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ERNANDO IGO TEIXEIRA DE ASSIS

AVALIAÇÃO DO POTENCIAL DA *CIMICIFUGA RACEMOSA* (L.) NUTT. EM REDUZIR OS EFEITOS ADVERSOS CAUSADOS PELA DEXAMETASONA E DOXORRUBICINA EM FOLÍCULOS OVARIANOS DE CAMUNDONGAS CULTIVADOS *IN VITRO*

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

Orientador: Prof. Dr. Anderson Weiny Barbalho Silva.
Co-orientadora: Prof.ª Dra. Alana Nogueira Godinho.

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BANCA EXAMINADORA

_____

Prof. Dr. Anderson Weiny Barbalho Silva (Orientador)

Universidade Federal do Ceará (UFC)

_____

Prof.ª Dra. Alana Nogueira Godinho (Co-orientadora)

Universidade Federal do Ceará (UFC)

_____

Prof. Dr. José Roberto Viana Silva (Examinador)

Universidade Federal do Ceará (UFC)

_____

Prof.ª Dra. Ana Liza Paz Souza Batista (Examinadora)

Universidade Federal do Ceará (UFC)

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RESUMO

Considerando a *Cimicifuga racemosa* (L.) Nutt. (CIMI) como um fármaco alternativo e natural no tratamento da menopausa, bem como a importância do estudo de redirecionamento de fármacos, esta proposta tem como objetivo investigar a ação da CIMI como um potencial fator capaz de reduzir os efeitos deletérios da dexameasona (DEXA) e da doxorrubicina (DOXO). Os estudos experimentais foram aprovados pela CEUA da Universidade Federal do Ceará *Campus* Sobral sob o protocolo N◦ 05/18. Para tanto, foram utilizados camundongos Swiss fêmeas (n=47) com ciclo estral regular. Em laboratório, os ovários foram coletados e cultivados individualmente em placa de 24 poços a 37,5° C, em 5% CO2, por 6 dias. Para o experimento 1 desta dissertação foram avaliados os seguintes grupos- (i) $DMEM^{+}$; (ii) CIMI [5 ng/ml]; (iii) DOXO [0,3 μg/ml]; (iv) CIMI [5 ng/mL] + DOXO [0,3 μg/ml]. Já para o experimento 2, foram investigados os seguintes tratamentos: (i) $DMEM^{+}$; (ii) CIMI [5 ng/ml]; (iii) DEXA [4 ng/ml]; (iv) CIMI [5 ng/ml] + DEXA [4 ng/ml]. Após o cultivo, os ovários foram destinados às análises de sobrevivência, ativação, crescimento e viabilidade folicular, MEC, taxa de apoptose e densidade do estroma. Os resultados de ativação, sobrevivência e crescimento foram comparados utilizando o Teste exato de Fisher, e as diferenças foram consideradas significativas quando $p<0,05$. No experimento 1, os resultados mostraram que a presença de DOXO em meio de cultivo, reduz significativamente o percentual de folículos morfologicamente normais. Por outro lado, a presença de CIMI [5 ng/ml] sozinha é capaz de manter o percentual de folículos morfologicamente normais similar ao grupo controle ($DMEM^{+}$) e quando adicionada ao meio contendo DOXO [0,3 μg/ml]. No experimento 2, a presença de DEXA [4 ng/ml] reduz o percentual de folículos morfologicamente normais quando comparado ao grupo controle, enquanto a presença de CIMI [5 ng/ml] é capaz de manter o percentual similar ao grupo controle e ainda atenuar os efeitos deletérios causados pela presença de DEXA em meio de cultivo. No tocante a ativação e desenvolvimento folicular, os ovários quando cultivados na presença de DEXA ou DOXO reduziram o percentual de folículos primordiais comparados ao grupo controle, enquanto a presença de CIMI [5 ng/ml] sozinha, manteve o percentual de folículos primordiais igual ao grupo controle. A presença de DEXA ou DOXO afetou negativamente a densidade do estroma, enquanto a presença de CIMI [5 ng/ml] no meio de cultivo protegeu contra os danos causados por estes fármacos. Além disso, a presença de DEXA ou DOXO aumentou a taxa de apoptose, enquanto a CIMI [5 ng/ml] no meio de cultivo reduziu a apoptose. Diante do exposto, podemos concluir que o extrato de CIMI [5 ng/ml] foi capaz de conter os efeitos deletérios causados por DEXA e DOXO ao final de 6 dias de cultivo.

Palavras-chave: foliculogênese; toxicidade; camundongas; fitomedicamento; glicocorticóide; cultivo *in vitro*.

ABSTRACT

Considering *Cimicifuga racemosa* (L.) Nutt. (CIMI) as an alternative and natural drug in the treatment of menopause, as well as the importance of the study of drug redirection, this study aims to investigate the action of CIMI as a potential factor capable of reducing the deleterious effects of dexameasone (DEXA) and doxorubicin (DOXO). The experimental studies were approved by CEUA of the Federal University of Ceará Campus Sobral under protocol N◦ 05/18. For that, Swiss female mice (n = 47) with regular estrous cycle were used. In the laboratory, the ovaries were collected and cultured individually in a 24-well plate at 37.5 ° C, in 5% CO2, for 6 days. For experiment 1 of this dissertation, the following groups were evaluated - (i) DMEM +; (ii) CIMI [5 ng/ml]; (iii) DOXO [0.3 μg/ml]; (iv) CIMI [5 ng/ml] + DOXO [0.3 μg/mL]. For experiment 2, the following treatments were investigated: (i) DMEM +; (ii) CIMI [5 ng/ml]; (iii) DEXA [4 ng/ml]; (iv) CIMI [5 ng/ml] + DEXA [4 ng/ml]. After culture, the ovaries were used for the analysis of survival, activation, growth and follicular viability, ECM, apoptosis rate and stroma density. The results of activation, survival and growth were compared using Fisher's exact test, and the differences were considered significant when $p < 0.05$. In experiment 1, the results showed that the presence of DOXO in culture medium, significantly reduces the percentage of morphologically normal follicles. On the other hand, the presence of CIMI [5 ng/ml] alone can maintain the percentage of morphologically normal follicles like the control group (DMEM+) and when added to the medium containing DOXO [0.3 μg/ml]. In experiment 2, the presence of DEXA [4 ng/ml] reduces the percentage of morphologically normal follicles when compared to the control group, while the presence of CIMI [5 ng/ml] can keep the percentage similar to the control group and still attenuate the deleterious effects caused by the presence of DEXA in culture medium. Regarding follicular activation and development, the ovaries when cultured in the presence of DEXA or DOXO reduced the percentage of primordial follicles compared to the control group, while the presence of CIMI [5 ng/ml] alone, kept the percentage of primordial follicles equal to group control. The presence of DEXA or DOXO negatively affected the stroma density, while the presence of CIMI [5 ng/ml] in the culture medium protected against the damage caused by these drugs. In addition, the presence of DEXA or DOXO increased the rate of apoptosis, while CIMI [5 ng/ml] in the culture medium reduced apoptosis. In view of the above, we can conclude that the CIMI extract [5 ng/ml] was able to contain the deleterious effects illustrated by DEXA and DOX at the end of 6 days of culture.

Keywords: folliculogenesis; toxicity; mice; phytomedication; glucocorticoid; *in vitro* culture.

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LISTA DE ABREVIATURAS E SIGLAS

ANOVA	Análise de Variância
Bcl-2	Célula-B de Linfoma 2
BMP-15	Proteína Morfogenética Óssea 15
BSA	Albumina Sérica Bovina
CAT	Catalase
CGPs	Células germinativas primordiais
CO^2	Gás Carbônico
CIMI	*Cimicifuga Racemosa* (L.) Nutt.
DEXA	Dexametasona
DMEM	Meio de EagleModificado de Dulbecco
DNA	Ácido desoxirribonucleico
DOXO	Doxorrubicina
ERO	Espécies reativas de oxigênio
ERON	Espécies reativas de óxido de nitrogênio
FGF-2	Fator de Crescimento Fibroblástico Básico 2
FOPA	Folículos ovarianos pré-antrais
FSH	Hormônio Folículo Estimulante
GDF-9	Fator de Diferenciação e Crescimento 9
GPx	Glutationa peroxidase
IL-1β	Interleucina 1 Beta
ITS	Insulina, Transferrina, Selênio
LH	Hormônio Luteinizante
MEC	Matriz extracelular
MET	Microscopia Eletrônica de Transmissão
NaCl	Cloreto de Sódio
O_2	Oxigênio
OMS	Organização Mundial da Saúde
PBS	Phosphatebuffered saline (Tampão fosfato salino)
pH	Potencial Hidrogeniônico
SOD	Superóxido dismutase
StAR	Proteína Reguladora Aguda da Esteroidogênese

TGF- β	Fatores De Crescimento Transformante Beta
TNF-α	Fator de Necrose Tumoral Alfa
H_2O_2	Peróxido de hidrogênio

LISTA DE SÍMBOLOS

<	Menor que
%	Porcentagem
µg	Micrograma
µm	Micrometros
g	Gramas
mg	Miligrama
mL	Mililitro
mm	Milímetros
mM	Milimolar
ng	Nanograma
ºC	Grau Celsius
α	Alfa
β	Beta

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1 INTRODUÇÃO

Os problemas de fertilidade têm aumentado significativamente nos últimos anos (BELLVER et al., 2019). Diante disso, a busca por biotécnicas alternativas que possibilitem a manutenção da fertilidade está cada vez mais evidente. Estudos *in vitro* estão sendo utilizados com sucesso como uma ferramenta de estudo da biologia reprodutiva e toxicologia em camundongos e humanos. No entanto, apesar da grande quantidade de informações produzidas nas últimas décadas, o entendimento completo dos mecanismos que controlam o desenvolvimento folicular ainda não está totalmente elucidado (SILVA et al., 2020).

O crescimento folicular é regulado por diversos fatores intra e extraovarianos, como fatores de crescimento, hormônios e glicocorticoides, como a Dexametasona (DEXA) (YE et al., 2019). Os glicocorticoides são mediadores hormonais de estresse amplamente utilizados como anti-inflamatórios e imunossupressores (BATU et al, 2019). Contudo, existem muitas reações adversas na prática clínica. Evidências sugerem que os glicocorticoides são prejudiciais para o sistema reprodutivo, influenciando o eixo hipotâmico-hipofisário-gonadal em todos os níveis (JEYAMOHAN et al., 2013; POULAIN et al., 2012; MILUTINOVIC et al., 2011). A morte folicular pode ser ocasionada por múltiplos fatores, incluindo regimes terapêuticos de patologias tais como o câncer. É bem conhecido que os agentes quimioterápicos usados no tratamento do câncer prejudicam a função ovariana e a fertilidade feminina (BEN-AHARON et al., 2010).

A Doxorrubicina (DOXO), que é amplamente usada para o tratamento de diferentes cânceres, incluindo sarcomas (MARUZZO et al., 2013), câncer de ovários (MORGAN et al., 2016) e tumores de mama (NCEC, 2015), possui efeitos deletérios sobre a fertilidade feminina, mesmo em doses baixas (NISHI et al., 2017). Os efeitos adversos dessa substância incluem toxicidade ovariana em camundongos, reduzindo a taxa de ovulação e o tamanho dos ovários (BEN-AHARON et al., 2010), bem como a menopausa precoce e aumento da taxa de infertilidade em mulheres que mantêm a atividade ovariana após quimioterapia (LETOURNEAU et al., 2012). Assim, a compreensão detalhada das macromoléculas envolvidas nas interações entre as células somáticas e germinativas no curso da foliculogênese poderá permitir o estudo de folículos ovarianos pré-antrais, que se encontram em maior número no ovário, como ponto de partida para a regulação da fertilidade dos mamíferos.

Estudos identificaram ainda receptores de glicocorticoides em células ovarianas (MILUTINOVIC et al., 2011; SAKUMOTO, ITO; OKUDA, 2008; TETSUKA et al., 1999) e que estes receptores podem atuar diretamente no ovário (IRAHARA et al., 1999; KOL et al., 1998). Jana e colaboradores (2005) relataram que a dexametasona induz um estado cístico no

ovário. Além disso, a queda do número de folículos primordiais por conta do uso da DEXA pode estar relacionada com uma deficiência na fertilidade (RISTIC et al., 2008).

Assim, a investigação acerca de substâncias que possam bloquear ou reduzir os efeitos adversos causados por quimioterápicos, como a DOXO, e glicocorticoides, como a DEXA, possui um grande potencial, podendo amenizar o impacto que esses fármacos têm na fertilidade feminina. Nesse sentido, o uso de produtos alternativos, como fitoterápicos, poderiam ser eficazes na prevenção da toxicidade causada pela DOXO e DEXA. Ademais, os fitoterápicos têm sido apontados como um recursso promissor para tal modalidade, como é o caso da *Cimicifuga racemosa* (L.) Nutt. (CIMI).

A CIMI é uma planta herbácea, pertencente à família Ranunculaceae, originária do Canadá e costa atlântica dos Estados Unidos, é um dos fitoterápicos mais populares. Comumente utilizada na medicina popular para aliviar dor e inflamação, para o tratamento de cólicas menstruais e nos sintomas da menopausa (CASTELO-BRANCO et al., 2021). Considerando a crescente consolidação da CIMI como fármaco alternativo e natural nestes tratamentos (MCKENNA et al., 2001; MORELLI et al., 2002), bem como a importância do estudo de redirecionamento de fármacos, é importante se avaliar o efeito sobre a fertilidade feminina quando administrado no período reprodutivo, e ainda se esta substância é capaz de reduzir os efeitos adversos causados pela administração da DOXO ou DEXA.

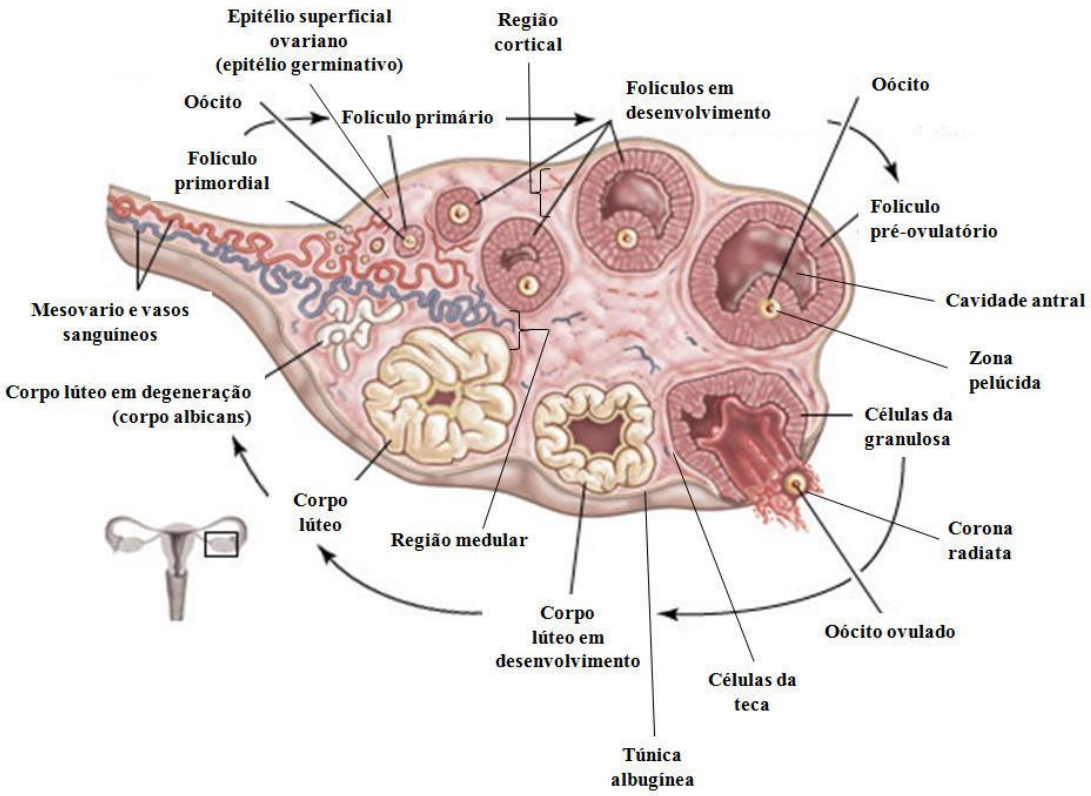
Para um maior esclarecimento da importância deste estudo, a revisão de literatura irá abordar aspectos relacionados aos ovários dos mamíferos, foliculogênese e desenvolvimento folicular, cultivo *in vitro* e avaliação toxicológica, efeito de glicocorticóides e agentes quimioterápicos na fisiologia reprodutiva de fêmeas e ainda sobre a CIMI e seus efeitos.

2 REVISÃO DE LITERATURA

2.1 Ovários dos mamíferos: estrutura e função

O ovário é o órgão central do sistema reprodutor feminino, cuja maior função é a diferenciação e liberação de um oócito maduro para fertilização e propagação das espécies, o qual é constituído de duas regiões distintas: a medular e a cortical (Figura 1). Na maioria das espécies a região medular localiza-se mais internamente no ovário, sendo composta de tecido conjuntivo, nervos, artérias e veias, responsáveis pela sustentação e nutrição deste órgão (FENG et al., 2018). O córtex ovariano por sua vez, é a porção mais externa do ovário e constitui a porção funcional, sendo composta por células germinativas (oócitos) e somáticas (células da granulosa - CG, da teca - CT e do estroma), as quais interagem e organizam a formação dos folículos ovarianos em suas diferentes categorias (MCGEE; HSUEH, 2000).

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



Fonte: Adaptado de: ENCICLOPÉDIA BRITÂNICA, 2013.

O ovário apresenta duas funções distintas: uma endócrina, caracterizada pela produção de hormônios e fatores de crescimento; e outra gametogênica, relacionada ao desenvolvimento e maturação dos oócitos. Estas funções são exercidas pela interação de dois fenômenos que ocorrem em seu microambiente, a oogênese e a foliculogênese; estes correspondem a processos biológicos complexos e coordenados que requerem uma série de

eventos que induzem as mudanças morfológicas e funcionais dentro do folículo, conduzindo a diferenciação de células e o desenvolvimento dos oócitos (BONNET et al., 2013).

O folículo ovariano é o centro de uma rede complexa de vias de sinalização sistêmica e local, por meio das quais o folículo cresce e o oócito adquire maturidade, o que é necessário para que a fertilização ocorra e um embrião viável se desenvolva (FREITAS et al., 2017). A maturação oocitária, em mamíferos é coordenada pela ação de diversas substâncias produzidas por oócitos, células somáticas (células da granulosa, células da teca) e estroma ovariano, as quais apresentam função endócrina, parácrina ou autócrina, ou ainda, por meio de comunicações tipo *gap junctions* em ambas as direções, onde um defeito nessas comunicações pode causar infertilidade (KIDDER; VANDERHYDEN, 2010).

A oogênese compreende o desenvolvimento e diferenciação das células germinativas primordiais (CGPs) até a formação do oócito haploide fecundado. Após o processo marcado pelo crescimento celular e pela redistribuição de organelas citoplasmáticas, as CGP, dentro do ovário, multiplicam-se ativamente e diferenciam-se em oogônias (RÜSSE, 1983). Somente após a fêmea atingir a maturação sexual 50 a 60 dias após nascimento, os oócitos primários retomarão sua divisão meiótica para tornarem-se oócitos secundários haplóides (n) ou maduros e, assim, atingirem a segunda parada da meiose, na fase de metáfase II, na qual estão aptos a serem ovulados e fertilizados. Esta fase ocorre na puberdade, por volta da 5° semana de vida dos camundongos (HUTT; ALBERTINI, 2007; SÁNCHEZ; SMITZ, 2012).

Na última replicação do DNA, antes de entrar na primeira divisão meiótica (PICTON; BRIGGS; GOSDEN, 1998), as oogônias aumentam de tamanho e se diferenciam em oócitos primários imaturos, que sofrem uma parada no seu desenvolvimento (SUH et al., 2006), em prófase I no estágio de diplóteno ou vesícula germinativa. Neste momento, é caracterizado o início da foliculogênese com a formação do folículo primordial (SILVA et al., 2002).

Apesar do conceito sobre o estoque finito e não renovável de células germinativas ser considerado uma premissa básica da fisiologia da reprodução há mais de 150 anos (BUKOVSKY et al., 2005), Johnson et al. (2005) apresentaram evidências para propor a revisão deste paradigma, mostrando indícios da continuidade da oogênese e foliculogênese no período pós-natal, pela atuação de células-tronco. Além disso, vem-se discutindo a relação da neo-ovogênese a um período reprodutivo específico das fêmeas, onde somente neste intervalo ocorreria a renovação e formação de folículos primordiais. Acredita-se, por exemplo, que 400-500 novos ovócitos são produzidos em fêmeas de camundongos adultos a cada ciclo estral, variando nas fases de metaestro/diestro, na vida reprodutiva das fêmeas uma parte dessa reversa

folicular é perdida após o recrutamento por falta de fatores de crescimento e nutrientes para todos os folículos em desenvolvimento (PINHO et al., 2011).

