figure 1). Fushii *et al* [22] recently showed that folliclescultured with FSH have formation of antral cavity one day earlier than those that did not receive this hormone, showing the importance of FSH on follicular development. Intraovarian regulators, like insulin-like growth factor (IGF), activin, oocyte-derived factors, and gap junction membrane channel protein, play a central role in the acquisition of FSH dependence at the early antral stage includes [13]. Theca-derived androgens bind to androgen receptors (ARs) in granulosa cells [23], thereby inducing FSHR expression and follicle growth during the preantral-to-antral transition [14,24,25]. AR deficiency in the mice ovary induces granulosa cell apoptosis, arrests antral follicle growth, and results in premature ovarian failure [26,27,28]. Thus, androgens play an important role in the growth, survival, and acquisition of FSH dependence in early antral follicles [13].

Anti-Müllerian hormone (AMH) is a product of granulosa cells of from small antral follicles onwards that has an inhibitory or retarding role in the development of antral follicles. AMH reduces follicle sensitivity to FSH, decreasing the expression of the FSH-stimulated FSH receptor (FSHR). AMH inhibits cyclic FSH-dependent recruitment and appears to play a role in all gonadotropin-independent follicular growth. Although there appears to be a regulatory relationship between androgens and AMH, there are conflicting data and it is not always possible to rule out that the observed effects may be mediated by estradiol, via testosterone aromatization [29].

Melatonin is endogenously produced by pineal gland that is found in follicular fluid of human antral follicles [30]. Melatonin receptors have previously been detected in granulosa cells of preantral and antral follicles [31]. Barros *et al* [32] demonstrated that this hormone is associated with meiotic competence of oocytes from early antral follicles. Melatonin maintains follicular survival, stimulates antral cavity formation and subsequent follicular and oocyte growth, as well as increases glutathione and metabolically active mitochondria levels after in vitro culture of sheep secondary follicles [32].

Figure 1. Development of pré antral follicles gonadotropin responsive stages. CNP is an intraovarian factor important for preantral and antral follicle growth as well as for oocyte maturation inhibition. Based on murine studies, CNP is secreted by granulosa cells of secondary and antral follicles in response to FSH stimulation. CNP acts through its receptor. NPRB, expressed in granulosa cells of secondary follicles, to increase cGMP production and to stimulate follicle development (Sato *et al*., 2012).



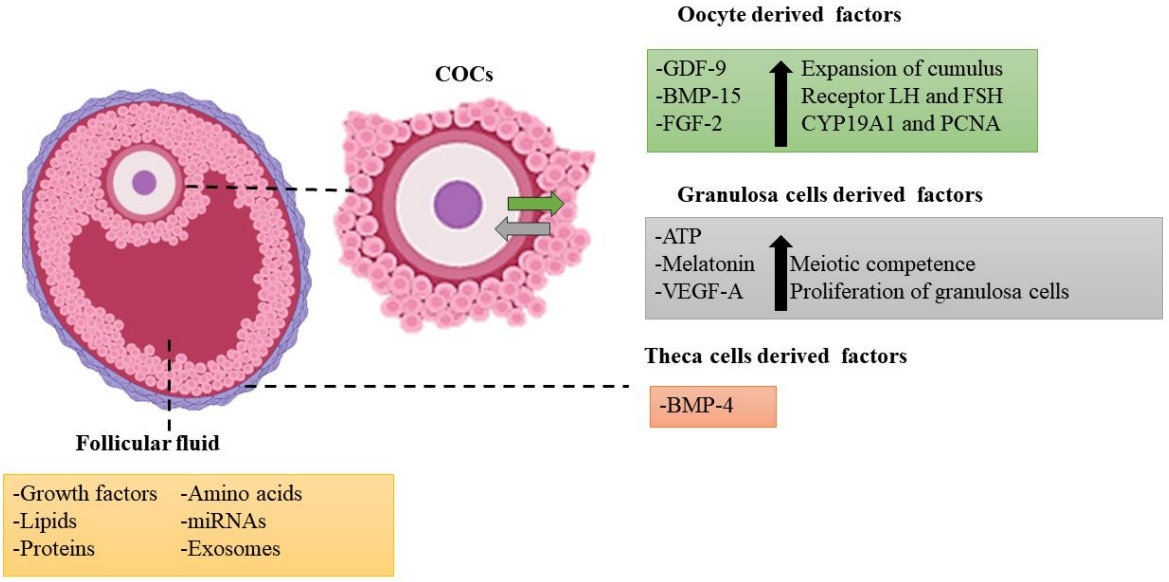
3. Oocyte-granulosa cell interaction during early antral follicle development

It is well known during follicular development; members of the transforming growth factor beta (TGFβ) family and their receptors are involved in the control of oocyte growth and granulosa cells proliferation. Oocyte-derived TGFβ family members, such as growth and differentiation factor – 9 (GDF-9) and bone morphogenetic protein-15 (BMP15), regulate granulosa cell proliferation and differentiation, as well as the development of the antral cavity [34,35]. In addition, recent studies indicate that these factors regulate expression of mRNAfor luteinizing hormone receptors in cumulus cells [36]. Oocyte-derived GDF-9 promotes the growth of COCs, while BMP-15 induces expression of choriogonadotropin receptor mRNA (LHCGR) in cumulus cells, and FSH receptor expression in follicles.Such factors contribute to follicular development and oocyte maturation [36,37]. GDF9 and BMP15bind to type II BMP receptor [38] and recruit type I activin-like kinase (ALK)5 [39] and ALK6 [40] to regulate downstream SMAD proteins in granulosa cells. Studies indicate that GDF-9 treatment enhances growth and differentiation of

preantral follicles in culture [41] and promotes theca cell androgen biosynthesis and proliferation [42]. In addition to these factors, R-spondin2 protein is also an important paracrine factor that can promote granulosa cell proliferation [43]. Fibroblast growth factor (FGF-2) and their respective receptors are also involved in early antral follicle development [44, 45, 35] FGF-2 alone or in association with VEGF-A influence steroidogenesis and proliferation of buffalo granulosa cells by regulating mRNA expression of CYP19A1, PCNA, and BAX [45,46].

Granulosa cells play a role in the development of antral follicles by promoting the development of the oocyte-granulosa cell complex and providing adenosine triphosphate (ATP) to the oocytes [47]. In addition, [48] demonstrated the influence of BPM-4, derived from theca cells, on steroidogenesis in early antral follicles. The CNP is also a stimulating factor for early antral follicles. In gene expression analyses indicated increases in transcripts for CNP receptors (NPP and NPRB) during early folliculogenesis in mice, in association with increases in ovarian CNP peptides [33]. Figure 2 exemplifies these interactions between oocytes and granulosa cells.

Figure 2. Oocyte-granulosa cell interaction during early antral follicle development.



4. Control of granulosa cell proliferation and estradiol production during early antral follicle development

In early antral follicles, granulosa cells are highly proliferative but susceptible to apoptosis. The factors secreted by the oocyte define the granulosa cell proliferation and survival [49]. Furthermore, it has been observed that recombinant GDF9 and BMP15 stimulate granulosa cell proliferation [50]. Furthermore, granulosa cell proliferation is dependent on cyclin D2 to activate cyclin-dependent kinase (CDK) family members CDK2, CDK4, and CDK6 [51]. In developing follicles, FSH stimulates granulosa cells proliferation and aromatization of androgens into estrogens. Estrogens also stimulate granulosa cell proliferation [52]. An increase in estradiol is associated with an increase in the expression of genes for aromatase, 3β-HSD and receptors for FSH and LH in granulosa cells [53].