2.2 Células germinativas primordiais, foliculogênese e população folicular

A foliculogênese é um processo fisiológico de ativação, crescimento e maturação do folículo ovariano (RYBSKA et al., 2018; FIGUEIREDO et al., 2019), iniciando com a formação do folículo primordial até o estágio de folículo pré-ovulatório (Figura 2) (MARTELLI et al., 2017). Esse processo é contínuo, com saída diária de folículos do "*pool*" de reserva, os quais crescem em direção à ovulação ou atresia (HAFEZ; HAFEZ, 2004).

Estudos em camundongos revelaram que durante a formação dos folículos, as células somáticas no ovário invadem aglomerados de células germinativas, e a quebra sincicial ocorre imediatamente antes da formação dos folículos primordiais (EPIFANO; DEAN, 2002). Uma vez formado, o conjunto de folículos primordiais serve como fonte de desenvolvimento de folículos e oócitos.

Durante a foliculogênese, oócitos e células da pré-granulosa sofrem alterações dinâmicas na expressão gênica que são reguladas por um conjunto de fatores de transcrição bem coordenados (CAI et al., 2020). Durante a fase de crescimento, um oócito de camundongo totalmente crescido acumula até ∼27 milhões de moléculas de RNA mensageiro (mRNA), enquanto uma célula somática média contém 100.000-600.000 mRNAs (KATARUKA et al., 2020).

O ovário de mamíferos é formado, ao nascimento, por milhares de folículos primordiais, os quais são considerados uma reserva dos folículos ovarianos. Em camundongos, as células germinativas primordiais (CGP) continuam a proliferar por mitose até 13,5 dias pós-coito, grupos de células germinativas conhecidas como cistos ou ninhos (oogônias). Aproximadamente 5 dias após o parto, as oogônias sofrem a primeira divisão meiótica e se transformam em oócitos, que ficam parados no estágio de diplóteno, da meiose I, sendo chamados de oócitos primários ou imaturos (células 2n) (JAGARLAMUDI; RAJKOVIC, 2012; RIMON-DAHARI et al., 2016).

A população folicular é bastante variável entre as espécies, sendo a população estimada em 1.500 em camundongas e 2.000.000 em mulheres (SHAW et al., 2000; BETTERIDGE et al., 1989; AMORIM et al., 2000; LUCCI et al., 1999; ERICKSON et al., 1986). Entretanto, o número e a distribuição dos diferentes tipos foliculares nos ovários de animais são marcados por uma grande variação individual, além de ser afetado pela raça, idade,

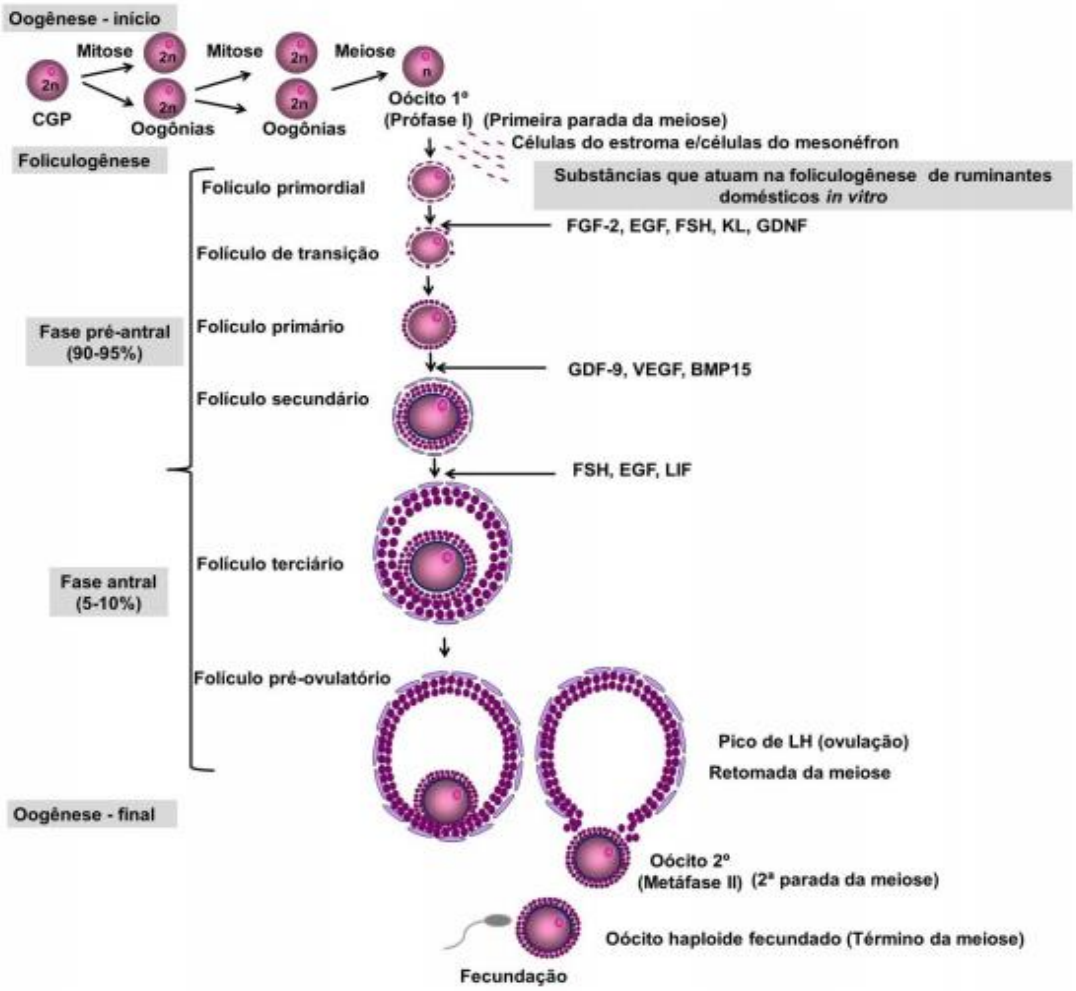
níveis hormonais, genética, bem como o status reprodutivo e nutricional do animal (IRELAND et al., 2007).

Quando a fêmea entra na vida reprodutiva, esses folículos primordiais crescem, amadurecem e eventualmente são eleitos para ovulação. Entretanto, mais de 99,9% destes folículos nunca atingem a ovulação, visto que a maioria morre por um processo fisiológico designado atresia folicular (MARTINS et al., 2008).

O folículo ovariano é composto por um oócito circundado por células da granulosa e/ou da teca, e é considerado a unidade estrutural e funcional básica do ovário, cuja função é proporcionar um ambiente ideal para o crescimento, sobrevivência e maturação do oócito (CORTVRINDT; SMITZ, 2001).

De acordo com o grau de evolução, os folículos podem ser classificados em pré-antrais ou não-cavitários, e ainda antrais ou cavitários. Os folículos pré-antrais são os folículos primordiais, primários e secundários. Já os folículos antrais são os folículos terciários e pré-ovulatórios (FIGUEIREDO et al., 2008).

Figura 2: Desenho esquemático da oogênese e da foliculogênese.



Fonte: Adaptado de: MENEZES, 2017.

Em roedores, os folículos primordiais são formados em torno do terceiro dia de idade gestacional e a primeira onda de recrutamento folicular acontece ao longo das três semanas subsequentes (HIRSHFIELD, 1989; RAJAH et al., 1992). Na fase pré-antral, ocorre a ativação dos folículos primordiais e o crescimento dos folículos primários e secundários. Já na fase antral, é possível observar o crescimento dos folículos terciários, com a formação de uma cavidade repleta de líquido denominado antro, e finalmente, a diferenciação destes em folículos pré-ovulatórios. Após sua formação, esses folículos podem imediatamente iniciar o seu crescimento ou permanecer quiescentes por dias, meses ou anos, dependendo da espécie, até serem estimulados a iniciar seu desenvolvimento. Este evento é denominado de ativação folicular e é marcado por alterações morfológicas como, por exemplo, o aumento do diâmetro oocitário, a proliferação das células da granulosa e a transformação do formato destas células de achatado para cúbico (VAN DEN HURK; ZHAO, 2005)

Quando o oócito é circundado por uma única e completa camada de células da granulosa de morfologia cúbica, os folículos são denominados primários (GOUGEON, 1996). Com a progressão da foliculogênese, ocorre a formação de duas ou mais camadas de células da granulosa de morfologia cúbica ao redor do oócito, dando origem aos folículos secundários. Além da proliferação das células da granulosa, observa-se crescimento oocitário, deposição da membrana basal, aparecimento da zona pelúcida, e a diferenciação das células do estroma, em torno da membrana basal, em células da teca (KNIGHT; GLISTER, 2006).

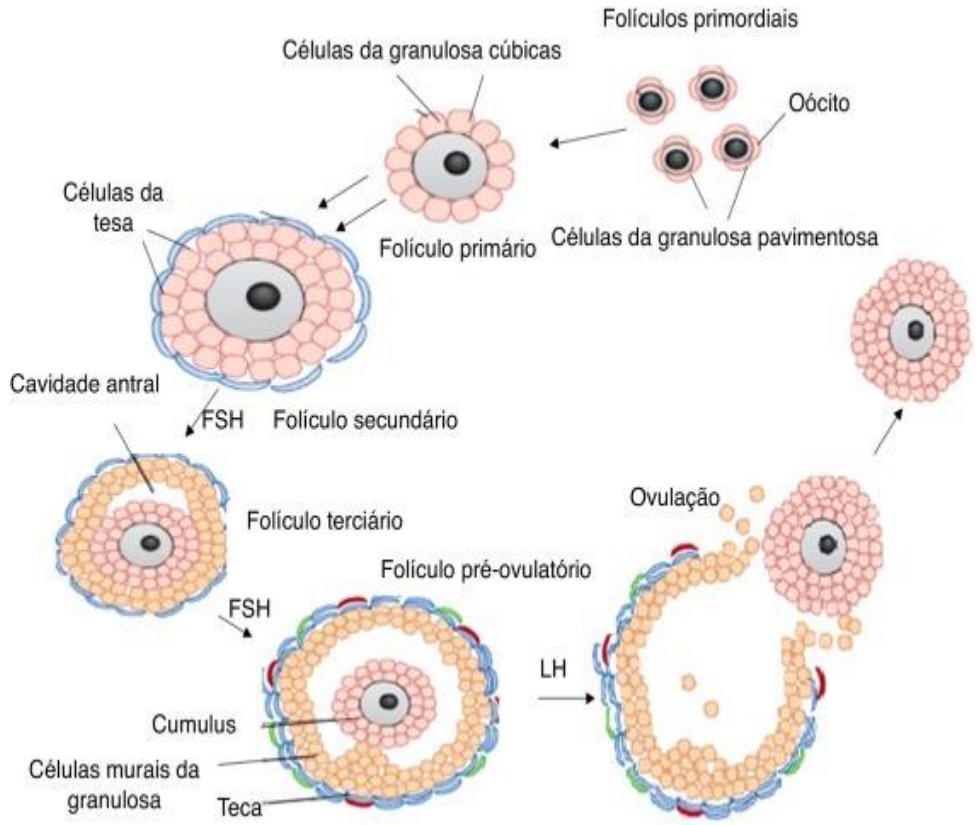
Quando os folículos começam a exibir espaços preenchidos por líquido entre as camadas de células da granulosa, os quais crescem formando a chamada cavidade antral, passam a ser denominados de folículos terciários e entram na fase antral. Neste estágio, pode-se observar também o aumento da vascularização da camada tecal, o crescimento oocitário contínuo e a proliferação das células da granulosa e da teca (OKTEM; URMAN, 2010) o fluido folicular que preenche esta cavidade contém água, eletrólitos, proteínas séricas e alta concentração de hormônios esteróides secretados pelas células da granulosa (BARNETT et al., 2006).

O volume do folículo murino aumenta ~ 45 vezes durante a transição do estágio primordial para o primário, enquanto o desenvolvimento até o folículo secundário abrange aumentos de 125 vezes no volume. A partir do início antral aos maiores folículos pré-ovulatórios, foram encontrados aumentos de ~ 15 vezes no volume, eles aumentam rapidamente com o contínuo acúmulo de fluido folicular (FENG et al., 2018). O fluido antral pode servir como importante fonte de substâncias modulatórias ou regulatórias derivadas do sangue e das secreções das células foliculares, como gonadotrofinas, esteroides, fatores de crescimento, enzimas, proteoglicanos e lipoproteínas (BARNETT et al., 2006).

2.3 Folículos primordiais e ativação folicular

O desenvolvimento dos folículos pré-antrais pode ser dividido em três estágios: ativação de folículos primordiais, transição de folículo primário para secundário e progressão de folículos secundários até o estágio antral (Figura 3) (FORTUNE et al., 2003). Em camundongo a duração do desenvolvimento folicular até a formação do folículo pré-ovulatório *in vivo* é de cerca de três semanas. A maioria dos folículos em desenvolvimento sofrerão atresia, porém alguns irão amadurecer até o estágio de folículos pré-ovulatórios (PEDERSEN, 1970).

Figura 3: Representação esquemática da folículogênese e dos estágios foliculares



Fonte: GUERREIRO et al., 2015.

Na ativação folicular, ocorre a saída dos folículos primordiais do seu estado de dormência, seguindo assim o seu crescimento, sendo esta uma etapa importante para a foliculogênese inicial (CORTVRINDT et al., 2001). Após a ocorrência deste evento, observa-se a transformação das células da pré-granulosa, com formato pavimentoso para formato cuboidal (FORTUNE et al., 2003). A ativação acontece de forma espontânea (FORTUNE et al., 2000), ou em virtude de estímulos hormonais e fatores de crescimento que são produzidos pelas células da granulosa e oócito, que agem de forma parácrina e autócrina no desenvolvimento dos folículos (ALBERTINI; BARRETT, 2003).

O processo de ativação é dinâmico e rigorosamente controlado e, apesar do enorme progresso realizado, muitos mecanismos a nivel molecular ainda não são totalmente abrangidos. Sabe-se, no entanto, que a via de sinalização PTEN/PI3K é uma via essencial para a ativação dos folículos primordiais. A ativação da via PI3K leva à ativação de seu componente AKT, uma proteína quinase serina/treonina que aumenta a proliferação e sobrevivência celular, enquanto o PTEN é um regulador negativo da PI3K. (GOUGEON et al., 2000; MATINS et al., 2008; MAIDARTI et al., 2019). A deleção do PTEN, PDK1, Rps6 ou Foxo3 em oócitos murinos induz uma dramática ativação e/ou perda prematura de folículos primordiais (JOHN et al., 2008; REDDY et al., 2008).

Após a ativação, quando uma camada completa de células da granulosa de formato cuboide circunda o oócito, são formados os folículos primários. Nesta fase do desenvolvimento folicular, além do núcleo do oócito encontrar-se em uma posição excêntrica, as organelas começam a mover-se para a periferia. O número de retículos endoplasmáticos lisos aumenta e a grande maioria das mitocôndrias apresenta-se alongada (ROSSETTO et al., 2011).

À medida que os folículos crescem, as proteínas que constituem a zona pelúcida começam a ser sintetizadas (PICTON, 2008). Por mecanismos independentes de gonadotrofinas, os folículos primários crescem até chegar ao estágio de folículos secundários pelo crescimento do oócito e por atividade mitótica entre as células da granulosa. Um folículo secundário é formado quando uma camada da teca é gerada a partir do tecido conjuntivo circundante (GRIVE, 2015). Nesta categoria folicular, os receptores de FSH (rFSH) tornam-se funcionais em ovários de ratas recém-nascidas com idade de quatro a cinco dias (SOKKA; HUHTANIEMI, 1990) e a sua transcrição atinge o ápice após 10 dias de nascimento em camundongos (O'SHAUGHNESSY et al., 1997).

Com o crescimento dos folículos secundários e organização das células da granulosa em várias camadas, ocorre a formação de uma cavidade repleta de líquido denominado antro. A partir deste estágio, os folículos passam a ser denominados de folículos terciários ou antrais, cujo diâmetro folicular aumenta acentuadamente devido ao crescimento do oócito, multiplicação das células da granulosa, da teca e aumento do fluido na cavidade antral (SILVA-SANTOS, 2013).

No entanto, durante a vida reprodutiva das fêmeas, apesar de muitos folículos serem ativados, apenas uma pequena parte (0,01%) é capaz de se desenvolver até a ovulação, a grande maioria (99,9%) é perdida por um processo natural conhecido como atresia (morte folicular) (FIGUEIREDO et al., 2008).

2.4 Regulação da ativação e supressão do desenvolvimento de folículos primordiais

O ovário dos mamíferos consiste em um depósito finito de células germinativas, que é estabelecido durante a oogênese e usado posteriormente para a foliculogênese. O ovário humano contém menos de 1 milhão de folículos no momento do nascimento (HANSEN et al., 2008). O "*pool*" de folículos pré-antrais existente nos ovários de mamíferos representa uma significativa reserva de material para estudos de manipulação genética em espécies domésticas, bem como para a preservação de espécies em extinção e preservação da fertilidade (PICTON, 2001).

Para ter sucesso na ativação e desenvolvimento folicular, é necessária uma complexa interação entre oócito e células somáticas do estroma ovariano, envolvendo diversos mensageiros, fatores de crescimento e hormônios. A interação harmônica entre estas substâncias é responsável pela saída dos folículos primordiais do estágio de quiescência e posterior crescimento dos folículos até o estágio pré-ovulatório (SILVA et al., 2016).

Na ativação folicular, o folículo primordial é ativado para sair da dormência e iniciar o crescimento folicular de maneira irreversível (SHAH et al., 2018). Estudos já demonstraram a participação de fatores de crescimento, como o fator de crescimento e diferenciação-9 (GDF-9), proteína morfogenética óssea-15 (BMP-15), fator de crescimento semelhante à insulina-1 (IGF-1) proteína morfogenética óssea-7 (BMP-7) fator inibidor de leucemia (LIF) fator de crescimento fibroblástico-2 e 10 (FGF-2; FGF-10) fator de crescimento e transformação beta (TGF-β) e kit ligand (KL). Ademais, alguns hormônios também são fundamentais para esse processo, como o hormônio folículo estimulante (FSH), estradiol (E2) progesterona (P4) hormônio do crescimento (GH) e Dehidroepiandrosterona. Além disso, as vias de sinalização para ativação folicular estão relacionadas com a fosfatidilinositol-3-quinase (PI3K) alvo mamífero da rapamicina (mTOR) e fator de transcrição Forkhead box L2 (FOXL2) (SILVA; VAN DEN HURK; FIGUEIREDO, 2016; RIMON-DAHARI et al., 2016).

Já a dormência dos folículos primordiais é mantida pelo equilíbrio das moléculas inibitórias e estimuladoras. Várias moléculas, como as proteínas de forkhead box (FOX) FOXL2 e FOXO3a, hormônio anti-Mülleriano (AMH), fosfatase homólogo à tensina (PTEN), inibidor de quinase dependente de ciclina 1B (p27Kip1) e o complexo de esclerose tuberosa (TSC) foram identificados como fatores inibidores (REDDY et al., 2010; KIM, 2012).

Com a influência de fatores inibidores intrínsecos e extrínsecos, a maioria dos folículos primordiais é mantida em estado quiescente. Mesmo que estejam dormentes, vários fatores são necessários para mantê-los vivos. As vias de sinalização PTEN/PI3K-PDK1-Akt-

S6K1-rpS6 e TSC/mTORC1-S6K1-rpS6 têm se mostrado essenciais para a sobrevivência do "*pool"* de folículos primordiais em repouso (HAO et al., 2019).

Quando o ovário é exposto a agentes quimioterápicos, a secreção de inibidores do folículo primordial diminui devido ao dano aos folículos em desenvolvimento, e isso acelera o recrutamento dos folículos primordiais e a redução da reserva ovariana resultando em um efeito de "*burnout*" (CHANG et al., 2015). Isso tem sido apoiado por vários estudos, que mostram números diminuídos de folículos primordiais e números aumentados de folículos em desenvolvimento precoce em grupos tratados com quimioterápico em comparação com grupos não tratados, usando camundongos fêmeas e tecido ovariano humano cultivado *in vitro* (CHANG et al., 2015; LANDE et al., 2017).

2.5 Atresia folicular: necrose, necroptose, autofagia e apoptose

Ao longo da vida das fêmeas de mamíferos há uma significativa redução na população de folículos pré-antrais (SHAW et al., 2000), devido ao processo fisiológico degenerativo irreversível denominado atresia, que acomete cerca de 99,9% dos folículos ovarianos (FIGUEIREDO et al., 2008). No folículo primordial, o processo de morte se origina no oócito, já nos folículos em crescimento, a atresia se origina nas células da granulosa e, subsequentemente, o oócito também é atingido (FINDLAY et al., 2019). A atresia folicular pode ocorrer pelas vias de necrose, necroptose, autofagia e apoptose (CHAUDHARY et al., 2018; FINDLAY et al., 2019; ZHOU; PENG; MEI, 2019). Quando o ambiente parácrino ou endócrino não é apropriado para suportar o crescimento e/ou diferenciação das células foliculares (SILVA et al., 2002).

A via de necrose e necroptose apresentam características morfológicas semelhantes e são caracterizadas por um aumento no volume celular, permeabilização e ruptura da membrana plasmática, que levam à morte celular (ZHOU; HAN; HAN, 2012; CHAUDHARY et al., 2018). Geralmente, a necrose é iniciada por mecanismos não celulares como isquemia, deficiência de níveis de adenosina trifosfato (ATP) e traumas, levando a danos irreversíveis na célula (MCCULLY et al., 2004). A necroptose é iniciada pelo fator de necrose tumoral-α (TNF-α) e operada através das proteínas quinase-1 e 3, que interagem com seus receptores RIPK1 e RIPK3, respectivamente, bem como pela proteína do tipo domínio da quinase de linhagem mista (MLKL) (ZHOU; HAN; HAN, 2012; CHAUDHARY et al., 2018; ZHANG et al., 2020). A acetilcolinesterase, as citocinas e o estresse oxidativo também desempenham papéis importantes na morte da célula da granulosa mediada por necroptose (CHAUDHARY et al., 2018, 2019). A necroptose das células da granulosa pode privar o oócito de nutrientes, fatores

de crescimento e fatores de sobrevivência. Sob essas condições, o oócito se torna mais suscetível à necroptose mediada por estresse oxidativo (CHAUDHARY et al., 2019).

A autofagia é um processo em que ocorre o sequestro de materiais celulares residuais, como proteínas e organelas danificadas, por autofagossomos, os quais, em seguida, se unem aos lisossomos que os degradam em monômeros reutilizáveis, como aminoácidos (LI et al., 2016, 2018). Essa via pode ser estimulada por várias formas de estresse celular, incluindo privação de nutrientes ou fator de crescimento, hipóxia, espécies reativas de ogixênio (ERO), danos ao DNA, agregados de proteínas, organelas danificadas ou patógenos intracelulares (KROEMER; MARIÑO; LEVINE, 2010). Vários fatores, incluindo as vias PI3K/PKA/mTOR, transdução do sinal de cálcio, mitocôndrias e alteração de citoesqueleto, participam da regulação da autofagia durante o desenvolvimento folicular (ZHOU; PENG; MEI, 2019).

Em folículos pré-antrais a autofagia ocorre principalmente em oócitos, enquanto em folículos antrais, ocorre principalmente nas células da granulosa e está intimamente relacionada à apoptose celular (ZHOU; PENG; MEI, 2019). O acúmulo de autofagossomos induz a morte celular apoptótica das células da granulosa por meio da expressão reduzida de proteínas antiapoptóticas do linfoma de células B-2 (bcl-2) e subsequente ativação da caspase (CHOI et al., 2011).

Segundo Barnett et al., (2006), a apoptose folicular ocorre de forma ordenada sendo geneticamente regulada pela expressão de genes específicos tais como *BAX* e caspases. Além disso, outro estudo relata que o desbalanço entre fatores pró e anti-apoptóticos são fatores determinantes na sobrevivência e desenvolvimento folicular (HSU; HSUEH, 2000). A apoptose é a principal responsável pela perda da reserva ovariana (GOUGEON, 2010). A ativação das caspases é um ponto chave para desencadear a apoptose. Estas podem ser ativadas por duas vias principais: a via extrínseca ou via do receptor de morte e pela via intrínseca ou via mitocondrial (BEATTIE, 2007).

A via de apoptose extrínseca é desencadeada por fatores de morte da família TNF (ligante FASL; TNF-α; ligante indutor de apoptose relacionado ao TNF [TRAIL]) (KRAMMER, 2000; NAGATA, 2018; STRASSER; JOST; NAGATA, 2009). Esses fatores são sintetizados como proteínas de membrana tipo II com uma estrutura homotrimérica e podem ser clivadas a partir da membrana para gerar uma forma solúvel (BLACK et al., 1997; SCHULTE et al., 2007). A via intrínseca, também chamada via mitocondrial, é regulada pelos membros da família bcl-2, que consiste em três subfamílias: membros pró-apoptóticos como as proteínas BH3 (Bim, Bid, Puma, Noxa, Hrk, Bmf e Bad), moléculas efetoras pró-apoptóticas (Bax e Bak) e proteínas anti-apoptóticas da família bcl-2 (bcl-2, bcl-XL, Mcl1, A1 e bcl-B) (CZABOTAR et al., 2014).

Estudos apontam que a expressão modulada de Bcl-2 em oócitos de camundongos resulta no aumento do número de folículos pré-antrais saudáveis e número reduzido de folículos pré-antrais atrésicos, sugerindo assim a regulação deste evento a partir da expressão de genes específicos (MORITA et al., 1999). Além disso, a observação de que camundongos Bax mutantes têm um número aumentado de folículos pré-antrais primários também demonstra que a apoptose é um processo regulador chave do desenvolvimento folicular (PEREZ et al., 1999).

2.6 Cultivo folicular *in vitro* como ferramenta para avaliar o efeito de substâncias contra agentes citotóxicos ao ovário

Em 1989, foi realizado o primeiro cultivo *in vitro* de folículos pré-antrais bem-sucedidos em roedores, levando ao nascimento de filhotes vivos desses folículos pré-antrais cultivados (EPPIG et al., 1989). A partir de então, esta técnica tem mostrado resultados satisfatórios com animais de laboratório, uma vez que Eppig e O' Brien (1996) obtiveram o nascimento de um camundongo a partir de folículos primordiais crescidos, maturados e fecundados *in vitro*. Posteriormente, esta mesma equipe, aperfeiçoando o protocolo utilizado anteriormente, relatou a produção de embriões e o nascimento de 59 camundongos, 14 saudáveis, a partir de folículos pré-antrais cultivados, maturados e fecundados *in vitro* (O'BRIEN et al., 2003).

Como mencionado anteriormente, o ovário é composto por uma grande população de folículos pré-antrais, a grande maioria morre e apenas uma pequena porcentagem alcança a ovulação, o que torna o rendimento ovariano (número de ovulações durante a vida em relação ao total de folículos presentes) muito baixo. Assim, com o intuito de evitar a grande perda folicular *in vivo* devido à atresia, o cultivo de folículos ovarianos *in vitro* vem sendo realizado na tentativa de: 1) elucidar os mecanismos envolvidos na regulação da foliculogênese inicial; 2) realizar testes *in vitro* da ação de fármacos (benéfica ou tóxica), radioatividade, vacinas imunoesterilizantes e nanopartículas sobre os oócitos, que se apresentam como uma opção ao uso de animais em experimentos; 3) produzir bancos genéticos (germoplasma); 4) aperfeiçoar a reprodução assistida de humanos e animais (FIGUEIREDO et al., 2007).

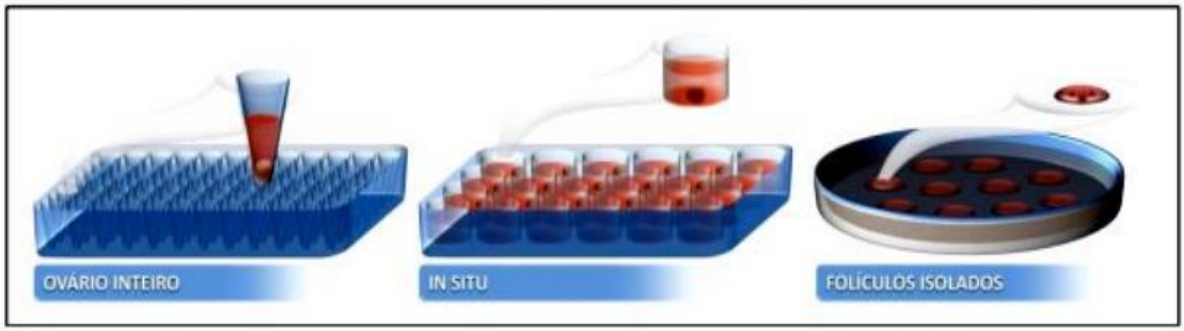
Estudos *in vitro* estão sendo utilizados com sucesso como uma ferramenta de estudo no desenvolvimento dos folículos em biologia reprodutiva e toxicologia em camundongos, ratos, bovinos, ovinos, suínos, primatas e humanos (XUYING et al., 2011). Os potenciais pontos de análise no cultivo *in vitro* na perspectiva da análise toxicológica, incluem o estudo dos mecanismos de ação dos produtos e como eles contribuem para o dano celular (oocitário

ou somático), análise da qualidade dos oócitos, efeitos no estabelecimento do *pool* de folículos primordiais e interações parácrinas (SUN et al., 2004).

A eficiência do cultivo *in vitro* pode ser afetada por fatores como o tipo de sistema de cultivo usado; a composição do meio de base; a concentração e associação de fatores adicionados ao meio de cultivo (FIGUEIREDO et al., 2007).

No tocante aos sistemas de cultivo, os folículos ovarianos podem ser cultivados inclusos no próprio tecido ovariano ou no ovário inteiro (cultivo *in situ*); ou ainda na forma isolada (Figura 4). O cultivo *in vitro* pode ser feito em dois passos, no qual é feito primeiramente o cultivo *in situ*, o que permite a ativação e o desenvolvimento do folículo primordial até estágio secundário, para posteriormente ser isolado e cultivado até o estágio antral (TELFER et al., 2008).

Figura 4: Desenho representativo dos tipos de cultivo *in vitro* de folículo ovariano.



Fonte: Adaptado de: SOUZA, 2013.

Além disso, já é conhecido que os sistemas de cultivo têm o potencial de revelar se os efeitos dos tóxicos afetam diretamente o ovário, tendo em vista que os efeitos observados *in vivo*, mas não *in vitro*, são presumivelmente indiretos (GANDOLFI et al., 2006). Os cultivos também podem revelar se os compostos direcionam os folículos em estágios específicos de desenvolvimento e permitem uma percepção mais profunda da maneira pela qual os agentes tóxicos podem afetar a integridade cromossômica do oócito, ou se eles têm a capacidade de alterar a sinalização hormonal dentro e/ou entre os folículos (SILVA et al., 2016).

Os métodos de cultivo disponíveis para a pesquisa toxicológica variam em termos de espécies, estágio folicular, tempo-curso e composição dos meios de cultivo. Cada sistema de cultivo tem suas próprias vantagens e desvantagens, todas exigindo uma análise cuidadosa antes da escolha do melhor método para um estudo de toxicologia (ARAÚJO et al., 2014).

2.7 O efeito de glicocorticoides na fisiologia reprodutiva de fêmeas

A região cortical da glândula adrenal, uma das estruturas essenciais para a função metabólica normal do organismo (BARLOW; ROSENTHAL, 1973), regula a produção e secreção de hormônios de natureza esteroide, os quais são classificados em mineralocorticoides e glicocorticoides (FELDMAN; NELSON, 1991; PARILLO, 1979).

Os glicocorticoides são lipofílicos e possivelmente passam pela membrana plasmática por difusão simples. Ao chegarem no citosol se ligam aos seus receptores intracelulares, seguem para o núcleo para interagirem com os genes no DNA onde os receptores se ligam as regiões promotoras dos genes alvos, exercendo ações de transativação, expressão de genes ou de transrepressão, repressão de genes (SMOAK; CIDLOWSKI, 2004).

Os glicocorticoides sintéticos são equivalentes aos naturais em relação a sua estrutura química, sendo a DEXA um exemplo bem conhecido, apresentando vinte vezes mais atividade farmacológica que o cortisol e com efeitos antinflamatórios e imunossupressor (BAVARESCO; BERNARDI; BATTASTINI, 2005). O uso dos glicocorticoides, porém, pode ter efeitos adversos, como apoptose em células do sistema imune, por isso a importância de estudos sobre suas ações metabólicas (TORRES; INSUELA; CARVALHO, 2012).

Os glicocorticoides, produzidos pela zona fasciculada do córtex adrenal, estão envolvidos em diversas funções fisiológicas e também na adaptação a situações de estresse (ANTI; GIORGI; CHAHADE, 2008). Os estudos das propriedades químicas dos esteroides adrenocorticais demonstraram uma forte correlação dos glicocorticoides com uma atividade anti-inflamatória, enquanto efeitos colaterais como edema e retenção de sódio estão relacionados com a atividade dos mineralocorticoides (FELDMAN; NELSON,1991).

A história dos glicocorticoides é inseparável da história da própria Medicina e sua importância está ligada não só à melhora da qualidade de vida, como também à sobrevida de pacientes com doenças que apresentam características imunológicas (ANTI; GIORGI; CHAHADE, 2008). Hidrocortisona, cortisona, prednisona, prednisolona e DEXA são exemplos de corticosteroides sintéticos (LIU et al., 2013), sendo a DEXA a droga de primeira escolha para a indução de uma terapia com corticoides (MULLER, 1989; REEDY, 1989).

Curiosamente, o primeiro relato de síndrome de Cushing, uma desordem endócrina causada por níveis elevados de glicocorticoides no sangue, em 1912, foi acompanhado de um quadro de amenorréia secundária. No entanto, os mecanismos moleculares pelos quais altos níveis de glicocorticoides suprimem a fertilidade são complexos e permanecem sem elucidação. Além disso, sabe-se que níveis basais de glicocorticoides são criticamente importantes para o estabelecimento e manutenção da fertilidade, como evidenciado em pacientes com

endocrinopatia caracterizada pela produção insuficiente de hormônios esteroides pelas glândulas adrenais, a doença de Addison, que se apresentam com insuficiência ovariana prematura (ERICHSEN et al., 2010). Notavelmente, um equilíbrio entre baixos e altos níveis de glicocorticoides diferencia entre fertilidade e infertilidade (WHIRLEDGE; CIDLOWSKI, 2017).

Os glicocorticoides sintéticos são comumente utilizados para tratar um amplo conjunto de condições clínicas (CALVERT; CORNÉLIUS, 1990), sendo geralmente prescritas para problemas inflamatórios agudos ou crônicos de severidade variada (STREETEN, 1975; FAUCI, 1976). Entretanto, os benefícios da medicação devem ser comparados com os riscos de sua utilização (CALVERT; CORNÉLIUS,1990).

Embora sua denominação tenha origem em seu efeito característico sobre o metabolismo dos carboidratos, os glicocorticoides atuam praticamente sobre todos os órgãos e tecidos (WRIGHT et al., 1993), haja vista a presença de receptores de glicocorticoides em quase todos os tipos celulares. As ações dos glicocorticoides são diversificadas, pois podem ser reguladas por diferentes fatores, como qual glicocorticoide está disponível e a sensibilidade da célula e do tecido alvo (LU; CIDLOWSKI, 2006).

Diversos efeitos podem ser causados, sendo um deles a depressão da função reprodutiva por supressão da síntese e liberação hipotalâmica de corticotrofina e gonadotrofina, supressão da síntese e liberação do cortisol e androgênios, bem como da síntese e ação do estrógeno e da testosterona (SILVA et al., 2017).

A administração de corticosterona em camundongas interrompeu a ciclicidade ovariana, em parte, pela ruptura do mecanismo de feedback positivo tanto a nível hipotalâmico quanto hipofisário, fator que seria necessário para a geração do pico pré-ovulatório de LH (LUO et al., 2016).

Os glicocorticoides atuam também sobre a função uterina, antagonizando a ação estrogênica. A DEXA, por exemplo, bloqueia o crescimento e proliferação celular uterina induzida por estrogênio em camundongas (RHEN et al., 2003; JOHNSON; DEY, 1980). A administração de DEXA em camundongos também regula a expressão de genes cujas funções estão relacionadas ao desenvolvimento celular, crescimento, proliferação e sinalização. No útero neonatal de camundongas, a DEXA inibe a proliferação de células epiteliais através de mecanismos independentes e dependentes de receptor de glicocorticoide (NANJAPPA et al., 2015).

No que diz respeito ao ovário, além das alterações cíclicas mediadas por seus efeitos sobre hipotálamo e hipófise, os glicocorticoides alteram a fisiologia ovariana através da regulação da função de células da granulosa, oócitos, células do *cumulus* e células luteais (LUO

et al., 2016). Em ratas, a DEXA aumentou a produção de progesterona por aumento da expressão de caspase-3 (YUAN et al., 2014), embora Huang e Shirley (2001) reportem decréscimo dos níveis de progesterona em células da granulosa. Tais diferenças podem ser reflexo da diferença de doses ou do estágio de desenvolvimento folicular, como será melhor abordado a seguir.

2.7.1 Dexametasona e desenvolvimento folicular

A DEXA, um membro da família dos corticosteróides é o análogo da prenidsolona (NAVARRO et al., 2020), tendo a capacidade de se comportar como glicocorticoide natural e consequentemente a mesma eficácia clínica (CHU et al., 2014). Estudos realizado por Ristic et al. (2008) constataram que a DEXA diminui o número de folículos primordiais, primários e secundários, como também o volume dos ovários de ratas tratadas com esse glicocorticoide. Animais tratados com DEXA requerem atenção, pois a queda do número de folículos primordiais por conta do uso da DEXA pode estar relacionada com uma deficiência na fertilidade.

Em testes *in vivo*, Tohei e Kogo (1999) comprovaram que ratas imaturas tratadas com DEXA apresentaram concentrações séricas de FSH elevadas ao mesmo tempo em que os níveis de inibina decresceram. Segundo seus estudos, esse aumento do FSH foi devido à inibição que a DEXA causou sobre a secreção da inibina. Por outro lado, no cultivo *in vitro* de folículos pré-antrais bovinos inclusos em tecido ovariano, a presença de DEXA (10 ng/mL) manteve o percentual de folículos morfologicamente normais ao final de 6 dias de cultivo e contribui para a manutenção da integridade ultraestrutural dos folículos ovarianos (SILVA et al., 2017).

Estudos recentes demonstraram que a DEXA pode influenciar negativamente a oogênese durante o desenvolvimento fetal em humanos (POULAIN et al., 2012). Além disso, já foi demonstrado que a DEXA regula o processo de apoptose nas células da granulosa de folículos pré-ovulatórios (YUAN et al., 2014). Em camundongos, Hułas-Stasiak et al. (2017) mostraram que a DEXA prejudica a foliculogênese e aumenta a atresia folicular por indução de autofagia e apoptose.

Com o desenvolvimento dos folículos ovarianos, as células da granulosa proliferam, diferenciam-se e passam por apoptose em torno do oócito, desempenhando um papel importante na reprodução pela produção de esteroides. A caspase-3 foi identificada como enzima chave naapoptose, sendo ativada em resposta ao LH e FSH (YACOBI et al., 2004).

Ainda no que diz respeito aos aspectos fisiológicos do desenvolvimento folicular, a biossíntese de hormônios esteroides gonadais (progesterona, estrógenos e andrógenos) começa com o colesterol. O passo agudamente regulado e limitante para síntese de hormônios esteroides é a entrada de colesterol na membrana mitocondrial interna onde é convertido em pregnenolona por clivagem de sua cadeia lateral por enzima do citocromo P450 (P450scc) (PRIVALLE; CRIVELLO; JEFCOATE, 1983; JEFCOATE et al., 1987). O transporte de colesterol em células esteroidogênicas é mediado pela Proteína esteroidogênica de regulação aguda (ou StAR) (CLARK et al., 1994), a qual foi identificada como uma fosfoproteína mitocondrial (STOCCO; CLARK, 1996).

Nesse contexto, pesquisas mostraram que a DEXA aumenta a indução de FSH por progesterona em células da granulosa de folículos pré-antrais de ratas (ADASHI; JONES; HSEUH, 1981). Ademais, tem sido relatado que os glicocorticoides podem potencializar o AMPc e a resposta esteroide ao LH e FSH em células granulosas de ratas e humanas que não luteinizaram *in vitro* (HSUEH; ERICKSON, 1978; BEN-RAFALL et al., 1988; HSUEH; ERICKSON, 1978; ADASHI, JONES; HSEUH, 1981).

Por outro lado, Huang e Li (2001), usando folículos pré-ovulatórios isolados de ovários de ratas adultas cíclicas, demonstraram que a DEXA é capaz de diminuir significativamente LH e forskolin ou os níveis de proteína StAR induzida por 8-BrcAMP, além de estar associada a diminuições na produção de progesterona com resposta dose-dependente. Os resultados dessa pesquisa indicam que a DEXA inibe a esteroidogênese do folículo pré-ovulatório por regulação negativa da expressão da proteína StAR.

Ainda, tem sido demonstrado que espécies reativas de oxigênio (ERO) danificam as mitocôndrias, resultando em desordens esteroidogênicas (QI et al., 2013; ZHOU et al., 2013). A DEXA, como agente indutor de apoptose, leva a um aumento da geração de ERO em uma variedade de células (PARK, et al., 2013; KAO et al., 2010).

2.8 Câncer e fertilidade: efeitos da quimioterapia na função ovariana

A taxa de sobrevivência após tratamento quimioterápico tem demonstrado um aumento para a maioria dos cânceres, incluindo câncer infantil e aqueles que afetam mulheres jovens. Entretanto, a infertilidade induzida por quimioterapia está afetando um número crescente de mulheres em nossa população (LOPES et al., 2020). Dessa forma, a qualidade de vida e a preservação da fertilidade são questões que devem ser cuidadosamente discutidas entre mulheres em idade reprodutiva submetidas ao tratamento de câncer. Ainda, essa busca em

amenizar o efeito de tais drogas sobre a fertilidade tem se tornado um dos grandes desafios da medicina (LEE et al., 2006).