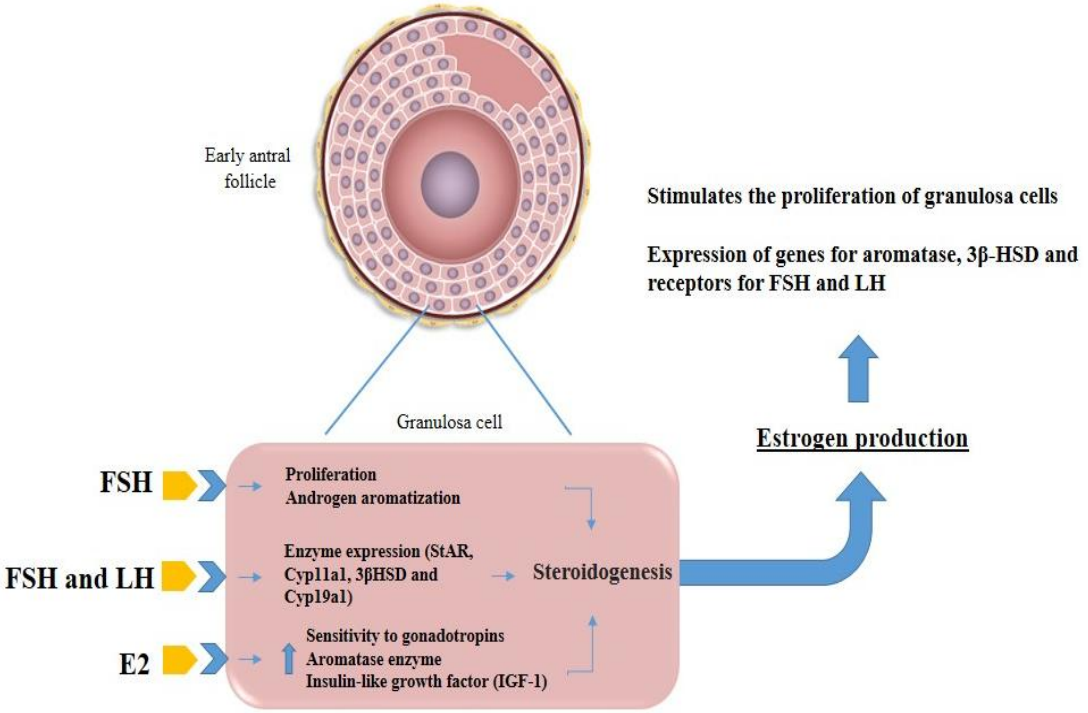
It was observed that neuronal neuropeptide Y (NPY) is strongly present in granulosa cells and the abundance of mRNA for NPY was higher in early antral follicles than in late antral follicles. In addition, NPY increased the proliferation of granulosa cells via NPY receptor Y5 (NPY5R) and mitogen-activated protein kinase (MEK) [54]. Baddela *et al.* [55] reported that in granulosa cells, hypoxia-inducible factor 1 (HIF1) transcriptionally regulates genes associated with steroidogenesis (StAR, HSD3B and CYP19A1) and proliferation (CCND2 and PCNA). The onset of expression of StAR mRNA occurs in early antral follicles of 1 mm in diameter [56]. Furthermore, FSH and LH, together with intraovarian cytokines, induce the expression of steroidogenic enzymes in granulosa cells, including StAR, Cyp11a1, 3βHSD and Cyp19a1, as show in figure 3 [57]. The expression of mRNA for LHR is found in granulosa cells from follicles smaller than 5 mm [58].

Follicular steroidogenic potential involves an extensive and highly coordinated series of developmental stages. During this process, after intense granulosa and theca cells proliferation (up to 100-fold), they differentiate into specialized endocrine cells. Ovarian steroids are synthesized by the cooperation of these cells. Theca cells synthesize androgens through the enzymatic activity of cytochrome P450 17A1 (CYP17A1) [52]. Follicles larger than 2 mm in diameter strongly

expressed LH-R and CYP17A1 mRNAs in most thecal cells [54]. Androgens are then converted to estrogens by aromatase (CYP19) produced by granulosa cells. Furthermore, progesterone is produced by granulosa cells and used by theca cells to synthesize androgens [59]. StAR, Cyp11a1 and Cyp19a1 are the key enzymes in the hormone synthesis process [60].

Granulosa cells express estradiol receptor, which contributes to follicular development [61]. Autocrine and paracrine activities of estradiol in granulosa cells stimulate aromatase enzyme activity, increasing gonadotropin sensitivity and IGF-1 [62]. In the ovary, IGF-I stimulate follicular steroidogenesis and increases estradiol production. The absence of IGF-I results in follicles stopped in the preantral/early antral stage and do not respond to gonadotropin [63,64]. In granulosa cells, the stimulatory effect of FSH on Cyp19 and AKT depends on IGF-I and on the expression and activation of IGF-IR [64]. Furthermore, FSH induces E2 production via FSHR-cAMP-dependent signaling to induce transcription of the CYP19A1 gene [65]. After follicle recruitment, gonadotropins gradually reduce granulosa cell proliferation and induce their differentiation to produces estradiol [66].

Figure 3. Influence of FSH, LH and E2 on granulosa cells to promote proliferation and production of enzymes involved in steroidogenesis**.**



5. Follicle atresia during development from early antral follicles

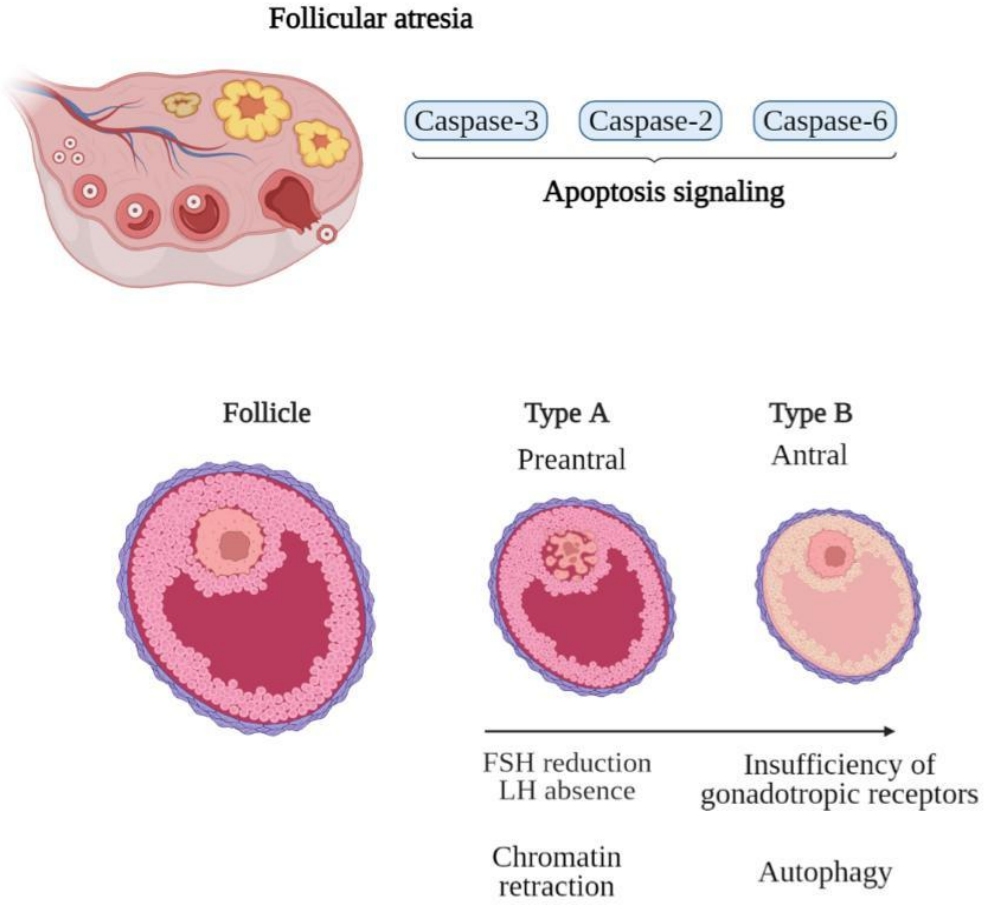
Spanel-Borowski *et al*. [67] reported two types of atretic patterns in ovarian follicles, namely type A, in which the oocyte degenerates while granulosa cells remain intact, and type B in which the granulosa cells show signs of extensive degeneration while the oocyte remains initially unaffected. These authors further showed that type A is the predominant form of atresia in preantral follicles, while in late antral follicles only type B is observed, being apoptosis of granulosa cells in the presence of a more or less intact oocyte, characteristic of atresia in large antral follicles [68]. In early antral follicles, the first changes that indicate atresia occur in the oocyte, such as nuclear chromatin retraction and oocyte fragmentation, while changes are rarely found in the granulosa cells present in these follicles [68].

Follicular atresia can occur through necrosis, necroptosis, autophagy and apoptosis pathways when the paracrine or endocrine environment is not suitable to support the growth and/or differentiation of follicular cells [69]. The necrosis and necroptosis pathway have similar morphological characteristics and are characterized by an increase in cell volume, permeabilization and rupture of the plasma membrane, which lead to cell death [69]. Generally, necrosis is initiated by non-cellular mechanisms such as ischemia, deficiency in ATP levels, and trauma, leading to irreversible cell damage [69]. Necroptosis is initiated by tumor necrosis factor-α (TNFα) and operated through protein kinase-1 and 3, which interact with its receptors RIPK1 and RIPK3, respectively, as well as by the domain-like protein of mixed lineage kinase (MLKL) [69]. Zhou *et al*. [70] showed that the process of autophagy is involved with atresia in secondary and early antral follicles. Autophagy is an evolutionarily conserved form of intracellular process that involves damaged proteins and organelles for degradation and recycling.

It is believed that granulosa cell apoptosis in late antral follicles is triggered by insufficient FSH levels or reduced numbers of FSH receptors [71]. The absence of LH and the decline of circulating FSH cause the subordinate follicles to decrease their growth and eventually, result in atresia [72] (Figure 4). FSH protects granulosa cells from oxidative damage and rescues granulosa cells from apoptosis. FSH is thought to rescue granulosa cells of antral follicles from apoptosis via

activation of the phosphatidylinositol 3 kinase (PI3K)–AKT signal transduction pathway. Activation of PI3K- KT via binding of FSH to its receptor leads to phosphoryla box O (FOXO) subfamily of forkhead transcription factors which influences, among other processes, survival of granulosa cells [73].