Embora o tratamento, mediante a administração de agentes quimioterápicos, venha aumentando a sobrevida dessas pacientes, também vêm ocasionando alterações não apenas nas células tumorais, mas em células normais do organismo, incluido as células germinativas, e assim, esgotando o "*pool"* folicular e aumentando o risco de falência ovariana e infertilidade (MEIROW et al., 2010). Isso tem sido apoiado por vários estudos, que mostram números diminuídos de folículos primordiais e números aumentados de folículos em desenvolvimento precoce em grupos tratados com quimioterápico em comparação com grupos não tratados, usando camundongos fêmeas e tecido ovariano humano cultivado *in vitro* (CHANG et al., 2015; LANDE et al., 2017). Segundo Soleimani et al. (2011), ainda não existem estudos com humanos que examinem a qualidade dos óocitos e dos embriões após o tratamento quimiorerápico. Porém, sabe-se que os agentes quimioterápicos podem causar efeitos gonadotóxicos, interrompendo processos celulares básicos, interferindo na proliferação celular.

Os agentes alquilantes, uma classe de quimioterápicos, podem criar ligações covalentes entre as cadeias do DNA, interferindo com a separação da dupla hélice do DNA durante o processo de replicação, interrompendo o ciclo celular. Além disso, o tratamento com os agentes alquilantes e a terapia com ciclofosfamida têm causado destruição dos oócitos, depleção folicular, fibrose cortical e danos aos vasos sanguíneos (FLEISCHER, et al., 2011; MEIROW et al., 2010).

Já os agentes quimioterápicos rotineiramente utilizados no tratamento do câncer são extremamente tóxicos para os ovários, particularmente para os folículos primordiais (GADDUCCI et al., 2008). Porém, esses quimioterápicos não exercem efeito citotóxico apenas sobre os folículos primordias, podendo atuar também sobre os folículos em crescimento. A presença dos folículos em crescimento inibe o recrutamento dos folículos primordiais, e a perda crescente dessa população folicular leva ao aumento na ativação de folículos primordiais, e assim, a perda da reserva folicular (MORGAN et al., 2012; CHANG et al., 2016).

Dados publicados por Kalich-Philosoph et al. (2013) sobre a ação de agentes quimioterápicos, como a ciclofosfamida e cisplatina, sobre a ativação folicular em ratas, sugerem que estas drogas ocasionam a ativação dos folículos primordiais quiescentes, evento que culminou no aumento precoce na quantidade dos folículos em crescimento e, simultaneamente, na morte dos folículos por apoptose, resultando na perda da reserva folicular ovariana inicial. Além disso, os quimioterápicos podem atuar diretamente sobre o oócito ou sobre as células da granulosa. Assim, a morte do oócito pode ocorrer de forma direta, ou resultar

da morte das células da granulosa, uma vez que a sobrevivência do oócito dependente de fatores secretados pelas células da granulosa (MORGAN et al., 2012).

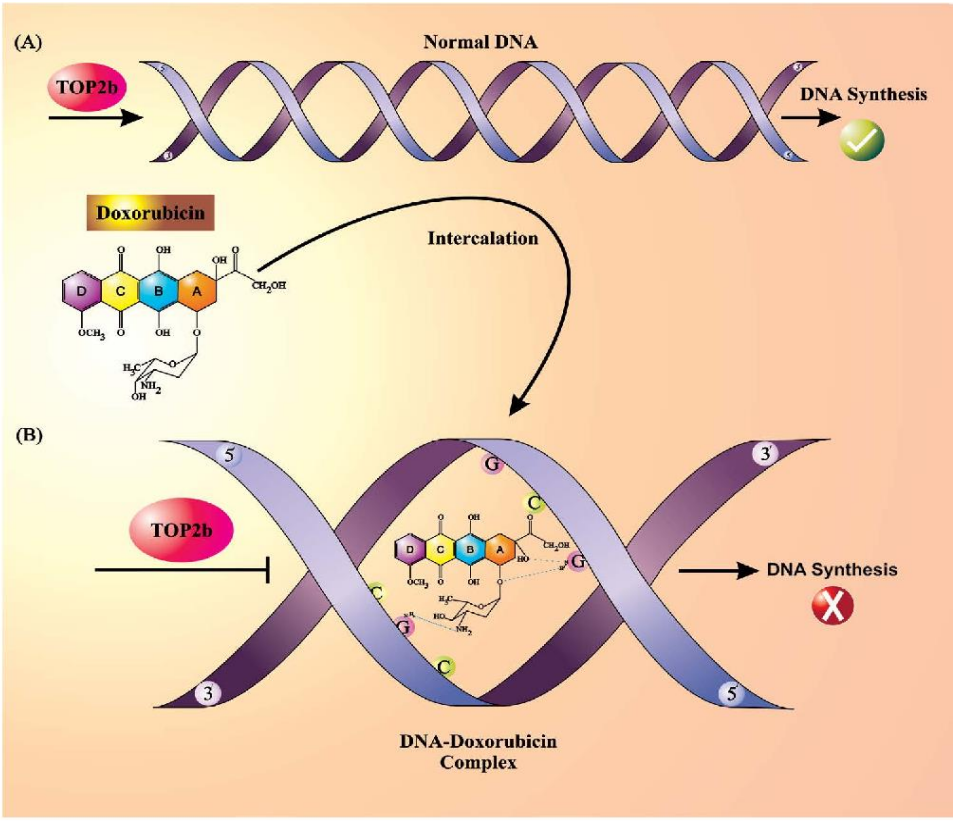
2.8.1 Doxorrubicina

A DOXO é um antibiótico antitumoral que pertence as antraciclinas, sendo utilizado na concentração de 0,3 μg/Ml. Atua como agente quimioterápico de primeira linha para o tratamento de diferentes cânceres (GUO et al., 2019), incluindo sarcomas (MARUZZO et al., 2013), ovários (MORGAN et al.,2016) e tumores de mama (NCEC, 2015), apresentando efeitos deletérios sobre a fertilidade feminina, mesmo em doses baixas (NISHI et al., 2017).

É um quimioterápico capaz de se difundir através da membrana mitocondrial e se acumular dentro dela, em seguida libera elétrons, produzindo ERO e perturbando a integridade funcional da cadeia respiratória, resultando em disfunção mitocondrial por diminuição de ATP e aumento de citocromo c, ocasionando o aumento da apoptose e alterações no metabolismo energético (CHEN et al., 2007; NAJAFI et al., 2020). Além disso, a DOXO também atua aumentando a inflamação e a autofagia e interfere na homeostase do cálcio (VARELA-LÓPEZ et al., 2019).

Nas células cancerígenas, a DOXO se intercala no DNA e interrompe a ação do reparo do DNA mediado pela topoisomerase-II (Figura 5) e promove a geração de radicais livres, danos às membranas celulares, ao DNA e às proteínas, dessa foma, como consequência, aumenta a apoptose (GEWIRTZ et al., 1999; SZYMANSKA et al., 2020; ZANDEN et al., 2020; VARELA-LÓPEZ et al., 2019). Em adição, a doxorrubicina também atua aumentando a inflamação e a autofagia e interfere na homeostase do cálcio (VARELA-LÓPEZ et al., 2019) Estudos mostram que a DOXO induz a apoptose ou morte celular programada em folículos ovarianos de camundongos (BENAHARON et al., 2010; XIAO et al., 2017; WANG et al., 2019).

Figura 5: Mecanismo de ação da doxorrubicina nas células. A) TOP2b relaxa o super-enrolamento do DNA para facilitar a replicação e a síntese do DNA; B) a doxorrubicina forma um complexo pelo DNA através das bases de G e C em ambas as cadeias de DNA e impede a atividade da TOP2b e a síntese de DNA. TOP2b: Topoisomerase 2b, G: guanina, C: citosina.



Fonte: Adaptado: MOBARAKI et al., 2017.

2.8.2 Mecanismos envolvidos na gonadotoxicidade induzida por doxorrubicina

Os efeitos adversos dessa substância incluem toxicidade ovariana em camundongos, reduzindo a taxa de ovulação, tamanho dos ovários e também danifica a microvasculatura ovariana e induz a necrose das células do estroma (LOPES et al., 2020; BEN-AHARON et al., 2010), bem como a menopausa precoce e aumento da taxa de infertilidade em mulheres que mantêm a atividade ovariana após quimioterapia (LETOURNEAU et al., 2012).

Além disso, Xiao et al. (2017) demostraram por meio de cultivo *in vitro* de folículos de camundongas a toxicidade dose-dependente de DOXO que afeta o crescimento folicular, sobrevivência e secreção hormonal. Verificaram ainda que mesmo em dose baixa, a DOXO afeta a maturação oocitária e resulta na anormalidade da divisão meiótica oocitária. Dessa forma, resultados como esses fornecem implicações significativas no entendimento de como o DOXO age de forma negativa nas funções ovarianas. É de suma importância fornecer proteção contra a fertilidade de pacientes em risco de disfunção reprodutiva e infertilidade durante o período de quimioterapia.

Estudos recentes mostraram que o tecido ovariano criopreservado de mulheres jovens submetidas à quimioterapia com este antineoplásico, apresentou um grande número de folículos atrésicos e após o cultivo *in vitro* desse mesmo tecido, observou-se uma diminuição na produção de estradiol e progesterona (PAMPANINI et al., 2019). Após o cultivo de folículos secundários de camundongos em diferentes concentrações de DOXO (20 e 200 nM) por 24 h, a concentração mais alta (200 nM) induziu dano ao DNA e apoptose folicular e oocitária, bem como a diminuição de estradiol, e a concentração mais baixa (20 nM) aumentou anormalidades na morfologia do fuso meiótico e desalinhamento cromossômico (XIAO et al., 2017). Além disso, a administração de uma dose única de DOXO (10 mg/kg) em camundongos interferiu na maturação meiótica (XIAO et al., 2017) e aumentou a apoptose, principalmente, em células da granulosa de folículos em crescimento e oócitos de folículos primordiais (WANG et al., 2019).

Com a finalidade de entender melhor o mecanismo molecular pelo qual a DOXO atua, alguns pesquisadores afirmaram que a perda folicular induzida por esses antineoplásicos é mediada por membros da via PI3K, como a proteína quinase B (AKT), a fosfatase e homólogo de tensina (PTEN) e o Forkhead box O3 (FOXO3a) (CHANG et al., 2015; JANG et al., 2016; WANG et al., 2019).

2.9 Defesa antioxidante no ovário

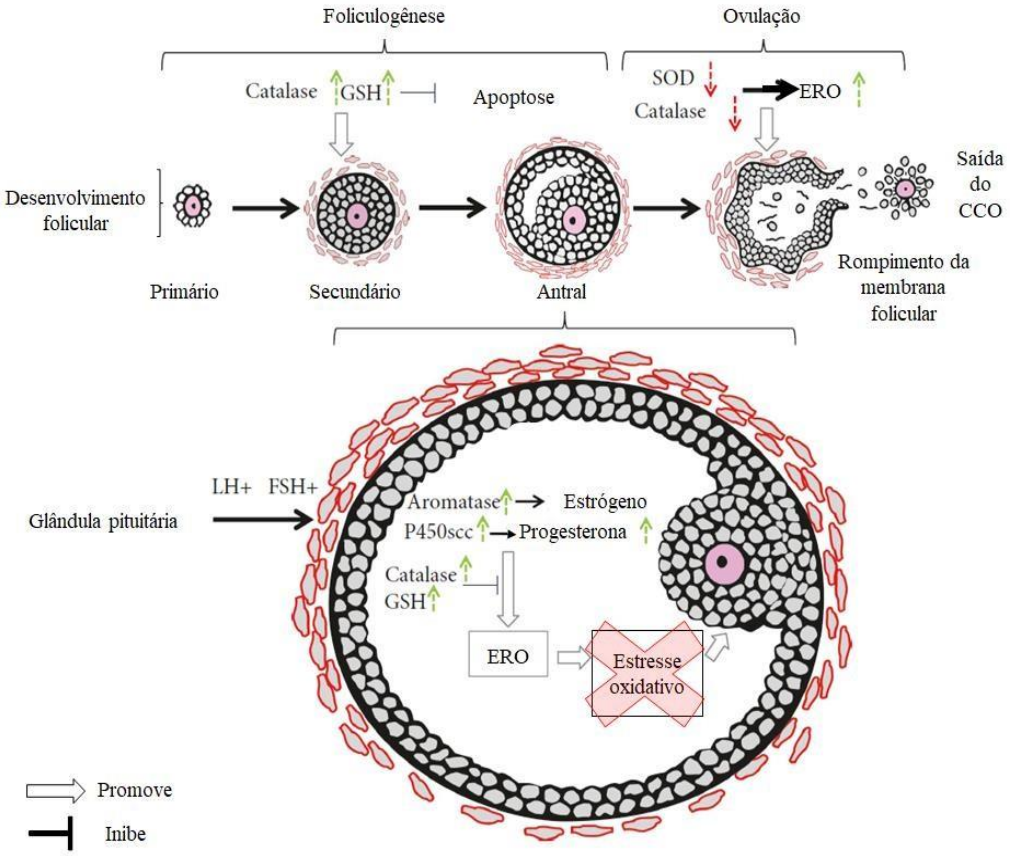
Os organismos são dotados de antioxidantes intracelulares e extracelulares cujo efeito é atrasar, prevenir ou remover o dano oxidativo de uma molécula alvo. As defesas enzimáticas são mais relevantes: superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPx). A SOD é considerada a primeira linha de defesa contra ERO, sua ação resulta em produção aumentada de peróxido de hidrogênio (H2O2), controlável pela atividade de CAT e GPx. A CAT localiza-se principalmente em peroxissomos, onde converter H2O2 em água e oxigênio molecular. Além disso, outros compostos antioxidantes não enzimáticos são sintetizados endogenamente ou adquiridos da dieta. Coenzima Q10 (CoQ) e ácido úrico (UA) são exemplos de antioxidantes endógenos (FREITAS et al., 2017; SIES, 2014). Dessa forma, como em outros tecidos, a defesa antioxidante celular do ovário é composta por uma série de enzimas citoplasmáticas e mitocondriais (MENG et al., 2016).

A relação entre ERO e sinalização antioxidante no ovário é complexa. As ERO ovarianas são mediadores importantes de vários processos, incluindo ovulação, formação de corpo lúteo e angiogênese ovariana, mas também podem ser prejudiciais aos processos ovarianos, como interações do complexo cumulus-oócito e maturação do oócito. Portanto, o

equilíbrio correto entre a geração e eliminação de ERO é essencial para a função ovariana ideal e fertilidade **(**KATZ-JAFFE et al., 2020).

Uma grande quantidade de ERO pode ser produzida durante a esteroidogênese, especialmente, durante a conversão de colesterol em pregnenolona. Neste caso, a CAT e a glutationa reduzida (GSH) protegem os oócitos do estresse oxidativo gerado pelos processos metabólicos fisiológicos, como a esteroidogênese no ovário. A GSH é considerada a principal representante dos antioxidantes não enzimáticos presentes em oócitos e embriões (BEHRMAN et al., 2001). Ao decorrer da foliculogênese, eventos consecutivos e sincronizados são acompanhados pela produção e eliminação de ERO. Os antioxidantes são fortemente modulados durante esse processo. A expressão da CAT e GSH nos folículos é aumentada com o crescimento folicular, enquanto a atividade da SOD é reduzida. Entretanto, ao final do desenvolvimento folicular, ocorre uma redução das enzimas antioxidantes e um aumento de ERO que irão contribuir para que ocorra o rompimento da membrana basal do folículo pré-ovulatório e, em consequência, a ovulação (Figura 8) (WANG et al., 2017).

Figura 6. Representação esquemática da regulação antioxidante durante desenvolvimento folicular. CCO: complexo cumulus-oócito.



Fonte: Adaptado de WANG *et al*., 2017.

Recentemente Rad et al. (2021) indicou que a administração do extrato de agrião *(Nasturtium officinale*) com propriedades antioxidantes, teve efeitos protetores em ovários de ratas contra a toxicidade induzida pela DOXO. O extrato melhorou a função ovariana com relação à produção de progesterona e estrogênio, e melhorou a divisão das células foliculares e da granulosa. Além disso, confirmaram que o extrato também teve efeitos protetores contra a peroxidação lipídica causada pela toxicidade da DOXO. Em um estudo realizado por KATZ-JAFFE et al. (2020) em Camundongos CF-1 fêmeas, com idade mais elevada, foi possivel observar que após a administração de antioxidantes, da polpa do açaí, o transcriptoma ovariano murino refletiu um ambiente com maior sobrevivência celular e sinalização β-adrenérgica, diminuição das vias pró-inflamatórias de sinalização apoptótica e sinalização antioxidante alterada em comparação com animais do grupo controle.

2.10 Antioxidades naturais

Os antioxidantes naturais estão presentes nas mais diversas espécies de plantas e são voltados para diferentes estudos, como extratos ou óleos essenciais, que possuem uma ampla variedade de compostos bioativos (CARNEIRO et al., 2017). Uma variedade de materiais vegetais são conhecidos por serem fontes naturais de antioxidantes, como ervas, especiarias, sementes, frutas e vegetais (LOURENÇO; MOLDÃO-MARTINS; ALVES, 2019). Existem várias classes de compostos fenólicos como ácidos fenólicos, flavonoides, lignanas, estilbenos e taninos, mas os flavonoides são os mais estudados por serem os compostos mais comuns e disponíveis nos vegetais (AMAROWICZ; PEGG, 2019).

Um antioxidante natural funciona direta ou indiretamente para minimizar os danos às biomoléculas causados por ERO e/ou espécies reativas de óxido de nitrogênio (ERON). Essa proteção ao estresse oxidativo pode ser realizada por um sistema enzimático ou não enzimático (UNUOFIN et al., 2020). Para prevenir a interação entre radicais e moléculas biológicas, os antioxidantes devem estar próximos ao local de formação do radical, competindo com o radical livre pelo substrato biológico, mantendo os níveis de EROs fisiologicamente adequados ao funcionamento celular. Se um antioxidante não estiver presente em quantidade suficiente para neutralizar ERO, ocorrerá oxidação de biomoléculas, como peroxidação lipídica, dano de proteína (carbonilação de resíduos de ácido aminoalcanóico), oxidação de nucleotídeos de DNA e RNA únicos, inibição de enzimas e ativação de apoptose (DUMANOVIĆ et al., 2021).

Dessa forma, vários compostos extraídos de plantas utilizadas na medicina herbal, principalmente aquelas com capacidade antioxidante, ganharam relevância significativa por seu

potencial no combate às espécies reativas de oxigênio durante o cultivo *in vitro* de folículos ovarianos, como por exemplo: a rutina (LINS et al., 2017); o resveratrol (ROCHA et al., 2018); o extrato de *Amburana cearensis* (GOUVEIA et al., 2016; MENEZES et al., 2018) e o extrato de *Justicia insularis* (MBEMYA et al., 2018). Esses compostos foram capazes de manter adequado equilíbrio entre a produção de ERO e os sistemas de defesa antioxidante favorecendo, dessa forma, a manutenção de taxas aceitáveis de folículos e oócitos viáveis. O uso do extrato da AV, que tem grande potencial antioxidante, foi utilizado na criopreservação de gametas masculinos de catetos e aumentou a motilidade espermática em 50% após a descongelação (SOUZA et al., 2016).

2.11 *Cimicifuga racemosa* (L.) Nutt.

O uso de plantas com a finalidade de tratamento e cura de enfermidades é quase tão antigo quanto a espécie humana e, por vezes, o conhecimento sobre a aplicabilidade de plantas medicinais pode representar o único recurso terapêutico de diversas comunidades e grupos étnicos. Indiretamente, a cultura medicinal pode despertar o interesse de pesquisadores em estudos multidisciplinares, que vise fontes naturais, seguras e de baixo custo (MACIEL et at., 2002; VALERIANO et al., 2019).

Ademais, a Organização Mundial da Saúde (OMS) recomenda a prática do uso de plantas medicinais nos programas de atenção primária à saúde como forma de diminuir os custos destes programas e ampliar o número de beneficiados, principalmente nos países subdesenvolvidos e em desenvolvimento onde persistem os grandes bolsões de pobreza (BIESKI, 2004).

A CIMI (Figura 6) é uma planta herbácea, pertencente à família Ranunculaceae, originária do Canadá e costa atlântica dos Estados Unidos (Nomenclatura botânica oficial: *Cimicifuga racemosa* (L.) Nutt. e nome popular: Cimicifuga, Black cohosh (BORRELLI; ERNST, 2008), a qual foi muito utilizada na medicina popular coreana para aliviar dor e inflamação, para o tratamento de cólicas menstruais e nos sintomas da menopausa (BOLLE et al., 2007). Os americanos também tinham o hábito de utilizar CIMI para aliviar a dor reumática crônica, tosse, resfriados (FOSTER, 1999).

No que diz respeito às suas propriedades, estudos atuais demonstram que a CIMI pode aliviar os sintomas da menopausa, de doenças cardiovasculares, reabsorção óssea (osteoporose) e até mesmo depressão (MCKENNA et al., 2001; MORELLI et al., 2002). Ainda, pode atuar como antioxidante (BURDETTE et al., 2002) e pode auxiliar em processos alérgicos (KIM et al., 2004).

Figura 7: Imagem representativa da *Cimicifuga racemosa* (L.) Nutt.

Fonte: Odla.nu

O mecanismo de ação da CIMI é controverso, visto que para a redução de ondas de calor, a CIMI modula as vias de sinalização do neurotransmissor no cérebro e não exerce efeito estrogênico (FRIEDERICHSEN et al., 2020). Estudos envolvendo camundongos ob/ob que possuem uma perda espontânea de função de um único gene, mutação que gera obesidade maciça, mostraram que quando tratatados com o extrato de CIMI foi possível obesrvar uma redução significativa da ingestão de água e uma normalização da resposta de glicose e insulina, assim a CIMI pode melhorar o controle metabólico via melhora da sensibilidade do receptor de insulina (MOSER et al., 2014; CASTRO et al., 2018).

Um dos estudos feito com extrato de CIMI, nomeado Ze 450, preservou a integridade mitocondrial e os níveis de ATP e impediu a formação de ERO mitocondrial e morte celular, dados obtidos por meio do cultivo *in vitro* de célula neurais e hepáticas. Algumas organelas presentes nas células são alvos de pesquisas a fim de compreender a funcionalidade celular. Dentre essas, a mitocôndria têm despertado maior atenção devido ao seu envolvimento direto no processo apoptótico. A taxa de acidificação extracelular revelou que o Ze 450 mediou uma mudança na respiração celular mitocondrial para a glicólise, e essa mudança metabólica foi um pré-requisito para os efeitos protetores contra danos oxidativos (RABENAU et al., 2019; OLIVEIRA et al. 2013)

Ademais, foi visto que a CIMI possui efeito protetor na periodontite através da inibição de citocinas (TNF-α), além de representar uma opção terapêutica segura no ensaio pré-clínico de periodontite em ratas (OLIVEIRA, 2013). Considerando que CIMI é um fitomedicamento que já está no mercado brasileiro com indicação para alívio dos

sinais/sintomas da menopausa, a possibilidade de uma nova indicação terapêutica para um fitomedicamento já disponível nas prateleiras poderá determinar uma considerável redução no tempo necessário para o desenvolvimento de novos medicamentos. No entanto, ainda não se sabe acerca de sua segurança (toxicidade) quanto ao uso do medicamento sobre a fertilidade feminina quando administrado no período reprodutivo.

Até a presente data, a investigação de sete espécies da planta CIMI levou à identificação de mais de 457 compostos. Estudos fitoquímicos revelaram que certos compostos de CR se destacam, incluindo glicosídeos triterpenos, constituintes fenólicos e formonectina (uma isoflavona) (CAMPOS et al., 2012). Anos de pesquisas farmacológicas provaram que os extratos brutos e certos compostos puros obtidos de CIMI apresentam efeitos benéficos no tratamento da síndrome da menopausa, anti-osteoporose, antivirais, antitumorais, antioxidantes e antiangiogênicas. Por outro lado, toxicidade induzida por plantas de CIMI sobre o fígado, coração e sistemas nervoso e periférico foram relatados. Portanto, a consideração de segurança deve ser colocada em alta prioridade para a terapia herbal com *Cimicifuga spp.* (GUO et al., 2017).

É importante enfatizar o potencial tóxico de muitos desses produtos na fisiologia reprodutiva (SINSEN, 2010), o que só vem a endossar a necessidade de estudos que fundamentem cientificamente tal prática a fim de minimizar ao máximo possíveis danos à reserva folicular presente no ovário. Portanto, projetos que visem estudar o perfil farmacológico de toxicidade de plantas medicinais comumente usadas pela comunidade devem ser considerados estratégias prioritárias pelas instituições nacionais de fomento à pesquisa.

Nesse sentido, nossa proposição é, portanto, avaliar a eficácia da CIMI, com efeitos já conhecidos de reposição hormonal na menopausa, sobre a prevenção dos possíveis efeitos adversos do uso crônico de DEXA e DOXO sobre a atividade reprodutiva.

3 JUSTIFICATIVA

Avanços recentes no tratamento de doenças neoplásicas aumentaram a sobrevida a longo prazo de pacientes submetidos a quimio-radioterapia (ROTI et al., 2012). Contudo, a utilização de medicamentos inibidores de topoisomerases, como, por exemplo, a Doxorrubicina (DOXO), usada na quimioterapia pode provocar danos nos ovários, levando a efeitos adversos na foliculogênese, afetando aspectos relacionados a saúde reprodutiva, incluindo o desenvolvimento folicular pré-antral. Os efeitos gonadotóxicos são particularmente importantes na fisiologia reprodutiva, pois a reserva folicular ovariana é limitada e não renovável, diminuindo ao longo dos anos até a completa depleção, o que caracterizaria o status de menopausa (RODRIGUEZ; OKTAY, 2014). Além disso, os medicamentos antineoplásicos, tendo a finalidade de parar ou inibir o crescimento e a disseminação de tumores, promovem parada na divisão ou morte celular, por interferirem na síntese de DNA, RNA, ou na replicação celular, podendo induzir a insuficiência ovariana independente da idade da paciente, culminando em fertilidade reduzida e amenorréia. (PARTRIDGE; RUDDY, 2007).

Estudos anteriores mostraram que o aumento induzido pelo estresse nos glicocorticóides circulantes ou a administração exógena do glicocorticóide sintético como a Dexametasona (DEXA) inibe as funções do hipotálamo e da hipófise, reprimem a transcrição do GnRH e, consequentemente, reduzem os níveis de FSH e LH (WHIRLEDGE; CIDLOWSKI, 2017). Estudos recentes demonstraram que a DEXA pode influenciar negativamente a oogênese durante o desenvolvimento fetal em humanos (POULAIN et al., 2012). Além disso, já foi demonstrado que a DEXA regula o processo de apoptose nas células da granulosa de folículos pré-ovulatórios (YUAN et al., 2014).

Diante do exposto, a investigação acerca de substâncias que possam bloquear ou amenizar os efeitos adversos causados por quimioterápicos, como a DOXO, e glicocorticoides, como a DEXA, possui um grande potencial. Nesse sentido, o uso de produtos alternativos, como fitoterápicos, tem sido apontado como uma alternativa promissora.

A *Cimicifuga racemosa* (L.) Nutt. (CIMI) é comumente utilizada na medicina popular para aliviar dor e inflamação, para o tratamento de cólicas menstruais e nos sintomas da menopausa (BOLLE et al., 2007). Considerando a crescente consolidação da CIMI no tratamento da menopausa (MCKENNA et al., 2001; MORELLI et al., 2002), bem como a importância do estudo de redirecionamento de fármacos, acredita-se que esta proposta seja valiosa e justificada frente à possibilidade da CIMI reduzir os efeitos deletérios da DOXO e da DEXA sobre a função folicular e reprodutiva, o conhecimento sobre plantas medicinais simboliza muitas vezes o único recurso terapêutico de muitas comunidades e grupos étnicos.

No entanto, ainda não se sabe acerca da segurança (toxicidade) do uso deste medicamento sobre a fertilidade feminina quando administrado no período reprodutivo e ainda se esta substância é capaz de amenizar os efeitos adversos causados pela DOXO ou DEXA. A investigação dos efeitos tóxicos de drogas quimioterapêuticas e glicocorticóides no tecido ovariano humano usando estudos *in vitro* é difícil de ser implementado devido à indisponibilidade de tecido ovariano. Portanto, estudos *in vitro* conduzidos utilizando modelos animais são amplamente encorajados (GANDOLFI et al., 2006).

A CIMI já é encontrada em medicamentos que controlam efeitos do climaterio (FRIEDERICHSEN et al., 2020), e o estudo da possível eficácia e segurança deste fitofármaco na redução dos efeitos adversos causados pela DOXO e DEXA no ovário, poderá contribuir para a identificação de um a nova abordagem terapêutica no tratamento da infertilidade. Além disso, a probabilidade de utilizar uma droga que já está no mercado poderá reduzir sobremaneira o grande intervalo de tempo inerente aos ensaios pré-clínicos e clínicos para o desenvolvimento de um novo fármaco, além de sugerir uma nova indicação terapêutica para um fármaco que já demonstrou ser eficaz e seguro.

4 HIPÓTESES

Diante do exposto, foram formuladas as seguintes hipóteses científicas.

- A adição *Cimicifuga racemosa* (L.) Nutt. ao cultivo *in vitro* de ovários de camundongas promove a ativação e desenvolvimento folicular mantendo a morfologia adequada das células foliculares e do tecido ovariano.
- A *Cimicifuga racemosa* (L.) Nutt. reduz os níveis de atresia induzda por Dexametasona e Doxorrubicina.
- A *Cimicifuga racemosa* (L.) Nutt. apresenta efeito protetor sobre o modelo de toxicidade reprodutiva induzida por Dexametasona e Doxorrubicina *in vitro*.

5 OBJETIVOS

5.1 Objetivo Geral

Avaliar a influência de diferentes concentrações de *Cimicifuga racemosa* (L.) Nutt. (5, 50, ou 500 ng/ml) sobre os efeitos da Dexametasona (4 ng/ml) e Doxorrubicina (0,3 µg/ml) no desenvolvimento de folículos ovarianos pré-antrais de camundongas cultivados *in vitro*.

5.2 Objetivos Específicos

i. Analisar os efeitos de diferentes concentrações de *Cimicifuga racemosa* (L.) Nutt. sobre a ativação, crescimento e sobrevivência de folículos ovarianos pré-antrais, bem como a ação na configuração da matriz extracelular (MEC) do ovário de camundongas após 6 dias de cultivo *in vitro*.

ii. Avaliar a influência da *Cimicifuga racemosa* (L.) Nutt. sobre a toxicidade induzida por Doxorrubicina (0,3 µg/ml) (capítulo 2) e Dexametasona (4 ng/ml) (capítulo 3) em ovários de camundongas após 6 dias de cultivo *in vitro*, analisando os seguintes parâmetros:

- Morfologia folicular (sobrevivência) por histologia clássica;
- Ativação de folículos primordiais por histologia clássica;
- Crescimento folicular por histologia clássica;
- Configuração da matriz extracelular (MEC) através dos níveis de colágeno;
- Densidade das células do estroma ovariano por histologia clássica;
- Apoptose folicular através da análise da expressão de caspase-3 ativada por imuno-histoquímica;
- Viabilidade de folículos ovarianos por microscopia de fluorescência.

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CAPÍTULO 1

Potential of natural antioxidants to minimize the adverse effects of chemotherapy on female reproductive system

Potential of natural antioxidants to minimize the adverse effects of chemotherapy on female reproductive system

Assis, E.I.T.[a, b]; Lima Neto, M. F.[a, b]; Costa, F.[a, b]; Godinho, A.N.[b]; Freire, J.M.O.[b]; Silva, J.R.V.[a,b]; Silva, A.W.B.[a, b]*.

[a] Laboratory of Biotechnology and Physiology of Reproduction -LABIREP - Biotechnology Nucleus of Sobral – NUBIS, Federal University of Ceara, Sobral, CE, Brazil.

[b] Nucleus of Research in Animal Experimentation – NUPEX, Federal University of Ceara, Sobral, CE, Brazil.

*Corresponding address (A.W.B., Silva): Biotechnology Nucleus of Sobral - NUBIS, Federal University of Ceara, Av. Comandante Maurocélio Rocha Ponte 100, CEP 62041-040, Sobral, CE, Brazil. Phone / Fax: +55 88 36954744 [andersonweiny@sobral.ufc.br]

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Abstract

Adverse effects of cancer chemotherapy tretments includes fertility problems in women. The antineoplastic agents induce oxidative stress in biological systems and can cause damage to female gametes and lead to a series of reproductive diseases. Natural extracts are increasingly being explored and applied in several research areas, mainly those that have antioxidant potential. *Cimicifuga racemosa* (L.) Nutt. (*C. racemosa*), which is already used to relieve menopausal symptoms, has several properties, including antioxidants activities. The purpose of this review is to evaluate the evidences of the effectiveness of *C. racemosa* as as an antioxidant and to provide updated information on the genus *Cimicifuga spp* and plants with antioxidant

properties capable of reducing the damage caused by chemotherapy. The relevant information about *C. racemosa* was collected from scientific databases (PubMed, Google Academic and Scielo). Chemical constituents from *C. racemosa* include triterpenoid glycosides, phenylpropanoids, nitrogen compounds, chromones, flavonoids and 4α-methyl steroid. So far, investigation of seven plants from *Cimicifuga spp*. genus led to the identification of more than 457 compounds with terapeutic action. Years of pharmacological research have proven that crude extracts and certain pure compounds obtained from this plant exhibited anti-osteoporosis, antiviral, anti-tumor, antioxidant and anti-angiogenic activities and have also been use for treatment of menopause syndrome, while can make changes in the serotonin pathways or even acting as a selective modulator of the estrogen receptor. Adverse effects are extremely uncommon and there are no known significant adverse drug interactions. *C. racemosa* has an excellent antioxidant potential against the negative effects caused by reactive oxygen species produced *in vivo* and *in vitro*. It is possible that *C. racemosa* may be able to reduce tissue damage and decrease the causes of infertility after cancer treatment.

Key-words: herbal medicine, *Cimicifiga racemosa*, ovary, fertility, reproduction

1. Introduction

In the current epidemiological scenario of cancer worldwide, 28.4 million new cases are projected to occur in 2040. This increase corresponds to 47% of the total of 19.3 million cases registred in 2020 (SUNG et al., 2021). Despite the major discoveries in cancer treatment nowadays, the rates of cancer-related mortality have remained relatively stable, patients who received chemotherapy have an increase in survival in different types of cancer (FENG et al., 2019; GAHAGAN et al., 2020). On the other hand, traditional and more recent modalities used to treat cancer such as surgery, radiation therapy, chemotherapy, targeted therapy and immunotherapy can cause side effects that negatively impact on quality of life and in reproduction. Therefore, the search for more effective and tolerable anticancer therapies has great potential for human medicine (WANG et al., 2021).

Women who have been treated with chemotherapy drugs for cancer have an increased possibility to exhibit fertility problems after treatment that affect the chance of a healthy pregnancy. The infertility disease caused by chemotherapy is affecting an increasing number of patients, given the improvement in survival rates for most cancers, including childhood cancers and those affecting younger women. High-dose of alkylating agents and abdominal or pelvic radiotherapy affect gonadal function in a dose-related manner, while old

patients showing more risk of suffering from an age-related decline in ovarian reserve. The gonadal lesion is clinically manifested as insufficiency of ovarian hormone (delayed or interrupted puberty, premature ovarian failure or premature menopause) and infertility (VAN DORP et al., 2018; LOPES et al., 2020). However, survival rates in these patients have been increasing over the years due to the greater effectiveness of new cancer treatments. The 5-years survival rates for pediatric cancer patients approaches 80% (MELAN et al., 2018), these treatments often result in impaired or absent reproductive capacity. In addition, only about half of the patients that reported reproductive problems, having previously been informed about therapeutic options for preserving fertility, which is pointed out as the second most important aspect among young patients after survival. In order to improve the quality of life and survival of patients, the improvement of techniques that optimize fertility preservation has become an important factor of reproductive biology research. *In vitro* and *in vivo* studies are of great relevance, since cultured cells or animal models can be exposed to a carcinogen to establish a neoplastic state before being treated with an anticancer agent and this provides evidence of the effect of an anticancer agent in the initial stages of cancer (MOORE et al., 2016).

Among the progress achieved by the research community to minimize the effects of chemotherapeutic treatments, the combination therapy has become an excellent strategy that serves as an important direction for the development of natural products in cancer therapy. Adjuvant treatment using natural products associated with current regimens could be beneficial in multiple aspects, including reducing the adverse effect, overcoming the drug resistance and improving the therapeutic response (HUANG et al., 2021).

Natural extracts of plants are increasingly being explored and applied in different areas of reproductive biology research, showing relevance for the treatment of many diseases. A plant extract that is increasingly proving efficient for the most diverse applications is the extract of *Cimicifuga Racemosa (L.) Nutt.* (*C. racemosa*), that is a species belonging to the Ranunculacea family and to the genus Actae, native of the United States. The rhizomes of *Cimicifuga* are important source of active coumpounds and have been widely used all over the world (HU et al., 2021). *C. racemosa* was already used by American Indians to treat rheumatism and gynecological disorders. Currently, extracts and preparations from your rhizome are mainly used to relieve climacteric complaints and are an effective and safe alternative to hormone replacement therapy. In its phytotherapic registration list, the Brazilian National Health Surveillance Agency (ANVISA) indicates *C. racemosa* for the treatment of climacteric symptoms (SANTOS and SANTOS, 2017; JÖHRER et al., 2020).

A *Cimicifuga racemosa* extract, called Ze450, showed pronounced effects on energy metabolism through activation of AMP-activated protein kinase in neuronalcells *in vitro*, and

beneficial anti-diabetic effects in vivo. Metabolic switch induced by *Cimicifuga racemosa* extract prevents mitochondrial damage and oxidative cell death, showing a promising therapeutic potential in age related diseases involving oxidative stress and mitochondrial damage (RABENAU *et al.,* 2019). A recent study investigated the immunomodulatory effect of *Cimicifuga heracleifolia* specie. HU et al. (2021) identified a triterpene glycoside as major bioavailable components. This component showed a strong anti-inflammatory activity on airway inflammation mice model by reducing the release of inflammatory cytokines and chemokines.

Oxidative stress is a key component in the process of carcinogenesis. Stimulated by endogenous and exogenous factors, such as reactive oxygen species (ROS), it induces cell damage (DAS et al., 2021). Although radiation produces ROS, some anticancer agents, such as alkylating agents and platinum and anti-tumor antibiotics, exert cytotoxicity by generating free radicals. Some endogenous antioxidant defense mechanisms, such as superoxide dismutase (SOD), glutathione peroxidysis (GPx) and catalase (CAT), can counterbalance oxidative microenvironments (VATNER et al., 2020). Exogenous non-enzymatic antioxidants, such as vitamins, minerals and polyphenols, also have the ability to minimize ROS activity. Therefore, antioxidant therapies can reduce the adverse effects of chemotherapy and/or radiotherapy, but they can antagonize the anti-tumor activity (YASUEDA et al., 2015).

The use of natural antioxidants against genotoxicity induced by antineoplastic drugs has become promising. Antioxidants can reduce the production of ROS, attenuating the activity of enzymes responsible for producing these oxidizable compounds and thus, increasing the activity of antioxidant enzymes, such as CAT, GPx, SOD and peroxiredoxins (HALLIWELL, 2006). In this context, the aim of this study is to show the evidences of effectiveness of *Cimicifuga racemosa* as a potent antioxidant and to provide updated information on the *Cimicifuga* and plants with antioxidant properties capable to reduce the damage caused by chemotherapy.

2. Adverse effects of chemotherapy on famele fertility

Advances in early detection of cancer and therapeutic treatments for this disease offered a greater chance of cure and survival of patients. However, despite these significant advances, survived pacients may have decreased quality of life because of side effects of treatments and their consequences in several fields, including reproductive health (BELLVER; DONNEZ, 2019). Chemotherapy uses drugs to kill or slow the growth of cancer cells. These are called cytotoxic drugs and they are designed to affect fastgrowing cells such as cancer cells.

This means they can also damage other cells that grow quickly, such as the reproductive cells. On this way, chemotherapy damages the gametes by enhancing apoptosis, and evidence suggests that targeted anti-apoptotic therapy may preserve fertility in patients receiving chemotherapy (LISA et al., 2018).

Updated data on cancer incidence and mortality show that breast cancer is the most commonly diagnosed, with an estimated 2.3 million new cases for year (SUNG et al., 2021). Tretament of this type of cancer involves the use of anthracyclines and taxanes, which have been reported to increase rates of amenorrhea in women undergoing chemotherapy for breast cancer (HAN et al. 2009). In addition to amenorrhea, more recent studies have highlighted the occurrence of chemotherapy-induced early menopause in pre-menopausal women after or during treatment of breast cancer (YEO et al. 2020; SHIN et al., 2019). These systemic effects and disorders in female reproductive physiology caused by antineoplastic therapy are justified by direct or indirect action of chemotherapeutic agents on the reproductive system.

It is accepted that the female reproductive system is more sensitive to various types of toxics and the antineoplastics drugs can affect uterus, ovaries and their functions. The gonadotoxic effects of antineoplastic agents on female reproductive system are widely studied and impair fertility through suppression of ovarian function. It causesg damage to ovarian follicles, by inhibiting cell division, functions of DNA, and causing mitochondrial damage and the formation of ROS (SPEARS et al., 2019; MORGAN et al. 2012). However, some individual factors influence this toxicity, such as age, the dosage of chemotherapeutic agents, time and type of treatment. Studies have shown that treatment with antineoplastics in women affects the uterus by promoting volume loss (VAN DE LOO et al. 2019; BENEVENTI et al. 2015). In addition, there is evidence of damage caused by chemotherapy to the uterine vascularization and to cells forming the endometrium and myometrium (GRIFFITHS; WINSHIP; HUTT, 2019). In a study using mice as an animal model, the chemotherapeutic agent doxorubicin, used to treat different types of cancer, directly reached the uterus and altered gene expression in response to estrogen synthesis (ANDERSEN et al. 2019). Besides, doxorubicin reaches the ovary and can decrease the ovarian follicular reserve by inducing atresia and over activation of follicles, especially the primordial and growing follicles (WANG et al. 2019; XIAO et al. 2017).