Figure 4. Follicular atresia patterns via oocyte degeneration and granulosa cell degeneration: Implicating factors during the process of autophagy and GCs retraction.



6. In vitro development of early antral follicles

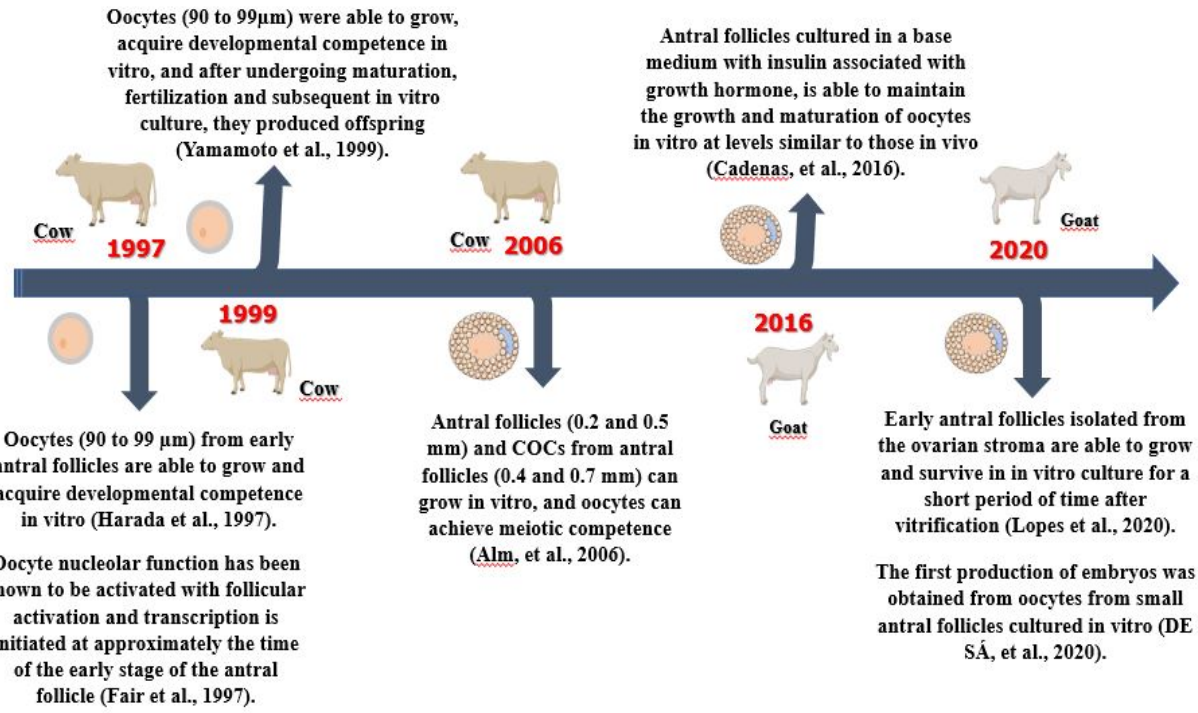
The culture of isolated early antral follicles may represent a promising alternative for the supply of competent oocytes for in vitro maturation protocols [74]. When comparing 2D and 3D culture systems for small antral follicles, He *et al*. [74] suggested that the 2D system is more suitable for culture lasting up to 4 days, while in culture periods longer than 4 days, the 3D system is more suitable.

Several studies have investigated the relationship between follicular and oocyte size with the acquisition of oocyte developmental competence in vitro, and many studies have focused on the development of culture protocols that can support the development of oocytes from small antral follicles (Figure 5). Harada, *et al*. [75] demonstrated for the first time that oocytes of 90 to 99 µm, from bovine early antral follicles (0.5 to 0.7 mm), can grow and acquire developmental competence in vitro, in the presence of hypoxanthine and FSH. Likewise, Yamamoto *et al.* [76] demonstrated that, in addition to being able to grow and acquire developmental competence in vitro, the oocytes (90 to 99µm) from bovine small follicles were able to produce offspring after undergoing maturation, fertilization and subsequent in vitro culture.

As demonstrated by Alm, *et al*. [77], antral follicles with a diameter between 0.2 and 0.5 mm, as well as COCs from bovine antral follicles (0.4 and 0.7 mm) can grow during in vitro culture, and oocytes can reach meiotic competence.

In goat species, in addition to obtaining an improvement in in vitro oocyte maturation, embryo production was reported from oocytes of small antral follicles cultured in vitro subjected to in vitro maturation followed by parthenogenetic activation or in vitro fertilization [78]. CADENAS *et al*. [79] found that early antral follicles of goats cultured in medium containing insulin (10 ng/mL) associated with growth hormone (50 ng/mL) are able to maintain the growth and maturation of oocytes in vitro at levels similar to those of their in vivo counterparts. Likewise, when observing the effect of stimulation of recombinant human FSH (hrFSH) in early antral follicles of goats, hrFSH improved the development of antral follicles in a concentration-dependent manner [80]. LOPES *et al.* [81] also demonstrated that early antral follicles isolated from goat ovarian stroma are able to grow and survive in vitro for a short period of time, after undergoing a vitrification process. However, Katska *et al*. [82] reported oocyte degeneration after culturing small antral follicles for 14 days, suggesting that the culture of oocytes isolated from initial antral follicles lasting longer than 7 days, must be adapted to decrease metabolic energy.

Figure 5. Schematic representation of the main advances in in vitro cultivation of early antral follicles.



7. Final considerations

The development of early antral follicles up to gonadotropin dependence involves a wide range of processes, which can be decisive for follicular growth, steroidogenesis and acquisition of oocyte competence. The mutual interaction between oocyte and follicular cells directly influences follicular and oocyte fate.

In addition, the in vitro culture early antral follicles opens perspectives to use of their oocytes for in vitro fertilization and to provide a better understanding of the mechanisms involved in the control of early antral follicles.

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9. Author Contribution Statement

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

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2.4 Crescimento de folículos antrais e maturação oocitária

Durante o crescimento dos foliculos antrais, ocorre uma intensa proliferação e diferenciação das células da granulosa, e o surgimento da cavidade antral (CHU, *et al*., 2018). Fatores de crescimento oriundos do oócito estimulam a produção de receptores de hormônio foliculo estimulante (FSH) nas células da granulosa (CONTO, 2018). Com o aumento do FSH ocorre o recrutamento de folículos antrais e a seleção de um folículo dominante, que também passa a ser receptivo ao hormônio luteinizante (LH) e se desenvolver até a ovulação (GOUGEON, 1986). A medida que o FSH diminui e com a ausência de LH os demais foliculos subordinados reduzem seu crescimento, resultando em atresia (LANDRY e SIRARD, 2018).

A interação mútua entre o oócito e células foliculares é fundamental tanto para o progresso folicular até a ovulação, como para a aquisição da competência e maturação oocitária (CHU, *et al*., 2018; ALAM e MIYANNO, 2020). Enquanto o oócito favorece o desenvolvimento folicular através do fornecimento de fatores de crescimento, as células foliculares fornecem um ambiente metabólico e hormonal favorável para a nutrição e maturação do oócito (BAUMGARTEN e STOCCO, 2018).

A maturação oocitária, compreende o conjunto de processos que permitem com que o oócito adquira competência para expressar seu potencial máximo de desenvolvimento após a fecundação e representa uma das fases mais importantes da produção *in vitro* de embriões (PIV), pois é o período em que o oócito alcança a capacidade para suportar os próximos eventos (GOTTARDI e MINGOTI, 2010). Essa competência oocitária é adquirida ao longo do crescimento folicular e está relacionada com modificações nucleares e citoplasmáticas ocorridas durante todas as etapas da maturação até o final do desenvolvimento oocitário, ou seja, refere-se à capacidade de completar a metáfase II (PEREIRA *et al*., 2016).

A maturação nuclear é caracterizada pelo processo de segregação cromossômica, envolvendo modificações estruturais e recombinação genética (CROCOMO *et al*., 2019). Em bovinos tem duração de 24 horas e inclui duas divisões consecutivas (fases M) na ausência de replicação do DNA (fase S) (HURK e ZHAO, 2005).

Os oócitos iniciam a meiose ainda durante a fase fetal. Em bovinos, ocorre por volta do dia 82 de gestação (GOESEELS, 2012). No entanto, os oócitos ficam bloqueados no estágio de diplóteno da prófase I, por ação de fatores inibidores da maturação, permanecendo assim até a puberdade, em que receberão estímulo das gonadotrofinas (CROCOMO *et al*., 2019).