In this sense, the ovaries are more affected by chemotherapy regimes because they are highly dynamic due to their functions of producing gametes and hormones. The primordial follicles form the ovarian reserve and are recruited and initiate to grow up to ovulation, but most of them are lost by atresia. Thus, antineoplastic drugs cause follicular death as granulosa cells of follicles in early stages of development have mitotic index. Studies in mice shows that antineoplastic agents cause the activation of primordial follicles through the

PI3K/PTEN/Akt/Foxo3a pathway, which can causes the depletion of the ovarian reserve (KALICH-PHILOSOPH et al. 2013; CHANG et al., 2015; WANG et al. 2019; GOLVEIA et al., 2021). However, a recent study showed that genotoxic chemotherapy with cyclophosphamide *in vivo* triggers loss of primordial follicles by apoptotic pathways without growth activation (TITUS et al., 2021). The primary mechanism of action of antineoplastic drugs is through damage to DNA, which consequently activates the cell apoptosis pathways. The interaction with DNA can be through alkylation, intercalation or complexation, for example. These interactions block normal DNA functions, such as transcription and replication. Studies have shown that Cisplatin (cis-diaminodichloroplatin II), one of the most potente antineoplastic drugs, reduces ovarian reserve by inducing oocyte DNA damage and increasing follicular apoptosis and reactive oxygen species (ROS) levels, as well as decreasing antioxidant enzyme expression and mitochondrial function (BARBERINO et al., 2017). It was recently demonstrated that Protocatechuic acid (PCA, 3,4-dihydroxybenzoic acid), a major metabolite of antioxidant polyphenols, found naturally in green tea, fruits, brown rice, nuts, and medicinal plants, can be used to protect mouse ovarian follicles during experimental treatment with cisplatin. PCA reduces follicular apoptosis, maintains cell proliferation and mitochondrial function, reduces ROS production, and increases GSH expression. The protective effect of PCA against cisplatin-induced damage in mouse ovarian follicles may be attributed to p-PTEN and p-FOXO3a proteins (GOVEIA et al., 2021). Lins et al. (2020) showed that the flavonoid rutin (quercetin-3-O-rutinoside) witch is predominantly found in the buckwheat, cranberries, mulberry and citrus fruits, has the potential to protect the ovarian follicles against ciscplatin-induced toxicity throught its antioxidante effects and PTEN/FOXO3a pathway.

Furthermore, there is evidence of indirect follicular death due to vascular and stromal damage by antineoplastic agents (SOLEIMANI et al. 2011) causing ischemia or necrosis of ovarian tissues in vitro, besides decreasing blood flow women (BEN-AHARON et al. 2012). Another damage mechanism is oxidative stress through the generation of ROS and decreased glutathione (GSH), since antineoplastic agents are associated with mitochondrial damage and oxidative stress pathways (LINS et al. 2020; GORINI et al. 2018). Additionally, mitochondrial damage can deregulate the respiratory chain by decreasing adenosine triphosphate (ATP) and losing its energy potential.

Due to the progress in assisted reproduction technologies, several fertility preservation methods have been developed and are being studied to help cancer patients to preserve their fertility. The highlight for cryopreservation of embryos and oocytes, cryopreservation of ovarian tissue, *in vitro* maturation of oocytes, artificial ovaries and technologies with stem cells and GnRH agonist therapy has been proposed. These techniques involve the removal of gametes

from women before cancer treatment and have been extensively reviewed in several studies. To understand the mechanisms involved in the damage caused by chemotherapy in women's reproductive health, several studies for the development and discovery of substances that limit the negative impact of chemotherapy in the ovaries have been conducted (CHO et al., 2020; HARADA; OSUGA, 2018)

Ovarian protection from toxic effects of antineoplastic agents can help to relieving symptoms associated with premature menopause. In this sense, the use of antioxidant substances to reduce the damage caused by chemotherapeutic drugs to ovaries and the reproductive system appers as alternative (BARBERINO et al., 2017; GOLVEIA et al., 2021; LINS et al., 2020).

3. Natural antioxidants and theiraction against gonadotoxicity caused by chemotherapeutic agents

To reduce the reproductive problems raised by gonadotoxicity of chemotherapeutic agents, many efforts have been spared and some progress has been achieved in the field of female fertility preservation. The development of substances capable of protecting oocytes from deleterious effects of chemotherapeutic drugs represents such a potential major improvement to preserve fertility for women (ROSSI et al. 2017). Thereupon, exploring mechanisms and identifying protective agents against ovarian toxicity during chemotherapy is a high priority. Considering that chemotherapeutic agents can promotes severe damage to normal physiology of the ovary by inducing oxidative stress, natural substances with antioxidant activity have been the target of several studies (MIKULA-PIETRASIK et al., 2019).

A recent study conducted by Lins et al. (2020) evaluated the beneficial effects of pre-treatment with rutin in ovaries of mice submitted to treatment with cisplatin. In this study, a dose of 10 mg/kg of rutin before cisplatin administration showed higher percentages of morphologically normal follicles and lower rates of apoptosis, in addition to increasing intracellular GSH levels and decreasing ROS production compared to treatment with cisplatin alone. In addition, it was found that the same dose was able to increase mitochondrial activity in the follicles. In the same study, in order to elucidate a possible molecular mechanism of the protective effect of rutin in ovarian injury induced by cisplatin, the authors evaluated the immunoreactivity for the proteins p-PTEN and p-FOXO3a, members of the PI3K pathway. It was found that treatment with cisplatin increased PTEN phosphorylation and decreased expression of phosphorylated FOXO3a with a suppressive effect on follicular activation. However, in treatment with rutin (10mg/kg), activation of the PI3K pathway increased

expression of p-FOXO3a and nuclear exclusion of p-FOXO3a, compared to treatment with cisplatin. Previous studies have already shown that deletion of specific PTEN in oocytes prevents follicles from undergoing apoptosis (REDDY et al., 2008; REDDY et al., 2009), thus these results reflect the possibility of direct effect of rutin in reducing apoptosis follicular by regulating the levels of p-PTEN and p-FOXO3a.

Melatonin is another compound with protective efficacy against ovarian damage induced by chemotherapy. Wang et al. (2020) showed that when melatonin was administered together with epirubicin, 60 mg/kg melatonin dose significantly restored serum levels of estradiol and progesterone in serum in mice. In addition, melatnoninprotected the pool of primordial and secondary follicles. Co-treatment with melatonin negatively regulated the expression of the pro-apoptotic genes *p53*, *Caspase 3*, *Caspase 9* and *Bax*, and conversely, significantly increased the expression of *Bcl-2* and *Bcl2l1*. Markers of antioxidant activity were also measured, showing that melatonin has a positive effect on the expression of genes of important antioxidant enzymes such as *SOD* and *CAT*. In addition, there was a reduction in ROS levels in the ovary treated with melatonin. Together, these findings indicate that melatonin can be used to reduce the side effects of epirubicin, acting as a combination chemotherapeutic agent for the treatment of young women with cancer (WANG et al., 2020).

Resveratrol is another compound that has been investigated in various studies. This phenolic substance present in fruits such as grapes is known for its antioxidant properties, and as an eliminator of reactive oxygen species, such as radicals induced by hydroxyl, superoxide and metal (BREUSS et al., 2019). In a model of Sprague-Dawley rats submitted to treatment with cisplatin, Chinwe et al. (2018) showed that treatment with resveratrol promoted better levels of GSH, SOD and CAT in ovarian tissues submitted to treatment with cisplatin. In that same study, resveratrol reduced the levels of malondialdehyde (MDA) and lipid peroxidation. Lipid peroxidation is the most notable sign of cell damage, causing the membrane to change polyunsaturated fatty acids into hydroperoxides and further degradation in low molecular weight species. Jiang et al. (2019) also showed negative regulation in the levels of MDA in ovarian cells of mice submitted to treatment with cyclophosphamide and busulfan. Taken together, these findings strongly suggest that resveratrol prevents cisplatin-induced ovarian toxicity in rats.

Quercetin is one of the flavonoids abundantly distributed in fruits, vegetables, olive oil, red wine, and tea. Recently, the positive effect of quercetin against damage caused by oxidative stress in ovaries of rats and *in vitro* culture of granulosa ovarian cells were reported by Wang et al. (2019). However, the antioxidant effect of this compound against the damages induced by cyclophosphamide in mouse BALB/c ovaries was explored by Elkady et al. (2019).

In this report, the authors found that quercetin, in a dose-dependent manner, significantly decreased oxidative stress in ovarian tissue. This was confirmed by a significant decrease in the levels of MDA a similar condition was observed in terms of GSH levels, high-dose quercetin significantly increased levels of reduced glutathione, but in low doses, no significant effect on GSH levels was observed. In addition, it was also found that high-dose quercetin promoted anti-apoptotic protection and anti-inflammatory effect, which was proven by verifying the decrease in the score of ovarian caspase 3 expression and decrease in NF-kB, respectively. Moreover, quercetin can be applied against the gonadotoxic effects of cadmium, carcinogenic element, in the ovaries of rats and mice, eliminated free radicals and activated activity of important antioxidant enzymes, such as CAT, SOD and GPx, thus improving the health of the ovaries and increasing hormonal secretion (estradiol, FSH and LH) (LI et al., 2017; NNA et al., 2017).

Azam et al. (2017) analyzed the protective effect of fennel (*Foeniculum vulgare*) on morphology, hormone production, and follicle condition in mouse ovary against the destructive effects of cyclophosphamide (CP). These authorsshowed that the fennel extract caused a significant increase in the serum levels of oestrogen and progesterone, and that this compound can reduce the negative effects caused by CP treatment that was acts on the number of primary, secondary, antral, and atretic follicles. So, these findings indicate a protective effect of fennel on mice ovarian tissue trated with CP.

Natural products are considered a rich reservoir of bioactive compounds with therapeutic potential. The extracts of roots and rhizomes of *C. racemosa* have been administered in traditional Asian medicines as antiinflamatory, antipyretic and analgesic medicines (FRIEDERICHSENet al., 2020).

Chemotherapy drugs are responsible for an excessive production of ROS and inflammatory mediators that trigger pathological mechanisms of cell damage, altering the normal physiological parameters of cells. In this way, antioxidants can play an important role in the control of ROS production (RAPA et al., 2021). Recently, it was reported that the extract of *C. racemosa*, improved the antioxidant status in the ovary of rats with letrozole-induced policistical ovary syndrome, restoring the normal activities of MDA and GSH in this organ. Hypothetically, these benefits could occur during the chemotherapy process that triggers high ROS production (AZOUZ et al., 2021).

C. racemosa contains in its chemical components: triterpene glycosides, actein, 12-acetyl-actein, 27-deoxyxactin; quinolizine alkaloids: N-methylcitisin, tannins, acidic compounds (acetic, butyric, formic, gallic, isoferulic, oleic, palmitic, salicylic); estrogenic principle: formononetine (isoflavone), bitter principles, resin (mixed complex actein) photoesterols, and essential oils (DIETZ et al., 2016). Scientific studies point to the following

active compounds with marked therapeutic effects: formonocetin which helps in regulating estrogen balance and suppression of excessive LH secretion, and 27-deoxyactein, which has an estrogenic activity in addition to its ability to reduce serum concentration do LH (FIRENZUOLI et al., 2011; F. BORRELLI and E. ERNST 2008; MOHAMMAD-ALIZADEH-CHARANDAB et al., 2013). Triterpene glycosides, characterized by a cycloartan framework and a pentose portion, exhibited relevant cytotoxic effects against human breast cancer cells (JÖHRER et al., 2020). Brazilian ANVISA registers isoferulic acid or 27-deoxyactein as a chemical constituent of *C. racemosa* standardization/marker (SANTOS and SANTOS et al., 2017).

According to clinical studies, the adverse effects associated with the use of *C. racemosa* were rare, mild and reversible. The information obtained in pharmacovigilance records made by 16 laboratories producing preparations based on *C. racemosa*, based on spontaneous reports from users (SILVA et al., 2009). It is one of the most controversial natural therapies used among breast cancer patients due to its ambiguous estrogenic or antiestrogenic activities. Under conditions where estrogen is in excess, the active ingredients of this plant can behave as estrogen antagonists by a mechanism of competitive estrogen receptor inhibition. However, in the presence of low concentration, active agents can act as weak agonists. (LOPES et al., 2017). The long-term effects of using chemotherapy can lead to a deficiency in estrogen production and a reduction of ovarian function (SPEARS et al., 2019). The phytoestrogens present in the rhizome of C. racemosa can act as a selective estrogen receptor modulator, thus, during chemotherapy, this estrogen deficiency could possibly be attenuated by C. racemosa (WOBSER et al., 2020). In addition, the use of this plant extract appears to be safe in cancer patients (WALJI et al., 2007). *C. racemosa* revealed an antiangiogenic effect by inhibiting proliferation and reducing endothelial cell migration and motility *in vitro*, whereas *in vivo* a oral administration of 10 mg/kg for 7 days inhibited blood vessel formation and oral treatments (CR 10-15 mg/kg) for 28 days resulted in a decrease in the size of 4T1 breast tumors and metastases to the lungs and liver (YUE et al., 2016; LOPES et al., 2017). Furthermore, it was demonstrated by Poschner et al. (2020) that *C. racemosa* root extract (BCE) and acteína, one of the main compounds of the extract, do not promote breast cancer cell human (MCF-7) growth or influence estrogen levels.

In vitro study using S30 cell line, showed that *C. racemosa* extract contain compounds like phenol group that are effective scavengers of ROS that can protect cellular DNA against oxidative damage (BURDETTE et al., 2003). Moreover, *in vivo* study with *C. racemosa* extract (BNO 1055) in women with symptomatic perimenopausal and arterial hypertension, showed that the *C. racemosa* can decrease the cystolic and diastolic blood pressure (GORACH et al., 2018). In this way, the mechanisms underlying the multiple therapeutic effects of *C. racemosa*

extract, e.g. reducing hot flushes and profuse sweating are not well defined. Friederichsen et al. (2019), in retrospective observational study found that both the *C. racemosa* dry extract (Ze 450) markedly reduced menopausal symptoms.

Other study with *C. racemosa* (Ze 450) investigated its effects on energy metabolism. Since mitochondria are the key regulators of cellular energy homeostasis, was observed that *C. racemosa* positively affects mitochondrial activitie while preserved mitochondrial integrity and ATP levels, and prevented mitochondrial ROS formation, loss of mitochondrial membrane potential and cell death (RABENAU et al., 2019). Additionally, studies show that chemotherapeutics such as Doxorubicin negatively affect mitochondrial function, generating high levels of ROS (KOLEINI; KARDAMI et al., 2017) which can induce an alteration of the normal funtions of cells (TILLY; SINCLAIR, 2013). Therefore, the extract of C. racemosa could possibly minimize the deleterious effects of this chemotherapy by protecting mitochondria, since a large part of ROS is produced during mitocondrial activitie (AVOLIO et al., 2020).

In ovariectomized (ovx) rats, *C. racemosa* extract (BNO 1055) was capable to reduce the high fat load of the body, serum leptin, cholesterol and glucose levels, wile reduced the fat tissue. This, the *C. racemosa* extract can be useful in preventing the metabolic syndrome (SEIDLOVA-WUTTKE et al., 2012). A number of clinical studies have demonstrated improvement in menopausal symptoms with isopropanolic and ethanolic extracts of *C. racemosa* (NAPPI et al., 2005). On the other hand, several *in vitro* studies have been performed to elucidate the drug mechanisms potentially involved in the *C. racemosa* action. In contrast, limited studies have investigated the antioxidant properties of this plant or its constituents in tissue and cells culture models.

5. Final considerations

The preservation of the fertility of young women during cancer treatment has become one of the great challenges of medicine, since most treatments can cause premature ovarian failure, due to the ovarian toxicity of chemotherapeutic agents. The likelihood of cancer survivors today is high and continues to grow progressively. Based on this sensitivity and with a view to reversing or minimizing the damage to reproductive cells resulting from chemotherapy, several studies and the research for options to preserve female fertility are becoming more frequent.

Medicinal plants are vegetables that contain substances in one or more of their organs with therapeutic capacity for various diseases. Thus, intervention strategies using bioactive

compounds derived from plants, have been studied as a way to preserve female fertility during cancer treatment. We believe that natural products can provide potential candidates for pharmacological protection of the ovary, considering that more studies are still needed. Understanding the exact mechanism of action of these plants, will allow the use of the nutural compound to block the pathway of toxicity or protect certain cells during the treatment of cancer resulting in greater effectiveness in preserving fertility.

Plants of the genus Cimicifuga have many important properties, like antioxidant activitie, antineoplastic and anti-inflammatory functions. Now a days, research using natural products, including plants of the Cimicifuga genus, with the objective of reduce the adverse effects caused to female reproductive system during cancer treatment are still scarce. Natural products like Cimicifuga racemosa extract, presents phytocomponents that can be important to prevent ovarian damage during chemotherapy. These components may can acts blocking or enhancing the deleterious action of chemotherapeutic agents in the ovary. Thus, it is necessary to conduct studies that can be able to answer these questions that have not yet been discussed.

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CAPÍTULO 2

Protective effect of *Cimicifuga racemosa* (L.) Nutt. extract on mice ovarian toxicity induced by doxorubicin

Protective effect of *Cimicifuga racemosa (L.)* Nutt. extract on mice ovarian toxicity induced by doxorubicin

Assis, E.I.T.[a,b]; Azevedo, V.A.N.[a,b]; De Lima Neto, M.F.[a,b]; Costa, F.C.[a,b]; Paulino, L.R.F.M.[a,b]; Barroso, P.A.A.[a,b]; Donato, M.A.M[c]; Peixoto, C.A.[c]; Do Monte, A.P.O.[d] ; Matos, M.H.T.[d]; Godinho, A.N.[b]; Freire, J.M.O.[b]; Souza A.L.P.[a,b]; Silva, J.R.V.[a,b]; Silva, A.W.B.[a,b]*

[a] *Laboratory of Biotechnology and Physiology of Reproduction -LABIREP - Nucleus of Biotechnology of Sobral – NUBIS, Federal University of Ceara, Sobral, CE, Brazil.*

[b] *Nucleus of Research in Animal Experimentation – NUPEX, Federal University of Ceara, Sobral, CE, Brazil.*

[c] *Laboratory of Ultrastructure, CNPqAM/FIOCRUZ, Federal University of Pernambuco, Recife, PE, Brazil*

[d] *Nucleus of Biotechnology Applied to Ovarian Follicle Development, Federal University of São Francisco Valley, Petrolina-PE, Brazil*

**Corresponding address (A.W.B., Silva): Nucleus of Biotechnology of Sobral - NUBIS, Federal University of Ceara, Av. Comandante Maurocélio Rocha Ponte 100, CEP 62041-040, Sobral, CE, Brazil. Phone / Fax: +55 88 36954744 [andersonweiny@sobral.ufc.br]*

This manuscript will be submit to ***Molecular Reproduction and Development*** *– this journal is interested in an integrated, systems-biology approach to understand the dynamic continuum of cellular, reproductive, and developmental processes.*

A1 stratum for CAPES / Impact factor: 3.113

Abstract

This study evaluated the potential of *Cimicifuga racemosa* (L.) Nutt. extract (CIMI) to reduce the deleterious effects of doxorubicin in mice ovaries after 6 days of *in vitro* culture. In experiment 1, to define the better concentration of CIMI on *in vitro* culture, the ovaries from mice with regular estrous cycle (n = 36) were cultured in $DMEM^+$ alone (control group) or

DMEM$^+$ supplemented with different concentrations of CIMI (5, 50 or 500 ng/ml). In experiment 2, the better concentration of the CIMI was tested in association with DOXO, in order to assess the potentiation or blocking the deleterious effects of doxorubicin. To this end, the mice ovaries (n = 32) were cultured in DMEM$^+$ alone (control group); CIMI 5 ng/ml; DOXO 0.3 μg/ml or CIMI 5 ng/ml in association with DOXO 0.3 μg/ml. After culture, the ovaries were analyzed by histology (survival, activation, and follicular growth; density of the stroma; extracellular matrix configuration (ECM), and immunohistochemistry for caspase-3) or fluorescence microscopy (folicular viability). The results showed that the presence of CIMI (5 ng/ml) can maintain the percentage of morphologically normal follicles similar to the control group (DMEM$^+$) and when added to the medium containing DOXO it is able to protect against the deleterious effects caused by DOXO, showing a percentage of normal follicles similar to the control group (DMEM$^+$). DOXO reduced the percentage of primordial follicles compared to the control group (DMEM), while the presence of CIMI (5 ng/ml) alone, kept the percentage of primordial follicles equal to the control group. There was no significant difference between groups in percentage of primordial follicles. The presence of CIMI (5 ng/ml) alone or in association with DOXO did not influence the follicular growth and the configuration of the ovarian ECM. In contrast, the presence of DOXO negatively affected the stroma density, while the presence of CIMI (5 ng/ml) was able to prevent the damage caused by this antineoplastic drug. The ovaries cultured with CIMI (5 ng/ml) alone or in association with DOXO, showed less immunoreaction for caspase-3 compared to DMEM or DOXO group. In addition, ovaries cultured with CIMI *plus* DOXO showed a follicular viability by fluorescence microscopy after culture. In conclusion, the extract of *Cimicifuga racemosa* (CIMI) was able to prevent the deleterious effects caused by doxorubicin in terms of maintaining follicular survival, as well as showing a protective effect on ovarian stroma and reducing the rate of apoptosis.

Keywords: folliculogenesis; in vitro culture; chemotherapy; fertility preservation; phytotherapic.

1. Introduction

The incidences of oncological diseases have increased in recent decades, with approximately 70,000 adolescents and adults diagnosed each year (FERREIRA; PÓVOA, 2020). Due to advances in treatments, survival rates are higher than 80% for various types of tumors, particularly for cancer patients in childhood or adolescence, contributing to increase cancer survivors in reproductive age (HUDSON et al., 2017). The effects of cancer and its treatment on female reproductive function are increasingly well documented. Overall, women

are 38% less likely to become pregnant after cancer diagnosis and treatment (SPEARS et al., 2019). Cancer-related infertility depends mainly on gonadotoxic treatments. Currently, fertile patients are directed to cryopreservation of oocytes and embryos or cryopreservation of fragments of ovarian cortex. However these methods have advantages and disadvantages that need to be considered (SILVESTRIS et al., 2020).

One of the main chemotherapeutic drugs known to decrease fertility in female patients is doxorubicin, an anthracycline antibiotic, which mainly acts by interfering with the synthesis of nucleic acids through a process called intercalation, which prevents the duplication and separation of chains of DNA and RNA. This process induces the production of reactive oxygen species, blocks protein synthesis, promotes oxidative damage and triggers cell death by apoptosis (TOKARSKA-SCHLATTNER et al., 2006). Doxorubicin is one of the most effective anticancer drugs in clinic and it is widely used in the treatment of various human tumors, such as neurofibromatosis, liver cancer, and breast cancer (XIANG et al., 2021).

Modern cancer treatments for young women have improved cure rates, but most often the price paid for survival is the loss of reproductive function due to gonadal toxicity (ROBERTS et al., 2015). Cancer treatments have side effects that can affect fertility and other areas of reproductive health such as pubertal development, hormonal production and sexual function (HUDSON et al., 2017). In a recent study, doxorubicin was able to promote loss of follicular reserve through atresia and overactivation of primordial follicles (WANG et al., 2019) and deletion of secondary follicles (XIAO et al. 2017). In this sense, oncofertility is in constant progress and evolves to develop new options for preserving fertility (LOPATEGUI et al., 2017).

When chemotherapeutic agents are used to treat various cancers, ovarian damage becomes detrimental to fertility. Protective agents that act independently of specific actions of chemotherapy drug have great value for human medicine. They act by blocking a common pathway of damage or by reducing the release of drugs to the ovary. Plant extracts have several active compounds, which are reported as attractive alternatives to protect the ovary against a specific chemotherapy or class of drugs (PARK et al., 2021). Some antioxidant substances are able to inhibit DNA damage in follicular cells when exposed to toxic drugs before apoptosis. In a recent study, *rutin*, a powerful natural antioxidant, was able to maintain mitochondrial activity and protect mouse ovarian follicles from oxidative damage caused by the cisplatin chemotherapy (LINS et al., 2020). In addition, a study has indicated that polyphenols in foods or spices, such as curcumin, gallate, epicatechin and resveratrol, have potent anti-tumor properties and are protective for the health of reproductive system in cancer patients, due to antioxidant agents that effectively inhibit carcinogenic properties of medications and protect cells from the harmful effect of reactive oxygen species (ROS) (RAD et al., 2021). *Cimicifuga*

racemosa (L.) Nutt., (Black Cohosh*; Cimicifuga racemosa*) is a plant belonging to the Ranunculaceae family. The root of *Cimicifuga racemosa* consists of more than 42 triterpene glycosides, 11 phenolic acids and more than 70 alkaloids and tannins. This plant has been traditionally used in China, Europe and North America for centuries to treat a variety of diseases, including symptoms of menopause and osteoporosis (PKHALADZE et al., 2020). *Cimicifuga racemosa* is effectiveness used in treating menopausal symptoms, and also exhibits antioxidant, anti-inflammatory, antidiabetic, antiviral, antiangiogenic, vasodilatory and immunosuppressive effects (GUO et al., 2017). In vivo, *Cimicifuga racemosa* extract was capable to improve antioxidant status, hormonal profile, lipid profile, glucose level and liver functions in rats with polycystic ovary syndrome (PCOS). In same study, this plant reduced the expression of Ki-67 in granulosa cells, teca cells layers and stromal cells (ASMA et al., 2020). When tested in vitro, *Cimicifuga racemosa* extract preserved mitochondrial integrity and ATP levels, whereas prevented the formation of mitochondrial ROS, loss of mitochondrial membrane potential and cell death after oxidative stress induced by erastin and RSL-3 in neuronal and liver cells (RABENAU et al 2019). In this sense, *Cimicifuga racemosa* extract can be able to reduce the damage in the ovary caused by doxorubicin through its antioxidant action. However, there are no reports about the effects of *Cimicifuga racemosa* when used alone or in association with doxorubicin and its potential mechanisms of action in ovarian tissues cultured *in vitro*.

The aim of this study was to investigate the effects of different concentration of *Cimicifuga racemosa* extract (CIMI) on mice ovarian tissue cultured *in vitro*, and to evaluate if CIMI prevents the ovarian damage caused by doxorubicin in cultured ovaries. In addition, it was proposed to analyze if this extract can preserve the ovarian follicular morphology and influences in the activation of primordial follicles, while can acts in the maintenance of ovarian stroma density, and in the configuration of extracellular matrix configuration (ECM), and follicular viability.

2. Material and methods

2.1. Animals

This study was conducted in accordance with the guidelines and normative resolutions of the National Council for Control in Animal Experimentation (CONCEA) and the ethical guidelines from Ethics Committee on the Use of Animals (CEUA) of Federal University Ceará (approved under protocol Nº. 05/18). Adult Swiss mice (*Mus musculus*) were used in this research, which were kept in polyethylene boxes lined with wood shavings (6 animals/box), with free

access to filtered water and feed (Nuvilab). The animals were kept at an average temperature of 22 ± 2°C, following 12h light-dark cycles. It is worth noting that the place where the animals were allocated, there is an exhaust system.

2.2. Evaluation of estrous cycle

All female mice from 18g and/or 2 months of age, had their estrous cycle evaluated once a day, for 15 days, between 9:00h and 10:00h a.m., by a single evaluator, as established by Marcondes et al. (2002). According to the observed cells, it was determined whether the female was in proestrus, estrus, metestrus or diestrus. Only females with a regular cycle, that is, lasting 4 to 5 days, were used to carry out the experiment.

2.3. Chemicals

Doxorubicin (CAS number: 25316-40-9, Sigma), *Cimicifuga racemosa* extract (CAS number: 8477626-1, Sigma), the culture medium and other chemicals supplements used in this study were purchased from Sigma Chemical Co. (St Louis, MO, USA), unless otherwise indicated in the text. The *Cimicifuga racemosa* extract was dissolved in 60% (v/v) ethanol for all experiments (RABENAU et al., 2018).

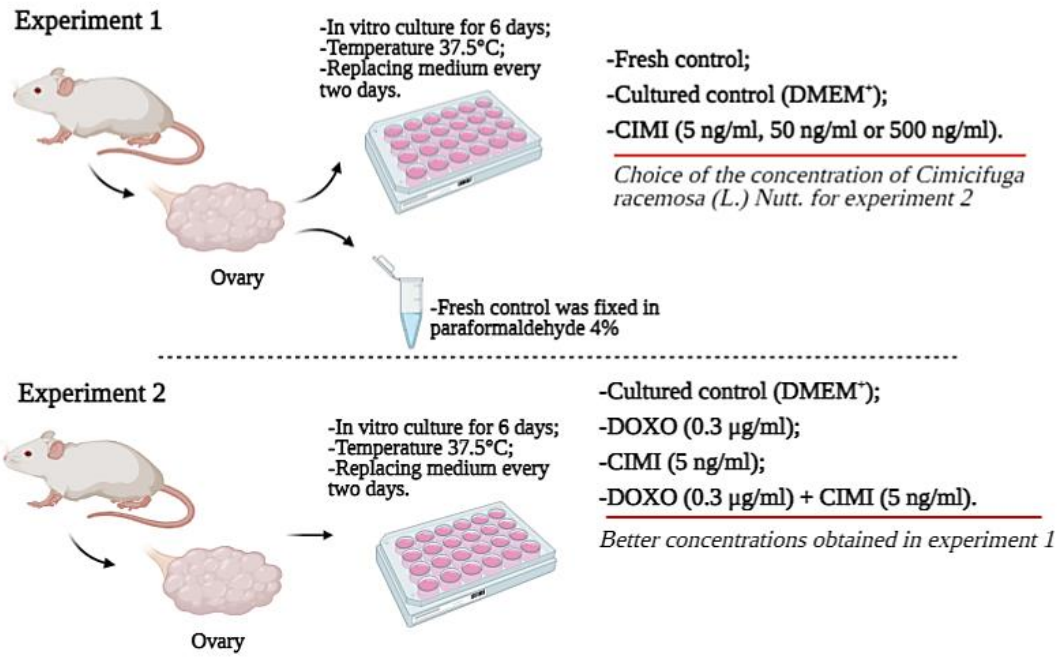
2.4. Experimental design

The ovaries were cultured according to the protocol described by O'Brien et al. (2003). In experiment 1, the ovaries (n= 36) were collected from euthanized mice and cultured individually in a 24-well plate containing 1 ml of DMEM/HAMS F12 supplemented with ascorbic acid (10 μg/ml), penicillin G (75 μg/ml), *Insulin-Transferrin-Selenium* (ITS) (10 μg/ml insulin, 5.5 μg/ml transferrin, and 5 ng/ml selenium) and *Bovine Serum Albumin* (BSA) (10 μg/ml). This base medium was called $DMEM^+$. Then, the ovaries were distributed in the following treatments: $DMEM^+$ alone (control group) or $DMEM^+$ supplemented with different concentrations *Cimicifuga racemosa* (5, 50 or 500 ng/ml) - CIMI group; and cultured at 37.5°C, in 5% CO2, for 6 days. Approximately half of the culture medium was replaced every 2 days. For the fresh control (D0), the ovaries were collected and fixed immediately in paraformaldehyde (4%) for 24 hours at 4ºC. The ovaries were used for analysis of survival, activation and follicular growth, as well as configuration of the extracellular matrix (ECM). After obtaining the results of this experiment, the better concentration of *Cimicifuga racemosa*

extract was identified and tested in association with doxorubicin, in order to assess the potentiation or blocking of the deleterious effects in the cultured ovaries.

In experiment 2, the mice ovaries (n = 32) were collected and cultured in (i) $DMEM^+$ alone (control group); (ii) $DMEM^+$ added with 5 ng/ml of *Cimicifuga racemosa* extract (CIMI group); (iii) $DMEM^+$ supplemented with 0.3 µg/ml of doxorubicin (DOXO group) or (iv) $DMEM^+$ containing 5 ng/ml *Cimicifuga racemosa* extract *plus* 0.3 µg/ml doxorubicin (CIMI + DOXO group). The in vitro culture was conducted at 37.5 ° C, in 5% CO_2 for 6 days. Approximately half of the culture medium was replaced every 2 days. After culture, the ovaries were destined for histological analysis to evaluate follicular survival, activation, growth, and viability, as well as ECM configuration, stromal density. Moreover, the protein expression for caspase-3 (CASP3) was evaluated by immunohistochemistry. The summary of experiment 1 and 2 could be view in the figure 1.

Figura 1. Experimental design: In experiment 1, the better concentration of Cimicifuga racemosa was chosen, while in experiment 2, the the best dose identified was used in combination with doxorubicin.



2.5. Morphological assessment of follicular development in cultured ovaries

The *fresh* control (uncultured ovaries - D0) and the cultured ovaries were fixed in paraformaldehyde (4% in phosphate buffered saline - PBS, pH 7.4) for 24 h and processed for classical histology. After fixation, the ovaries were dehydrated in gradual series of ethanol, clarified with xylol and embedded in paraffin wax. For each ovary, 7 µm sections were mounted

on slides and stained using the Hematoxylin-Eosin (HE) method. Quantitative analysis of slides was carried out by an experienced researcher who is unaware of the treatments of each group under analysis. The slides were examined by microscope (Nikon) at 400x magnification. The follicular development stages were classified as primordial (1 layer of flattened or flattened granular cells around the oocyte) or growing follicles (primary, 1 layer of cuboidal granular cells; secondary, 2 or more layers of cells of the cuboidal granulosa around the oocyte). These follicles were individually classified as histologically normal when an intact oocyte was surrounded by granulosa cells well organized in 1 or more layers, and which had no pycnotic nucleus. Degenerate follicles were defined as those with retracted oocyte, with pycnotic nucleus and/or surrounded by disorganized granulosa cells, detached from the basement membrane (GOUVEIA et al., 2021). The evaluation of follicular activation and growth was performed by quantifying the follicles in the different follicular development classes (primordial, primary and secondary) and by measuring the follicular and oocyte diameter before and at the end of each culture period, respectively. Overall, 130 follicles were evaluated for each treatment.

2.6. Analysis of follicular viability after ovarian culture by fluorescence microscopy

For a better evaluation of follicular viability, after 6 days of culture, part of the ovaries cultured *in vitro* were destined for follicular isolation for viability analysis. Follicles in different stages of development were mechanically isolated using 26-G needles from the ovaries (five ovaries per treatment). After isolation and selection, the follicles were transferred to 100 µl drops of $DMEM^{+}$ medium mounted on glass slides with 4 mM of calcein-AM and 2 mM of homodimer ethidium-1 (Molecular Probes, Invitrogen, Karlsruhe, Germany), followed by an incubation at 37°C for 15 min and, finally, the follicles were examined under a fluorescence microscope. The fluorescent signals emitted by calcein-AM and homodimer ethidium-1 were collected at 488 and 568 nm, respectively. While the first probe detects intracellular esterase activity of viable cells, the second probe marks the nucleic acids of non-viable cells due to the rupture of the plasma membrane. Granulosa oocytes and cells were considered alive when the cytoplasm was positively marked with calcein-AM (green) and if the chromatin was not marked with homodimer-ethidium-1 (red) (SILVA et al., 2017). On the other hand, cells with their chromatin labeled with ethidium homodimer-1 (red) were considered non-viable.

2.7. Detection of apoptotic cells by Immunohistochemistry assay

Immunohistochemistry was performed as previously described by Lins et al. (2020). The sections (5 µm thick) of each block were cut using a microtome (EasyPath, São Paulo, Brazil) and mounted on Starfrost glass slides (Knittel, Braunschweig, Germany). The tissue sections were incubated in 0.01 M citrate buffer (pH 6), heated in the microwave (20 min, 90 W, 96ºC) and cooled for 20 min (RT) to recover the antigenicity of activated caspase-3. Sections were washed twice for 5 min with 0.05 M Tris buffered saline (TBS) (pH 7.4). Subsequently, endogenous peroxidase activity was depleted by incubating the sections in 3% (v/v) H2O2 in TBS (10 min, RT). Non-specific staining was blocked with normal goat serum (NGS) (1/4, Dako, Glostrup, Denmark) diluted in TBS, enriched with 0.3% Triton-X 100 (v/v) (Tx - Sigma) and 1% (w/v) BSA (Sigma) for 30 min at room temperature. Subsequently, the sections were incubated overnight (RT) with a primary antibody directed against activated caspase-3 (host: rabbit, polyclonal, Sigma) diluted in TBS, enriched with 0.3% (v/v) Tx and 5 % (w/v) BSA (1/225). The following day, following two washing steps of 5 min each with Dako Wash Buffer (Dako), the sections were incubated for 30 min in RT with anti-rabbit Envision (Dako). Following another two washing steps for 5 min each with TBS and one step with distilled water, a positive immunostaining was visualized by incubating the sections for 10 min (RT) with the chromogen 3-amino-9-ethylcarbazole (Dako). The sections were contrasted with Carazzi's hematoxylin (Klinipath, Olen, Belgium), rinsed and mounted with Medium Faramount (Dako). Using an optical microscope (Nikon) connected to a computer equipped with Image-Pro Plus® software (Media Cybernetics) five photos were taken at the same magnification (100x) and analyzed quantitatively using the Gimp 2.6 software program (GNU Manipulation Program images, UNIX platforms).

2.8. Analysis of the extracellular matrix and evaluation of density of ovarian stroma

To assess collagen fibers of the extracellular matrix of ovarian cortex, staining with Picrosirius Red (Abcam Kit) was performed following the methodology described by Vanié (2017) with modifications. Ovarian sections of 7 µm were dewaxed in xylol and incubated in Sirius Red solution (0.1%) for 1 hour at room temperature. Then, the excess dye will be removed with acetic acid solution (0.5%) and the sections were dehydrated and subjected to slide assembly with subsequent observation under an optical microscope (Nikon, Eclipse, TS 100, Japan) under magnification 400x. For each treatment, the percentage of the area occupied by collagen fibers in ten different fields was measured with the aid of a DS Cooled DS DS-Ri1 camera attached to a microscope (Nikon, Eclipse, TS 100, Japan), the microscope and the images were analyzed by Image J Software (Version 1.51p, 2017) with 400x magnification.

Only the collagen fibers were marked in red with the picrosirius color, while the follicles remained colorless (white). The analyzer software automatically excludes the circumference of unstained follicles from the total area marked in red. The Image J Software was used to quantify the percentage of collagen fiber in uncultured and cultured tissues. The staining intensity of collagen fibers was determined by measuring the average pixel intensity of the total area imaged after background subtraction.

The evaluation of cell density of ovarian stroma was carried out after 6 days of culture in the different treatments: (i) control group ($DMEM^+$); (ii) DOXO (0.3 μg/ml), (iii) CIMI (5 ng/ml); (iv) CIMI +DOXO (5 ng/ml+0.3 μg/ml), where the number of stromal cells was calculated in an area of 100 μm^2. For each treatment, ten fields from different sections were evaluated. The average number of stromal cells per field was calculated as previously described (CAVALCANTE et al., 2019). All evaluations and measurements were performed by a single operator.

2.9. Statistical analysis

The statistical analysis was performed using the computer program GraphPad Prisma (5.0). Data that did not show normal distribution, selected by the Shapiro-Wilk test, underwent logarithmic transformation. The parametric variables of the average percentage of normal follicles and follicular activation in each treatment were evaluated by Fisher's exact test and the comparison between treatments using Kruskal-Wallis. For the analysis of collagen fibers, the data were analyzed by the Kruskal-Wallis test, followed by the Dunn comparison. The results were expressed as mean and standard error (mean ± S.E.M). Differences were considered significant when $p < 0.05$.

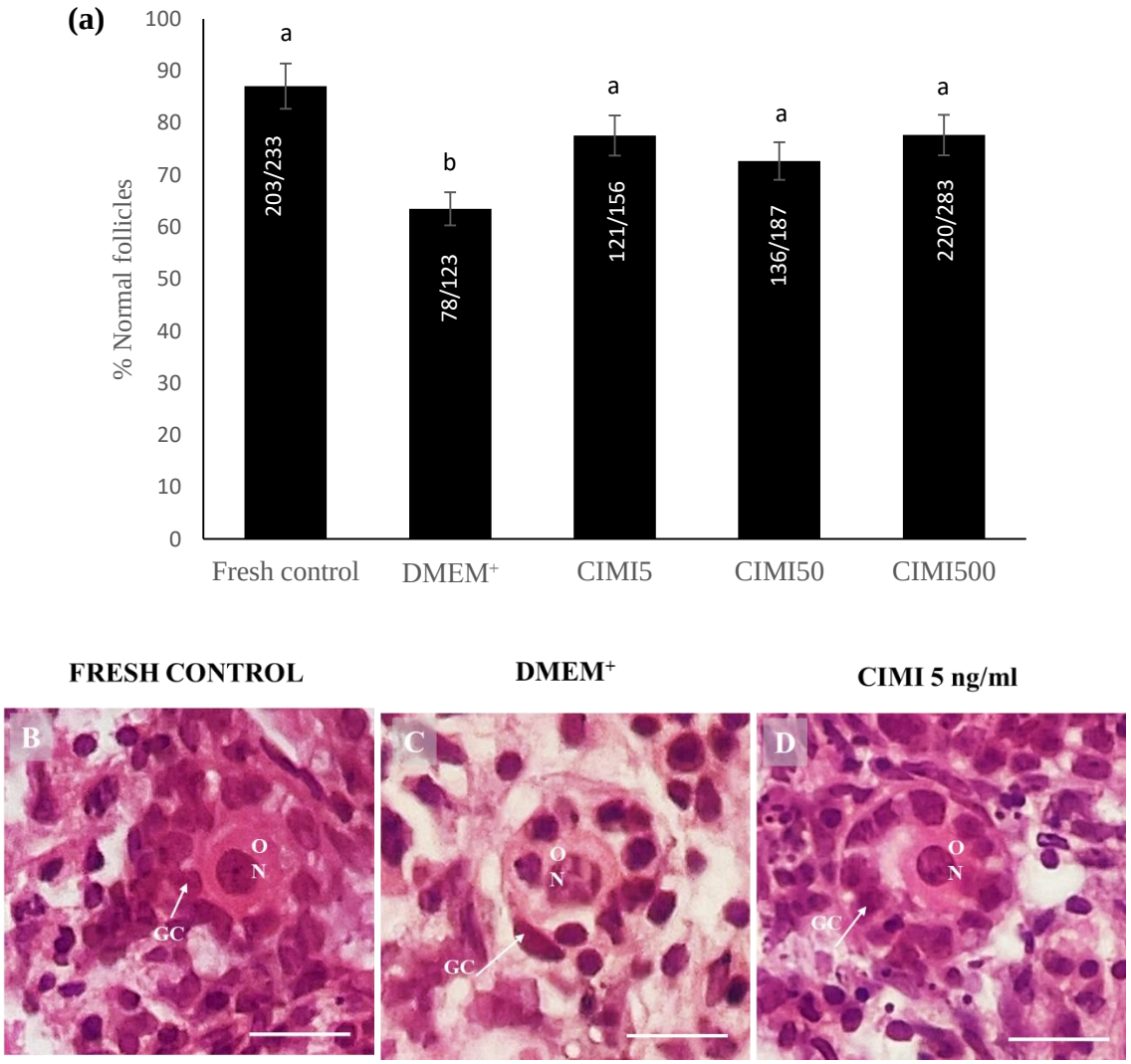
3. Results

3. 1. Experiment 1 – Effect of CIMI on follicular morphology, activation and development after in vitro culture

Ovaries cultured in medium supplemented with CIMI (5, 50 or 500 ng/ml) showed a significant increase ($P < 0.05$) in the percentage of morphological normal follicles when compared to control ($DMEM^+$) (Fig. 2A). Moreover, the ovaries cultured in medium enriched with CIMI (5, 50, or 500 ng/ml) maintained a percentage of morphologically normal follicles

(P <0.05) compared to fresh control (uncultured control) (Fig. 2A). Overall, the mice preantral ovarian follicles from fresh control (Fig. 2B) and those cultured in presence of CIMI 5 ng/mL (Fig. 2C) had centrally located oocytes and granulosa cells surrounded by normal intact basement membrane, while atretic follicles with retracted oocyte and disorganized and swollen granulosa cells were commonly observed after culture in cultured control ($DMEM^+$).