Em consequência da influência do LH, acontece a ruptura da vesícula germinativa e a retomada da meiose, que inicia com a formação do primeiro fuso meiótico, e é concluída com a expulsão do primeiro corpúsculo polar (DANG DO *et al.*, 2018). Ao retomar a meiose, o oócito progride da prófase I, através da metáfase I, anáfase I, e telófase I, até a metáfase II

(MII) (ADONA *et al*.,2020). Os oócitos permanecem presos nessa fase até receberem um estímulo de ativação fornecido pela penetração do espermatozoide, desencadeando a conclusão do ciclo meiótico e início do desenvolvimento embrionário (VAN DEN HURK e ZHAO, 2005). A maturação citoplasmática pode ser caracterizada como um conjunto de processos necessários para que a célula se torne apta a ser fecundada e oferecer suporte ao desenvolvimento embrionário inicial (CHAVES *et al.*,2010). Ela envolve a redistribuição de organelas citoplasmáticas, o acúmulo de RNA mensageiro (RNAm), proteínas e fatores de transcrição necessárias para que ocorra a maturação oocitária. (FERREIRA *et al*., 2009).

Durante o desenvolvimento do oócito ocorre uma complexa organização citoplasmática, incluindo o aumento da reserva lipídica, redução do complexo de golgi redistribuição das organelas celulares, com a migração das mitocôndrias para a posição perinuclear e dos grânulos corticais para a periferia citoplasmática (PICTON *et al*.,1998; DIELEMAN *et al*., 2002). Essas mudanças ocorridas durante a maturação citoplasmática são fundamentais para que o oócito obtenha condições para o bloqueio da polispermia, assim como também, para descondensar o núcleo do espermatozoide e formar o pró-núcleo masculino após a fecundação (CHAVES *et al.*, 2010). Além disso, a transcrição de proteínas e o seu armazenamento no citoplasma durante essa fase é essencial para garantir o progresso inicial do embrião até o estágio de oito células (bovinos), momento em que ocorre a ativação do genoma e, possibilitando a expressão de genes determinantes no sucesso da embriogênese na pré- implantação (FERREIRA *et al.*, 2009).

Durante o crescimento do oócito ocorre um estoque de mRNAs e proteínas que impulsionam o desenvolvimento inicial dos embriões em bovinos (FAIR *et al*., 2007; REYES e ROSS, 2016), formando um grande estoque materno. Essa transcrição de RNAm ocorre enquanto o núcleo encontra-se em quiescência meiótica, e cessa quando ocorre a retomada da meiose, assim que cromossomos se condessam ficando “inativos” (CROCOMO *et al.*, 2011).

Bezerra *et al*. (2019) observaram alterações nos níveis de RNAm para o fator de crescimento e diferenciação-9 (GDF-9), Fator de maturação de oócitos (cMOS), ribonuclease específica poli (a) (PARN), fator de iniciação da tradução (eIF4E), Ciclina B1 (CCNB1) e histona com ligante específico para oócito (H1FOO) durante o crescimento de folículos secundários bovinos até folículos antrais pequenos, médios e grandes (Figura 3). MAMO *et al*. (2011) também identificaram um grande número de diferentes genes em oócitos bovinos, dentre os quais 75%foram mais expressos em oócitos imaturos.

Dentre os transcritos mencionados o GDF-9 possui um papel regulador no desenvolvimento oocitário (DONG *et al*., 1996), estimulando o crescimento de folículos pré-

antais, a proliferação das células da granulosa (SPICER *et al*., 2006), bem como, induzindo a transição de folículos primários para secundários(SOUSA *et al*., 2008) e a formação da cavidade antral (VASCONCELOS *et al*., 2013), permitindo a manutenção da integridade folicular.

Outra importante proteína estocada pelo oócito, é a ciclina B, que juntamente com Cdc 2 quinase formam o fator promotor de maturação (MPF), que é responsável pela regulação dos ciclos celulares de meiose e mitose (BILODEAU-GOESEELS, 2012). O c-MOS também desempenha um papel essencial no controle da divisão celular meiótica em mamíferos, atuando através de proteínas quinases ativadas por mitógenos (MAPKs) e regulando a sobrevivência ou apoptose das células (JALOCHA *et al*., 2010). Além desses transcritos estocados no oócito, a histona H1FOO também já foi identificada no núcleo oocitário bovino em concentrações mais altas no estágio de vesícula germinativa, diminuindo gradualmente durante o desenvolvimento embrionário, e pode estar envolvida com a condensação da cromatina e ativação ou repressão de genes (MCGRAW *et al.*, 2006).

Embora já se tenha conhecimento acerca desses transcritos estocados pelos oócitos durante a maturação, os mecanismos envolvidos no controle do desenvolvimento de folículos antrais e maturação oocitária ainda não são bem elucidados. Assim, o cultivo folicular *in vitro* para se melhor compreender a foliculogênese e a oogênese. No entanto, o ambiente *in vitro* pode favorecer um aumento das espécies reativas de oxigênio (EROs), induzido o estresse oxidativo e consequentemente a atresia celular (OTALA *et al*., 2002).

2.6. Estresse oxidativo durante o cultivo folicular *in vitro*

As espécies reativas são átomos, moléculas ou íons derivados do oxigênio, que possuem pelo menos um elétron desemparelhado em seus orbitais externos, altamente reativos. Elas constituem três classes; espécies reativas de oxigênio (EROs), espécies reativas de enxofre (EREs) e espécies reativas de nitrogênio (ERNs) (MARTELLI e NUNES, 2014).

As EROs possuem maior relevância, pois são produzidas naturalmente pelo metabolismo fisiológico, e quando estão em baixa concentração,desempenham um importante papel na competência dos oócitos (GUÉRIN *et al*., 2001). No entanto, seu excesso apresenta efeitos prejudiciais, tais como a peroxidação dos lipídios de membrana e agressão às proteínas dos tecidos e das membranas, àsenzimas, aos carboidratos e ao DNA (BARREIRO *et al.*, 2006).

Durante o cultivo *in vitro*, as concentrações de EROs aumentam devido as altas concentrações de oxigênio (O2), consideravelmente maiores em comparação as condições *in vivo*, e pela a exposição a luz e a ausência da proteção antioxidantes materna (ALVES *et al*., 2019, SADEESH *et al*., 2014), levando ao acúmulo intracelular de EROs, e consequentemente, induzindo o estresse oxidativo (EO). Esse desequilíbrio intracelular das EROs tem sido relatado como um dos principais limitantes do êxito do cultivo *in vitro*, pois seu excesso nas células da

granulosa resulta em apoptose, e consequentemente atresia folicular (SAEED-ZIDANE *et al*., 2017).

Em condições normais as células possuem dois sistemas de proteção antioxidante para manter o equilíbrio das EROs; o sistema antioxidante enzimático e não enzimático. O sistema enzimático inclui enzimas superóxido dismutase (SOD), catalase (CAT), peroxirredoxinas e o sistema glutationa redutase/peroxidase (GPX) (BARBOSA *et al*., 2010). Essas enzimas atuam controlando a formação dos radicais livres, e a perfeita interação entre elas é importante para a manutenção da integridade celular (SCHNEIDER e OLIVEIRA, 2014). Já o sistema antioxidante não enzimático, inclui compostos de baixo peso molecular como o ácido ascórbico, tocoferol, selênio, zinco, taurinas, hipotaurinas, caroteno, ácido lipóico e outros compostos tióis como: cistina, cisteína, cisteamina e beta-mercaptoetanol (CROCOMO *et al.,* 2012)**.**

Devido à redução dessa proteção antioxidante no cultivo *in vitro*, antioxidantes como a transferrina, selênio e ácido ascórbico tem sido frequentemente adicionado em meios de cultivo, no entanto, outras substâncias que apresenta em potencial antioxidante, vem sendo testadas (LINS *et al*., 2017).

2.7 N-acetilcisteína

A N-acetilcisteína (NAC) é um composto antioxidante amplamente utilizado para diminuir o estresse oxidativo causado pelas espécies reativas de oxigênio (EROs). Ela é formada pelo aminoácido L-cisteína ($C_3H_7NO_2S$) com a adição de um grupo acetil ($-CO-CH_3$) (ONDANI *et al*., 2011). Seu potencial antioxidante pode ocorrer de forma indireta, através da regulação dos níveis de glutationa (GSH) que é um dos principais antioxidantes endógenos. Em alguns casos, os efeitos da NAC podem ocorrer de forma direta devido a capacidade do grupo tiol livre (-SH) de interagir diretamente com os grupos eletrofílicos dos radicais oxidantes (ALDINI *et al.*, 2018).

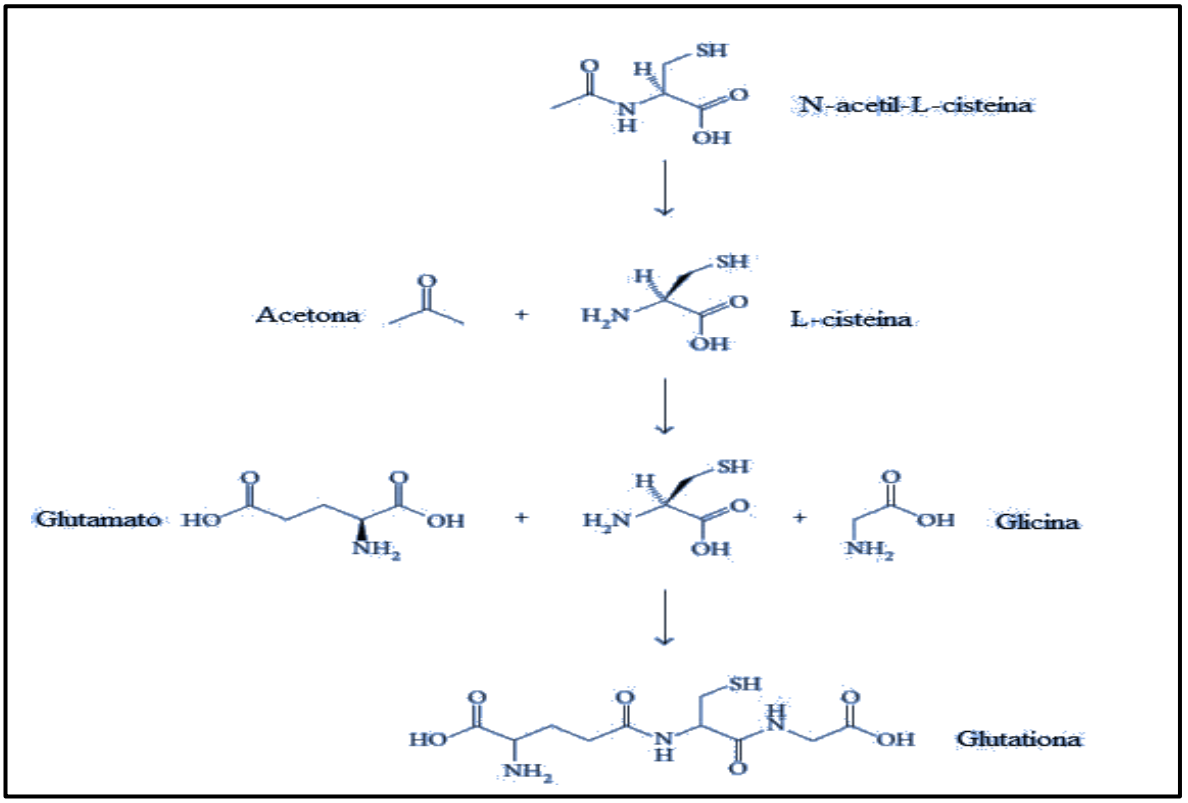
Os benefícios da NAC durante o cultivo *in vitro* têm sido relacionados principalmente ao fornecimento de cisteína necessária para a síntese e reposição de glutationa (GSH) (ATKURI *et al.*, 2007). Quimicamente a NAC assemelha-se a cisteína, no entanto a porção acetil reduz a atividade do tiol, tornando-a menos tóxica, menos suscetível a oxidação e mais solúvel em água (BORGSTRÖM *et al*., 1986; ATKURI *et al.,* 2007), consequentemente, sua estrutura molecular permite que ela penetre facilmente na membrana celular e possa ser desacetilada em L-cisteína, a qual combinada com o glutamato e glicina formarão a GSH no interior das células (Figura 4) (GRINBERG *et al*, 2005).

Neste sentido a NAC apresenta grande importância para o fornecimento de GSH

intracelular. Oócitos e embriões precoces por exemplo, só podem sintetizar uma quantidade limitada de GSH, sendo necessário a absorção de tióis pelas células do cumulus para a síntese de GSH (LI *et al*, 2018; SUN *et al*, 2021). Sun *et al*, (2021) demonstraram que a suplementação com NAC durante o cultivo *in vitro* de oócitos e embriões bovinos, diminuiu significativamente a proporção de blastômeros apoptóticos, resultando em maior capacidade de incubação e taxas de desenvolvimento.

Liu *et al.* (2012) demonstraram que a NAC melhora a qualidade de oócitos de camundongos fertilizados e o desenvolvimento inicial de embriões, além de aumentar a atividade dos telômeros. Em oócitos suínos, a presença de 1,5 mM de NAC no meio de maturação, aumentou a percentagem de embriões viáveis que atingem o estágio de desenvolvimento dos blastocistos (WHITAKER e KNIGHT, 2010). No tecido ovariano de ratas, também foi relatado que a administração de NAC melhora os efeitos deletérios da acrilamida (composto tóxico e cancerígeno) de maneira dependente da dose, melhorando a foliculogênese e reduzindo o nível de apoptose (NAIMI *et al*., 2020).

Figura 3: Fórmula química da N-acetilcisteína e sua conversão em glutationa.



Fonte: PEI *et al.*, 2018.

Em um trabalho realizado com mulheres portadoras da síndrome dos ovários policístos (SOP), as quais foram tratadas com NAC, houve uma redução do número de oócitos imaturos

e com anormalidades morfológicas. Essa melhora na qualidade dos oócitos foi relacionada principalmente ao forte efeito antioxidante da NAC, o qual também modula a expressão de c-Kit e GDF-9 aumentando as taxas de maturação oocitária (CHERAGHI *et al.*, 2018).

3. JUSTIFICATIVA

A escolha do modelo experimental justifica-se pelo fato de que a produção pecuária bovina, exerce grande impacto no setor do agronegócio no Brasil, proporcionando geração de emprego e aumento da renda no país (MOREIRA *et al*., 2019; SOARES *et al*.,2019). Dessa forma, estudos que propiciem uma melhor compreensão dos mecanismos que envolvem a foliculogênese ovariana *in vitro* pode contribuir para a multiplicação de animais geneticamente superiores, favorecendo o desenvolvimento da economia nacional.

A melhoria dos meios de cultivo *in vitro* por meio da utilização de substâncias com atividade antioxidante pode amenizar ou prevenir os danos causados pelo estresse oxidativo durante o cultivo de folículos ovarianos. A investigação do desenvolvimento de folículos antrais iniciais *in vitro*, é de grande relevância para favorecer o entendimento da foliculogênese trazendo novas perspectivas para o resgate de oócitos aptos a serem ultilizados em protocolos de maturação *in vitro*. A NAC tem grande potencial para melhorar o cultivo de folículos antrais iniciais bovinos, tendo em vista que essa substância tem reduzido o estresse oxidativo durante o cutivo de tecido ovariano (FABBRI *et al*., 2015; LI *et al*., 2019). Além disso, a maioria dos trabalhos envolvendo o cultivo folicular *in vitro* tem se limitado a folículos secundarios, havendo poucos estudos envolvendo o cultivo *in vitro* de folículos antrais iniciais em bovinos (ARAÚJO *et al*., 2015). Estes folículos possuem morfologia bem desenvolvida e consequentemente podem apresenta melhor aptidão para suportar o cultivo *in vitro* (HE *et al*., 2020).

4. HIPÓTESES

- A NAC favorece o crescimento e a manutenção da morfologia folicular de folículos antrais iniciais bovinos durante o cultivo *in vitro*.

- A NAC promove proteção antioxidante e reduz os níveis de EROs em COCs de foliculos antrais iniciais cultivados *in vitro*.

- A NAC melhora a viabilidade oocitária e parada meiótica oócitos de foliculos antrais iniciais bovinos cultivados *in vitro*.

5. OBJETIVOS

5.1. Objetivo Geral

Avaliar os efeitos de diferentes concentrações de NAC durante o cultivo *in vitro* de folículos antrais iniciais (~500 μm).

5.2. Objetivos específicos

- Avaliar os efeitos da adição de NAC sobre o crescimento e morfologia de foliculos antrais iniciais bovinos.
- Analisar os efeitos da NAC sobre os níveis intracelulares de EROs de oócitos de foliculos antrais iniciais bovinos cultivados *in vitro*.
- Analisar os efeitos da NAC sobre a viabilidade oocitaria e progreção meiótica de foliculos antrais inciais cultivado *in vitro*.

ARTIGO 2

Effects of N-acetylcysteine on growth, viability and levels of reactive oxygen species in small antral follicles cultured in vitro

Effects of N-acetylcysteine on growth, viability and levels of reactive oxygen species in small antral follicles cultured in vitro

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ABSTRACT

This study investigates the effects of different concentrations of N-acetylcysteine (NAC) on follicular growth and morphology as well as on the viability, reactive oxygen species (ROS) levels and meiotic progression of oocytes from in vitro cultured bovine early antral follicles. To this end, isolated early antral follicles (~500 μm) were cultured in TCM-199+ alone or supplemented with 1.0, 5.0 or 25.0mM NAC at 38.5°C with 5% CO2 for 8 days. Follicle diameters, were evaluated at days 0, 4 and 8 of culture. At the end of culture, the levels of ROS, chromatin configuration and viability (calcein-AM and ethidium homodimer-1 staining) were investigated in the COCs. Comparisons of follicle diameters between treatments were performed using the Tukey test. Data on percentages of morphologically normal follicles, growth rates and chromatin configuration were compared using Fisher's exact test ($P < 0.05$). The results showed an increase in follicular diameters after culture in all treatments, except for follicles cultured with 25mM NAC. Fluorescence microscopy showed that oocytes cultured in all treatments stained positively with calcein-AM, and that 5.0 mM reduced fluorescence for the ethidium homodimer-1. Intracellular levels of ROS in oocytes from follicles cultured with 1.0mM NAC showed a significant reduction compared to the other treatments. The presence of NAC in culture medium did not influence the rates of oocyte at the germinal vesicle stage. In conclusion, NAC at concentrations of 1.0 and

5.0nM reduces ROS levels and staining for the ethidium homodimer-1, respectively, but 25mM NAC reduces follicular growth and the percentages of continuously growing follicles.

Keywords: Antioxidant. Bovine. ROS. Antral follicles.

1. Introduction

The growth of preantral and early antral follicle is a key step of follicular development associated with intense transcriptional activity and acquisition of oocyte competence [1]. Some in vitro studies report that oocytes from small antral follicles, between 1 and 2 mm, have significantly reduced competence when compared to those from larger antral follicles (>3 mm), which have the ability to complete nuclear maturation [2,3]. Several molecular events and the bidirectional communication between the oocyte and surrounding granulosa cells trough transzonal projections (TZPs) coordinate the development of early antral follicles up to ovulation [4].

In the last decades, in vitro culture of ovarian follicles has contributed for a better understanding of the roles of hormones and growth factor during follicular development [5,6], but in vitro growth of follicles up to maturation was still not reported for human and domestic animals [7,6]. In domestic animals, the in vitro development of secondary follicles during 18 days of culture is generally associated with increased degeneration rate at early antral follicle stages [8]. Under normal physiological conditions, the cells produce variable levels of ROS, but their excess can cause oxidative stress and compromise cell development [9,10]. This is one of the main factors associated with the low quality of in vitro cultured ovarian follicles [6,11]. Therefore, adding substances with antioxidant activity in the culture medium can prevent or mitigate these damages [12].

N-Acetylcysteine (NAC) is a precursor of cysteine and reduced glutathione that has an important role in the control of oxidative stress [13]. Fabbri *et al.* [14] reported that NAC and FSH improved preantral follicle growth and viability in cultured ovarian tissues. Furthermore, NAC

increased the content of GSH and reduced ROS levels in mice COCs matured in vitro [15]. In porcine COCs, NAC also reduced the levels of ROS, and increased cumulus cell expansion during in vitro maturation [16]. However, the effects of NAC during the in vitro growth of bovine early antral follicles are still unknown. Studying early antral follicle growth in vitro can contribute for the elucidation of the mechanisms that regulate their growth, and consequently enable the use of their oocytes in in vitro maturation protocols.

This study aims to investigate the effects of different concentrations of NAC on the growth and morphology of early antral follicles and on the viability, ROS levels and meiotic progression of oocytes from small antral follicles cultured in vitro.

2. Materials and methods

2.1. Source of ovaries

Ovaries (n=150) from adult cows were collected from a local slaughterhouse immediately after slaughter. The ovaries were washed in 70% ethanol, followed by two rinses in TCM-199 buffered with HEPES and supplemented with penicillin (100IU) and streptomycin (0.1mg/mL). The ovaries were transported within 1h to the laboratory in TCM-199 at 4°C.

2.2. Follicle isolation and in vitro culture

In the laboratory, the ovarian cortex (1–2 mm) was fragmented with a sterile scalpel blade and placed in TCM-199 medium supplemented with Hepes. Antral follicles of approximately 500μm in diameter were manually dissected from the strips of cortical tissues 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 the presence of an antral cavity were selected for culture. The follicles were individually cultured in 150μL of culture medium under mineral oil in Petri dishes (60 × 15mm, Corning, USA). The control culture medium, called TCM-199^{+}, consisted of TCM-199 (pH 7.2-7.4) supplemented with FSH (50

μg/mL), insulin (50 μg/mL), transferrin (50 μg/mL) and selenium (50 μg/mL) (ITS), 0.015 mg/mL of bovine serum albumin (BSA), penicillin (100IU), streptomycin (0.1mg/mL), glutamine (50 μg/mL), and hypoxanthine (50 μg/mL) (HE *et al*. 2020; ALM, *et al*. 2006). For the treatments, the follicles were randomly cultured in TCM-199^{+} alone or supplemented with 1.0, 5.0 or 25.0mM NAC [17,18]. Follicles were cultured at 38.5° with 5% CO2 in air for 8 days (HE *et al*. 2020). At day four of culture, 75 μL of medium was replaced by fresh medium.

2.3. Assessment of follicular morphology and growth

The morphological evaluation of follicles was performed with the aid of a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan) at days 0, 4 and 8. Morphologically normal, follicles had spherical oocyte surrounded by homogeneous granulosa cells, intact basement membrane and external stromal-thecal layer. Follicles with dark oocytes and cumulus cells were considered degenerated. In addition, perpendicular measurements were performed on normal follicles at days 0, 4 and 8.

2.4. Assessment of oocyte viability by fluorescence microscopy

After culture, COCs were extruded with the aid of 25 G needles and incubated with 100 μL of TCM-199 containing 4 mM calcein-AM and 2 mM ethidium-1 homodimer (Molecular Probes, Invitrogen, Karlsruhe, Germany) at 37°C for 15 min. Then, they were washed three times in TCM-199 and examined under fluorescence microscope (Nikon, Eclipse, TS 100., Japan). Oocytes and cumulus cells were considered viable if cytoplasm was stained positively with calcein-AM (green) and the chromatin not labeled with ethidium homodimer-1 (red; VAN DEN HURK *et al*., 1998).

2.5. Evaluation of ROS levels in COCs from cultured follicles

Oocytes were washed in 0.1% polyvinyl alcohol in phosphate-buffered saline (PBS-PVA) and incubated with 6-carboxy-2,7-dichlorodihydrofluorescein diacetate (H2DCFDA, Molecular Probes®, Eugene, OR) at 38.5°C for 30 min, in the dark. Then, oocytes were washed with PBS-

PVA and placed on glass slides with ProLong® Gold (Molecular Probes, Eugene, OR). The slides were evaluated under an epifluorescence microscope (Nikon, TS100) at a wavelength of 460 nm. The fluorescence intensity of ROS saturation was analyzed individually using Image J software (version 1.46; National Institutes of Health, Bethesda, MD). The relative fluorescence intensity was considered directly proportional to the ROS concentration**.**

2.6 Evaluation of chromatin configuration

To assess chromatin, cumulus cells were removed by vortexing and the oocytes fixed in 4% paraformaldehyde for 15 minutes and transferred to 0.1% Triton X-100. The chromatin configuration was evaluated after adding 10 μg/mL of Hoechst 33342 on an inverted epifluorescence microscope (Nikon, TS100). Oocytes were classified according to the stages of nuclear maturation, i.e., as germinal vesicle (GV), germinal vesicle breakdown (GVBD) or degenerated.

2.9. Statistical analysis

Statistical analyses were performed using GraphPad Prism software, version 9.0. Data of follicular diameter and the levels of ROS, calcein and ethidium homodimer-1 staining were initially subjected to normal distribution analysis using D'Agostinho & Pearson Test. Comparisons between treatments were performed by ANOVA and Tukey test. Data of percentages of morphologically normal follicles, growth rates and chromatin configuration were compared using Fisher's exact test. Differences were considered significant when $P < 0.05$.

3. Results

3.1. Effects of NAC on follicular morphology and growth

A progressive and significant increase in follicular diameters was observed with the increase of culture period from 0 to 4 and 8 days in all treatments, except for follicles cultured with 25.0mM NAC that did not have significant growth between days 4 and 8 (Table 1). After 8 days of culture, follicles cultured with 25mM NAC had significantly lower diameters than those cultured in other

treatments (Table 1).

Table 1. Diameters (mean ± SEM) of early antral follicles after 0, 4 and 8 days of culture in TCM-199^{+} alone or supplemented with different concentrations of NAC.

Treatment	Day0	Day4	Day8
TCM-199^{+}	534.23±47.25 ^{Aa}	634.68±86.04 ^{Ab}	711.69±89.69 ^{Ac}
NAC 1.0 mM	531.08±69.70 ^{Aa}	620.76±81.63 ^{Ab}	687.45±77.95 ^{ABc}
NAC 5.0 mM	530.87±68.42 ^{Aa}	625.89±80.90 ^{Ab}	696.63±82.92 ^{ABc}
NAC 25.0 mM	529.62±60.19 ^{Aa}	609.50±76.44 ^{Ab}	653.98±83.79 ^{Bb}

Lowercase letters (a, b, c) represent statistically significant differences between culture days ($P < 0.05$). Capital letters (A, B, C) represent statistically significant differences between treatments ($P < 0.05$).

Table 2 shows follicular growth in the different periods of culture (from day 0 to day 4 and from day 4 to day 8). Different from follicles cultured with 1.0 or 5.0mM NAC, 25.0mM NAC a significantly reduced follicular growth rate between the periods of 4 and 8 days of culture (Table 2).

Table 2. Follicular growth (mean ± SEM) in the different periods (days 0 to 4 or days 4 to 8) of follicle cultured in TCM-199^{+} alone or supplemented with different concentrations of NAC.

Follicular growth at different intervals (four days) of culture		
Treatment	**Day 0-4**	**Day 4-8**
TCM-199^{+}	100.44 ± 64.49	77.01±58.80
NAC 1.0 mM	89.21±56.36	66.46±50.64
NAC 5.0 mM	94.38±53.47	70.54±58.40
NAC 25.0 mM	79.87±45.37	62.17±32.52*

*Significant difference between culture intervals (D0-D4 and D4-D8) ($P < 0.05$).

The presence of 25mM NAC in culture medium significantly reduced percentage of growing follicles after 4 and 8 days, when compared to other treatments. In presence of 25.0mM NAC, the percentages of growing follicles were reduced, when compared with growing follicles in the period between days 0 and 4 (Table 3).

Table 3. Percentage of continuously growing follicles after 4 and 8 days of in vitro culture in TCM-199+ alone or supplemented with different concentrations of NAC.

Treatment	Day4	Day8
TCM-199^{+}	90.00% (90/100)^{a}	60.00% (60/100)^{a}*
NAC 1.0 mM	86.36% (95/110)^{a}	63.63% (70/110)^{a}*
NAC 5.0 mM	85.84% (91/106)^{a}	60.37% (64/106)^{a}*
NAC 25.0 mM	74.03% (77/104)^{b}	41.34% (43/104)^{b}*

Lowercase letters (a, b) represent statistically significant differences between treatments ($P < 0.05$). * Represent statistically significant differences between the culture periods (D4 and D8).

3.2 Viability of oocytes from cultured follicles

Oocytes from follicles cutured with 5.0 mM NAC showed a significant reduction in fluorescence intensity for ethidium homodimer-1 when compared to those cultured in control medium. In each treatment, significant lower fluorescence for ethidium-1 homodimer in relation to calcein-AM was observed in oocytes from follicles cultured with 5 and 25mM NAC (Figures 1 and 2). However, when comparing the fluorescence intensity of calcein-AM in oocytes from follicles cultured in the different treatments, there was no significant difference.

Figure 1. Oocytes from bovine antral follicles cultured for 8 days after staining with calcein-AM (green) and ethidium homodimer-1 (red). Oocytes from antral follicles cultured in TCM-199^{+} alone (A, E) or supplemented with 1.0 (B, F), 5.0 (C, G) or 25.0 mM NAC (D, H).

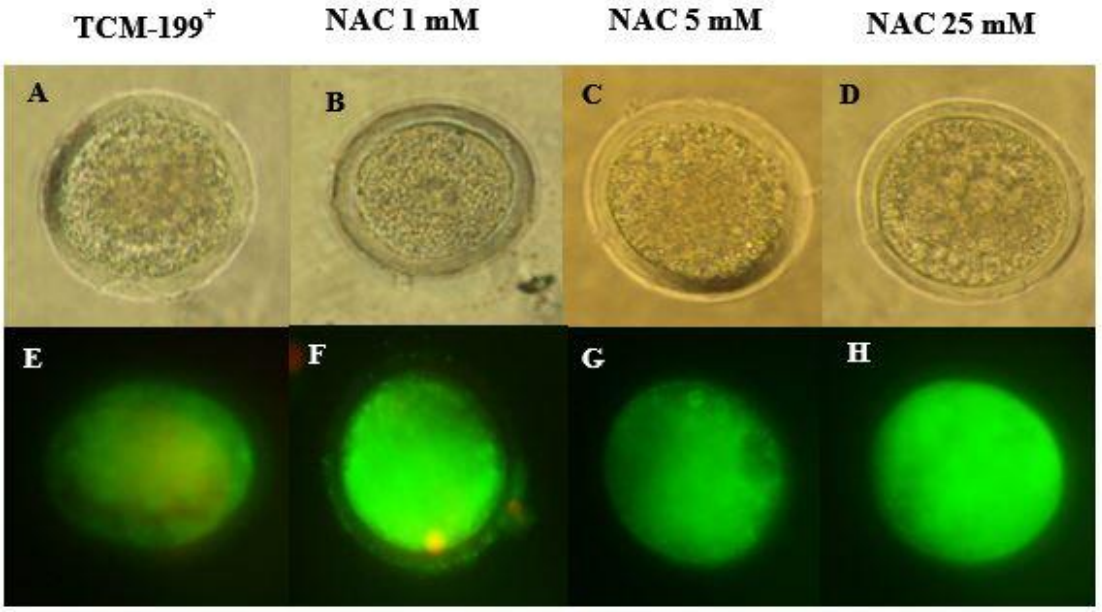
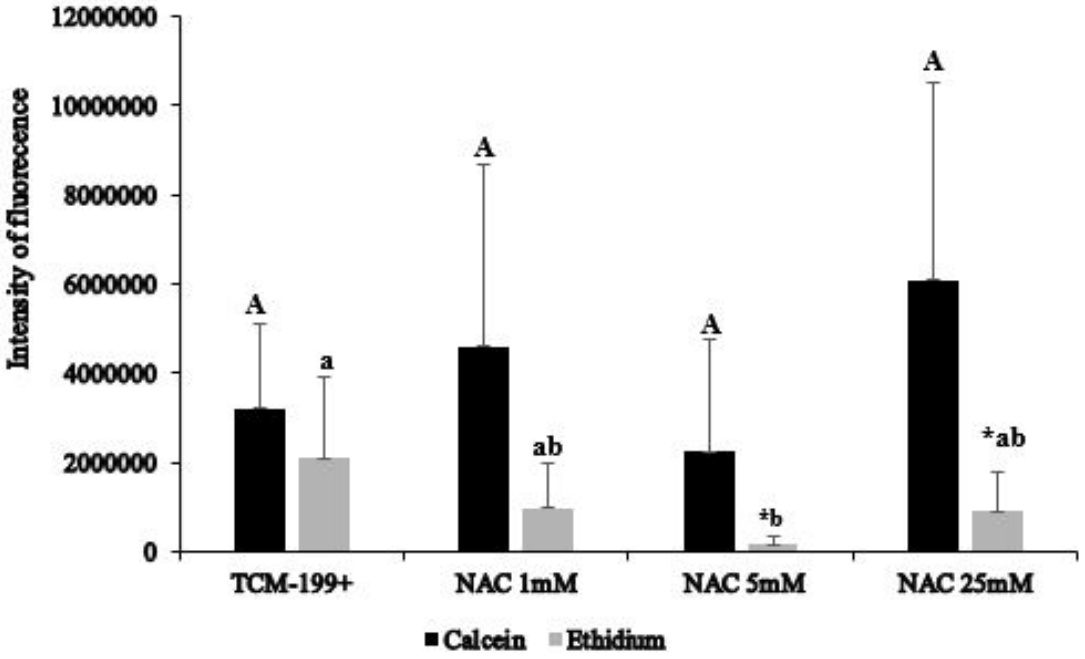


Figure 2. Fluorescence intensity staining for calcein-AM (green) and ethidium-1 homodimer (red) in oocytes from follicles cultured in TCM-199^{+} alone or supplemented with different concentrations of NAC. Capital letters (A) represent statistically significant differences for calcein - AM (black columns) between treatments ($P < 0.05$). Lower case letters (a, b) represent statistically significant differences for etidium homodimer-1 (grey columns) between treatments ($P < 0.05$). * Represents the significant difference between calcein-AM and etidium homodimer-1 within the same treatment.



3.3. Evaluation of ROS levels in oocytes from cultured follicles

Oocytes from follicles cultured with 1.0 mM NAC had significantly lower levels of ROS than those cultured in control medium alone or with 5.0 and 25.0mM NAC (Figure 3 and 4).

Figure 3 Oocytes stained with H2DCFDA for detection of ROS. (A) oocytes cultured in control medium, (B) or with 1., (C) 5.0, (D) or 25.0 mM NAC.

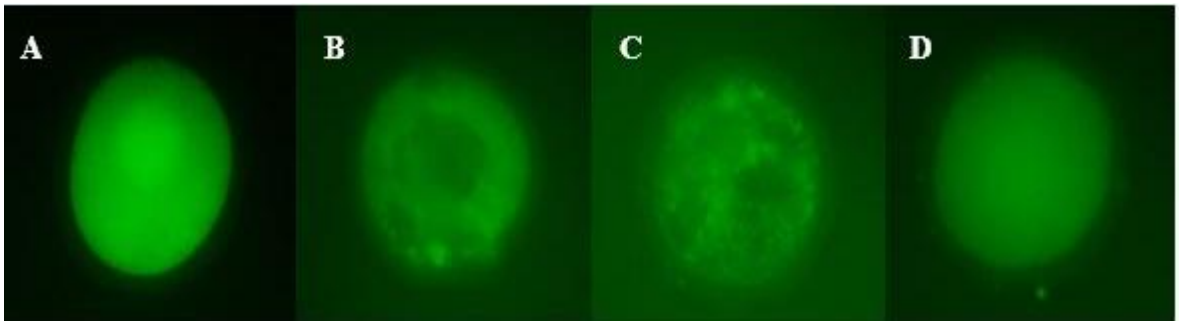
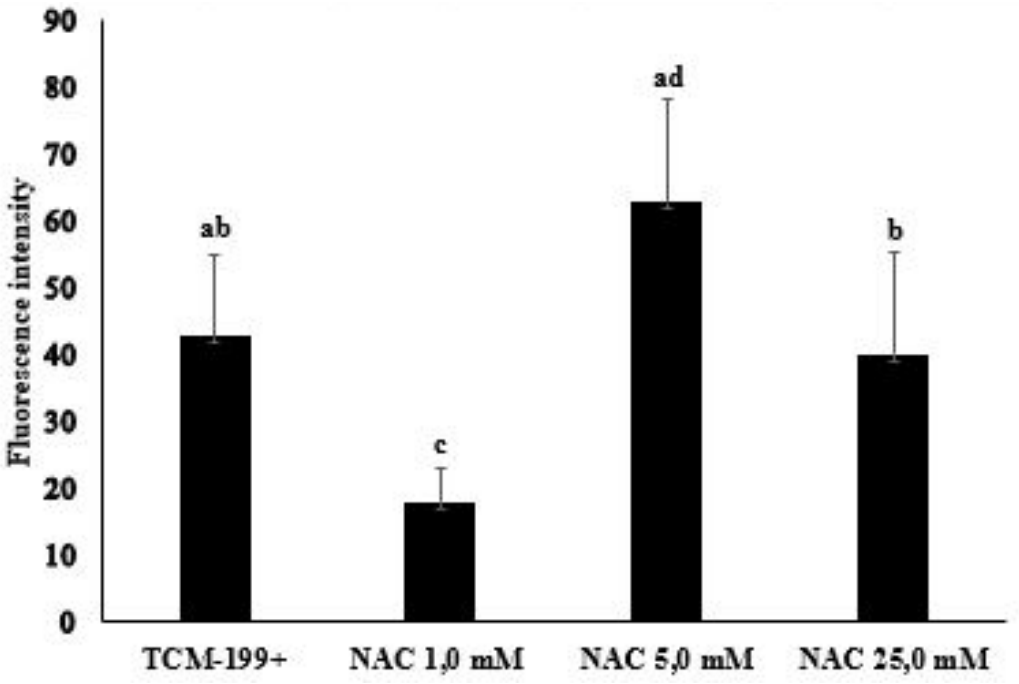


Figure 4. Fluorescence intensity for H2DCFDA (ROS) in oocytes from follicles cultured in TCM-199^{+} alone or supplemented with different concentrations of NAC. Lowercase letters (a, b)

represent significant differences between treatments.



3.4. Evaluation of chromatin configuration in oocytes from cultured antral follicles

Most of the oocytes from antral follicles cultured in TCM199^{+} alone or supplemented with different concentrations of NAC were in GV stage, being their percentage higher than those at GVBD. The percentages of oocytes with degenerated chromatin did not differ among treatments. Oocytes from follicles cultured in TCM199^{+} alone and with 5.0 mM NAC had lower percentages of oocytes at GVDB stage than at GV stage (Table 4).

Table 4. Percentages of oocytes at GV and GVBD stages and with degenerated chromatin after culture of antral follicles in TCM199^{+} alone or supplemented with different concentrations of NAC.

	Oocyte chromatin configuration			
Tratamentos	**Total (n)**	**GV (%)**	**GVBD (%)**	**DEG (%)**
TCM – 199^{+}	32	78.12%^{Aa}	15.6%^{Ab}	6.25%^{A}

		(25/32)	(5/32)	(2/32)
NAC 1.0mM	30	63.3%^{Aa} (19/30)	36.6%^{Aa} (11/30)	0%^{A} (0/30)
NAC 5.0mM	31	67.7%^{Aa} (21/31)	19.3%^{Ab} (6/31)	12.9%^{A} (4/31)
NAC 25.0mM	24	62.5%^{Aa} (15/24)	29.16%^{Aa} (7/24)	8.3%^{A} (2/24)

Capital letters (A) represent statistically significant differences between treatments ($P < 0.05$). Lowercase letters (a, b) represent statistically significant differences between GV and GVBD in the same treatment ($P < 0.05$).

4. Discussion

This study shows that 1.0mM NAC reduces the levels of ROS in oocytes from early antral follicles cultured in vitro. It is known that the accumulation of ROS is one of the limiting factors during in vitro culture, causing oxidative stress and various harmful effects to cells [19,20]. Likewise, SUN *et al*. [21] showed that 1.0 mM NAC attenuated ROS levels during in vitro oocyte maturation. In addition, many other studies have also demonstrated the antioxidant effects of NAC [22,23,24,25]. The antioxidant potential of NAC is mainly due to its ability to easily penetrate the cell membrane and be deacetylated producing cysteine, for the intracellular synthesis of glutathione [26,27].

The presence of 5.0 mM NAC in culture reduced the fluorescence intensity for ethidium homodimer-1 in oocytes, showing that NAC helps maintain oocyte membrane integrity during culture. When there is loss of integrity, the ethidium homodimer-1 can easily enter the cell, and due to the high affinity with nucleic acids, it binds to the DNA emitting red fluorescence [28], allowing to verify the physical and chemical changes in the cell membrane. In contrast, calcein can passively cross the cell membrane, and within cells it is converted by intracellular esterases to

a lipid-insoluble polar product (calcein) that is retained by cells with intact membranes, producing intense green fluorescence [29], thus allowing the verification of cell viability. In caprine granulosa cells, 5.0 and 10.0 mM NAC reduced the genotoxicity caused in response to exposure to methoxychlor [17], while in fibroblasts, 5.0 and 10.0mM NAC reduced the production of ROS and the toxicity induced by 2-hydroxyethyl methacrylate, consequently reducing cell death and restoring mitochondrial activity [30].

In our study, 25.0mM NAC reduced follicular growth and the percentages of continuously growing follicles. Recently, Sun *et al*. [21] reported that NAC at concentrations above 10.0 mM causes a reduction in the pH of the culture medium and is harmful to oocyte growth during in vitro maturation. The reduction in the pH of the medium may explain the lower follicular growth of early antral follicles cultured with 25mM NAC. Clinical studies have shown that the plasma concentration of NAC ranges from 300 to 900 mg/L, which is equivalent to 1.8–5.5 mM [31]. Furthermore, previous studies have suggested that NAC at higher concentrations may have a pro-oxidant effect. Sprong *et al* [32] showed that high doses of NAC (550 and 950 mg/kg in 48 h) in rats increase oxidative stress and toxicity induced by lipopolysaccharide (LPS), but low doses of NAC are highly effective against LPS toxicity. Morais *et al* [33] also observed that the increase in the plasma concentration of NAC, as a result of its association with dapsone, potentiated the adverse effect of depsone in rats.

Regarding to chromatin configuration, NAC did not influence the percentages of oocytes that remained in the GV stage. The experimental model used with the culture of the intact antral follicle maintains communication between the oocyte and granulosa cells, allowing the supply of follicular components to the oocyte and the conservation of high levels of cAMP, consequently preventing the spontaneous resumption of meiosis [34,35], and guaranteeing the time necessary for the oocyte to acquire the developmental competence to proceed with the following stages of its growth [36]. In addition, evidences suggest that the control of meiotic resumption or arrest can be influenced by ROS levels, and the presence of antioxidants in the culture medium is very important to inhibit the spontaneous resumption of meiosis under in vitro culture conditions [20].

5. Conclusion

In conclusion, 1.0mM NAC reduces ROS levels and at concentration of 5.0mM, NAC reduces the fluorescence intensity for ethidium homodimer-1 in oocytes from cultured follicles and helps to maintain oocyte membrane integrity, 25mM NAC, however, reduces follicular growth and the percentages of continuously growing follicles during in vitro culture of bovine early antral follicles.

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CONCLUSÕES GERAIS

Este estudo investigou a ação antioxidante da NAC durante o cultivo *in vitro* de folículos antrais iniciais bovinos. Com base nas avaliações realizadas observou-se que baixas concentrações de NAC reduz os níveis de EROs e a intensidade de fluorescência para homodímero-1 de etídeo (NAC 1,0mM e NAC 5,0mM, respectivamente). No entanto, concentrações elevadas de NAC (25mM) pode ser prejudicial para o crescimento folicular *in vitro.*

PERSPECTIVAS

A N-acetilcisteína já tem sido relatada por apresentar um vasto potencial biotecnológico em diversos estudos. Por tanto sua utilização durante o cultivo *in vitro* de folículos bovinos, abre perspectivas para o melhoramento dos meios de cultivo e o consequente sucesso dessa biotécnica reprodutiva. Os resultados obtidos nesse estudo podem contribuir com a compreensão acerca da atividade antioxidante da NAC sobre o cultivo folicular *in vitro*, no entanto, torna-se necessário novas investigações para uma compreensão mais detalhada dos mecanismos de atuação da NAC durante o cultivo de folículos antrais iniciais bovinos.

Além disso, o cultivo de folículos antrais iniciais intactos, pode contribuir com um melhor entendimento dos mecanismos da foliculogênese nessa fase folicular e uso de folículos de tamanhos diferentes em biotécnicas reprodutivas.

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