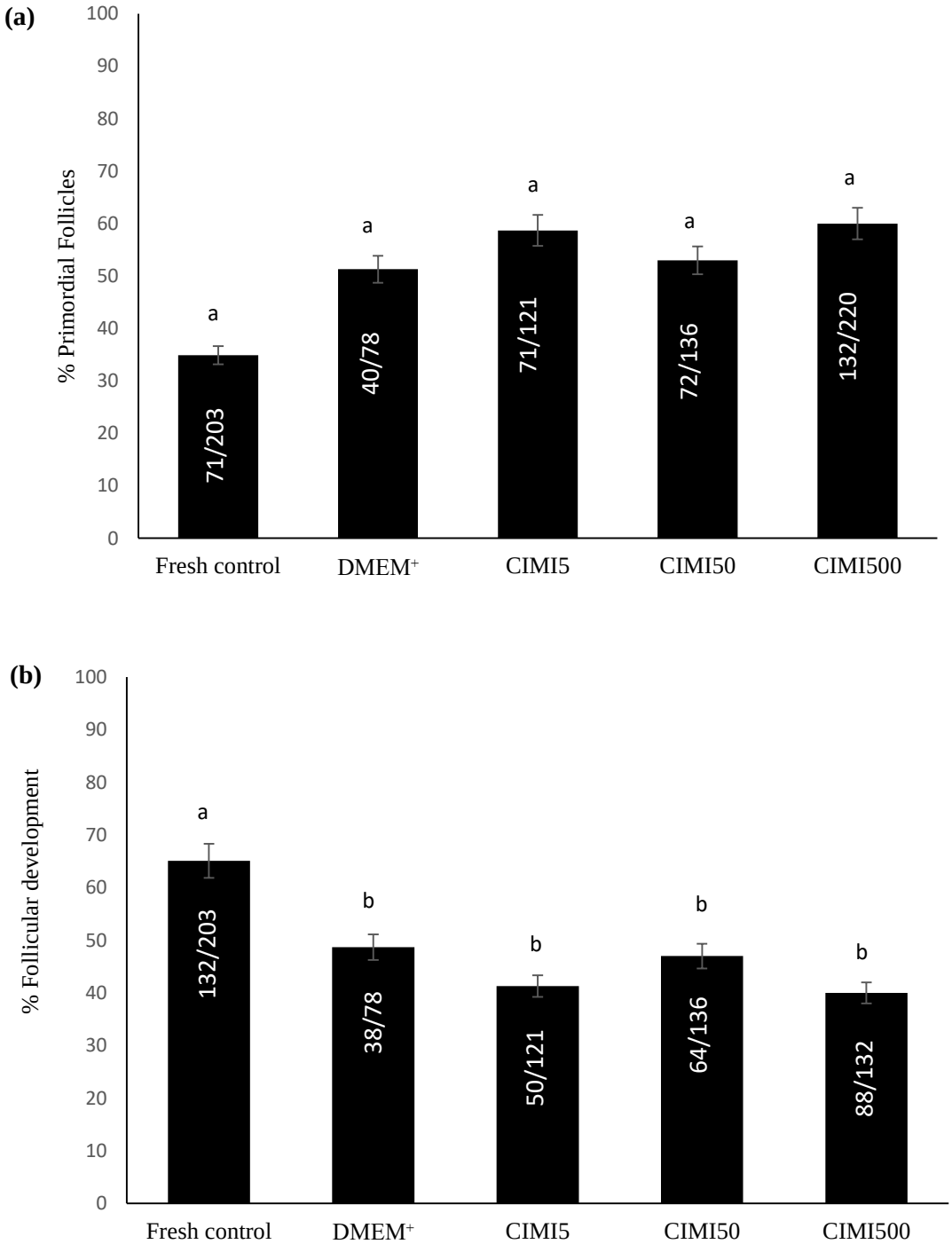
Figure 2. (A) Percentage (mean ± SE) of normal follicles evaluated at the end of 6 days of culture by classical histology, hematoxylin and eosin staining, the ovaries were divided between the fresh control (uncultivated), control group ($DMEM^+$) or $DMEM^+$ supplemented with CIMI (5 ng/ml, 50 ng/ml or 500 ng/ml). a and b different lowercase letters indicate statistically significant differences between treatments (P <0.05). (B) Histological sections of normal preantral follicle from fresh control; (C) Degenerated follicle cultured in $DMEM^+$. (D) Normal preantral follicle cultured in the presence of CIMI (5 ng/mL). O: oocyte, GC: granulosa cells; N: nucleus. Scale bar: 100 µm (400x).



To verify if any follicular category is more susceptible to atresia, the different developmental stages were compared in each treatment concerning to follicular activation. So, after *in vitro* culture of the ovaries in the presence of CIMI (5, 50, or 500 ng/ml), a similar

pattern ($P < 0.05$) was observed in activation of primordial follicles compared to *fresh* control. On the other hand, the treatment groups with CIMI (5, 50, or 500 ng/ml) maintained the percentage of primordial follicles statistically similar to control group ($DMEM^{+}$) and fresh control. In addition, the presence of 500 ng/ml of CIMI in culture medium reduced ($P < 0.05$) the percentage of developing follicles when compared to fresh control. However, this treatment showed a similar percentage of follicular development ($P < 0.05$) when compared to tissues cultured in control medium ($DMEM^{+}$) alone or containing CIMI at 5 or 50 ng/ml.

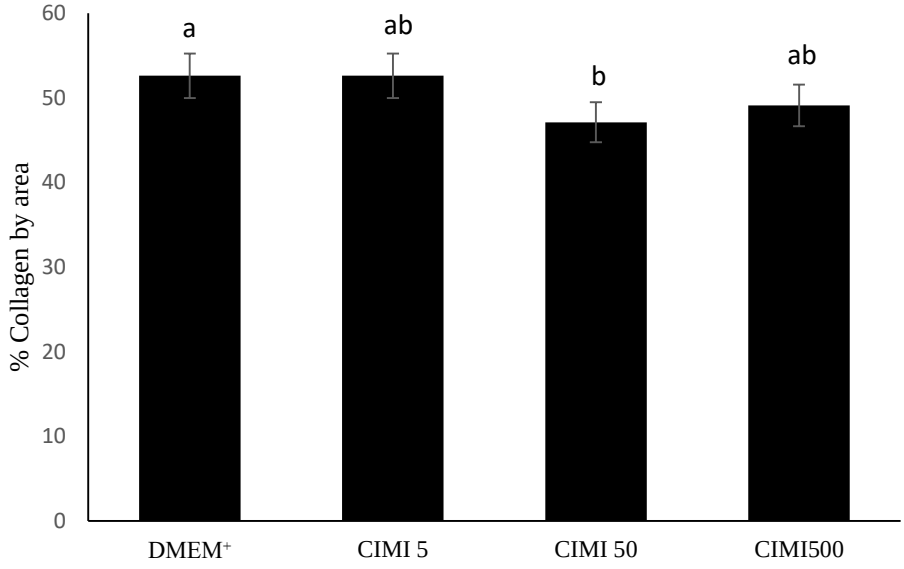
Figure 3. Percentage (mean ± SE) of primordial follicles (a) and developing follicles (b) evaluated at the end of 6 days of culture by classical histology, hematoxylin and eosin staining. The ovaries were divided in different groups: fresh control (uncultured controle); DMEM$^+$ (cultured control group) and CIMI at different concentrations (5 ng/ml, 50 ng/ml or 500 ng/ml). a and b different lowercase letters indicate statistically significant differences between treatments ($P < 0.05$).



3. 2. Evaluation of the ovarian extracellular matrix after in vitro culture

At the end of culture it was analyzed the extracellular matrix maintenance by picro-sirius red. It was observed that the treatments containing CIMI (50 ng/mL) reduced the percentage of collagen fibers when compared to DMEM^{+} (control group). In the order hand, when ovaries were cultured in the presence of CIMI 5 or 500 ng/ml, the tissue maintained their percentage of collagen fibers similar to control (DMEM^{+}) ($p < 0.05$). However, no significant difference were observed between treatments groups ($p > 0.05$).

Figure 4. Percentage of maintaining collagen fibers levels (mean ± SD) in the mouse ovary analyzed by the picro-sirius red kit and observed under optical microscopy (400x). The ovaries were cultured for 6 days in DMEM^{+} (control group) or CIMI (5 ng/ml, 50 ng/ml or 500 ng/ml). a and b different lowercase letters indicate statistically significant differences between treatments ($p < 0.05$).



3. 3. Experiment 2 – Assessment of Cimicifuga racemosa (L.) Nutt on reducing damage caused by doxorubicin on in vitro culture of mouse ovaries

The results showed that the presence of DOXO (0.3 μg/ml) in culture medium significantly reduces the percentage of morphologically normal follicles. On the other hand, the presence of CIMI (5 ng/mL) alone is able to maintain the percentage of morphologically normal follicles similar to the control group (DMEM^{+}). When added to medium containing DOXO (0.3 μg/ml), CIMI (5 ng/mL) is capable of protect against the deleterious effects caused by this chemotherapy (DMEM^{+}) (Figure 5). In the figure 6 it's possible to see a morphologically normal follicles (Fig 6A, B and C) with a visible and intact oocyte, surrounded by granulosa

cells organized in one or more layers and degenerated follicles showing a swollen and disorganized granulosa cells, and shrunken oocyte (Fig 6D, E and F).

Figure 5. Percentage (mean ± SE) of normal follicles evaluated at the end of 6 days of culture by classical histology, hematoxylin and eosin staining, the ovaries were divided between the control group ($DMEM^{+}$) and subjected to treatments with DOXO (0.3 µg/ml), CIMI (5 ng/ml) and CIMI+DOXO (5 ng/ml + 0.3 µg/ml). a and b different lowercase letters indicate statistically significant differences between treatments ($P < 0.05$).

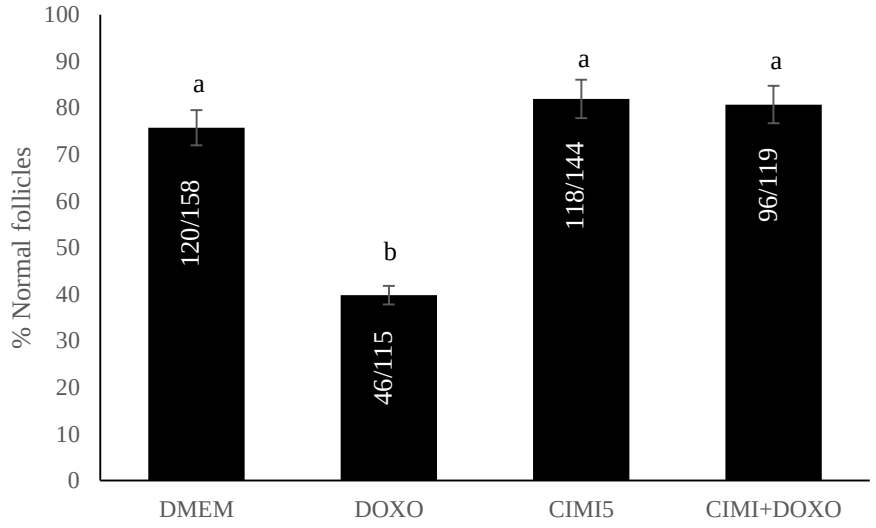
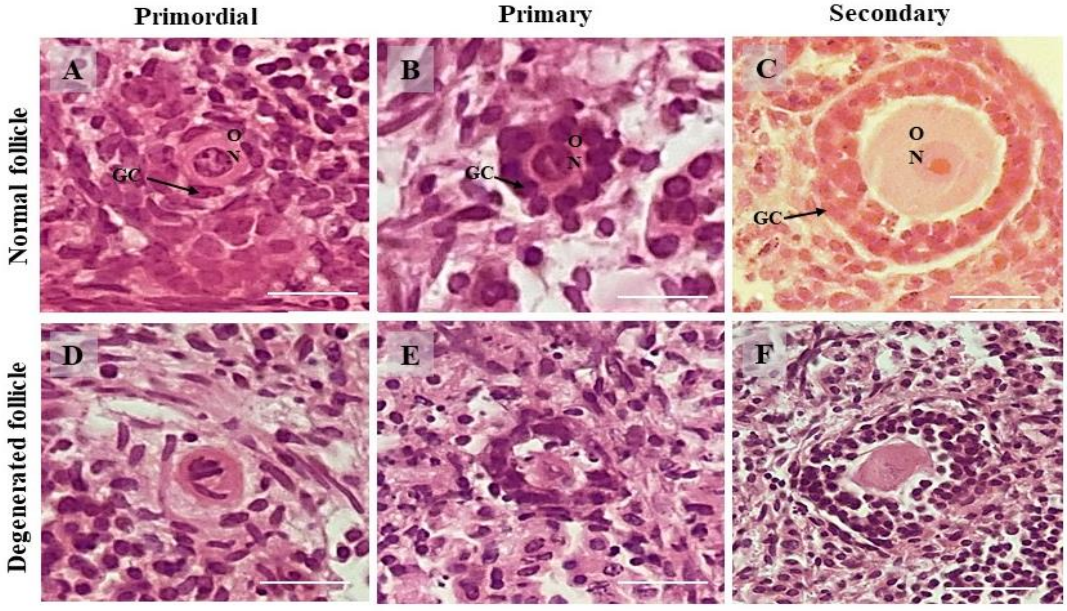
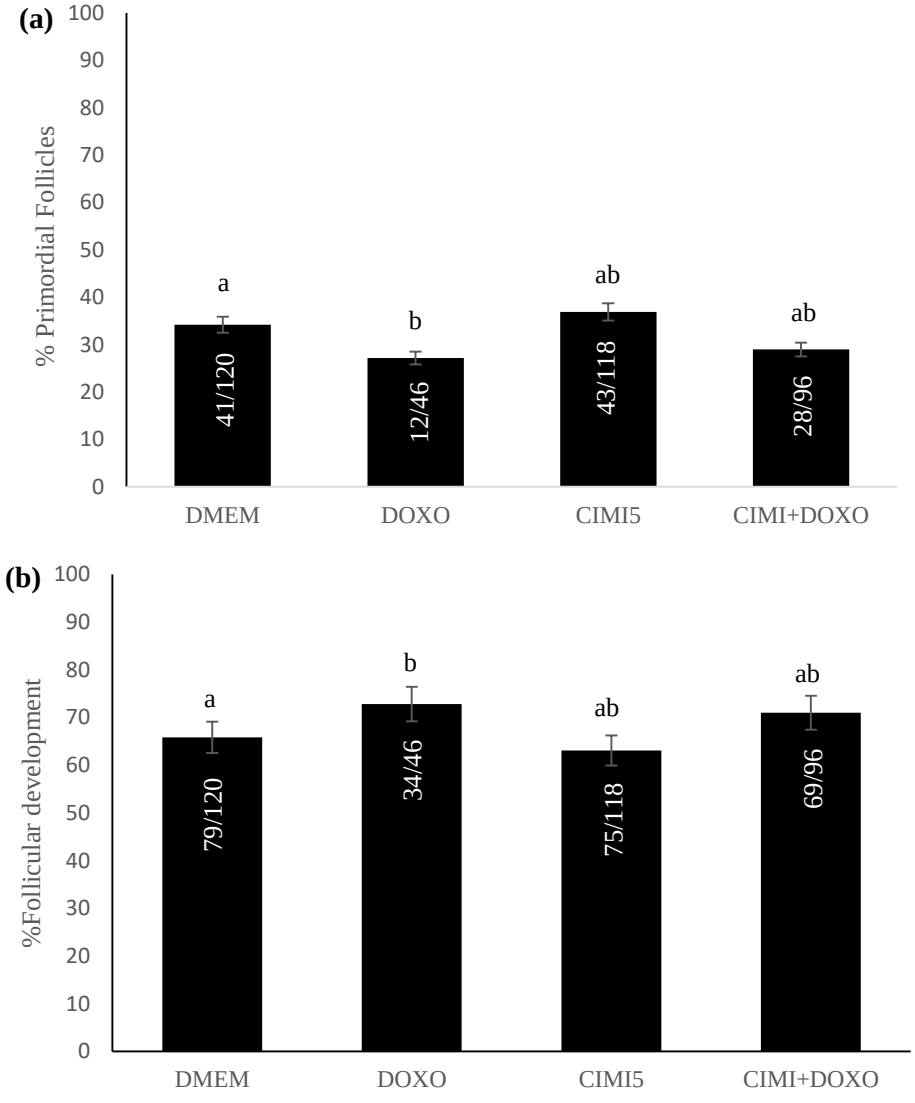


Figure 6. Representative images of ovarian sections of mouse cultured *in vitro* for 6 days showing a morphologically normal (a-c) and atretic (d-f) follicles from different stage of development stained with hematoxylin and eosin. (A) Normal and (D) degenerated primordial follicle; (B) Normal and (E) atretic primary follicle; (C) Normal and (F) apoptotic secondary follicle. Granulosa cells (CG); Oocyte (O); Oocyte nucleus (N). (400x - Scale bar: 100 µm).



The ovaries cultured with CIMI (5 ng/ml) and CIMI + DOXO (5 ng/ml + 0.3 µg/ml) showed a similar percentage of primordial and developing follicles, compared to control group ($DMEM^+$). However, when the ovaries were cultured in the presence of DOXO (0.3 µg/ml), they had reduced percentage of primordial and developing follicles, compared to control group ($DMEM^+$). Nevertheless, no significant difference was observed between treatments ($P < 0.05$) (Fig. 7).

Figure 7. Percentage (mean ± SE) of primordial (a) and developing (b) follicles evaluated at the end of 6 days of culture by classical histology, hematoxylin and eosin staining. The ovaries were cultured in control group ($DMEM^+$); DOXO (0.3 µg/ml); CIMI (5 ng/ml) or CIMI+DOXO (5 ng/ml + 0.3 µg/ml). a and b different lowercase letters indicate statistically significant differences between treatments ($P < 0.05$).



3. 4. Quantitative analysis of activated CASPASE-3

To examine the molecular mechanisms for the protective effect of CIMI on doxorubicin-induced ovarian damage, we evaluated CASPASE-3 immunostaining in ovaries cultured in $DMEM^{+}$ or supplemented with CIMI (5ng/ml), DOXO (0.3 μg/ml) or CIMI (5ng/ml) *plus* DOXO (0.3 μg/ml).

The immunohistochemical results showed that the brown color representing positive staining for caspase-3 was more expressed in ovaries from control group ($DMEM^{+}$) and DOXO (0.3 μg/ml) (Fig. 8). In the groups treated with CIMI (5 ng/ml) alone or associated with DOXO (CIMI5 *plus* DOXO), immunostaining for CASPASE-3 was lower, compared to other groups (Fig. 9). Therefore, CIMI (5 ng/ml) alone or associated with DOXO (0.3 μg/ml) was able to reduce the immunostaining for CASPASE-3 in the ovarian cells, suggesting a reduction in the apoptotic profile of these cells.

Figura 8. Quantification profile of the immunostaining to capase-3 in mouse ovaries after *in vitro* culture in the presence or absence of *Cimicifuga racemosa (L.) Nutt* and doxorubicin.

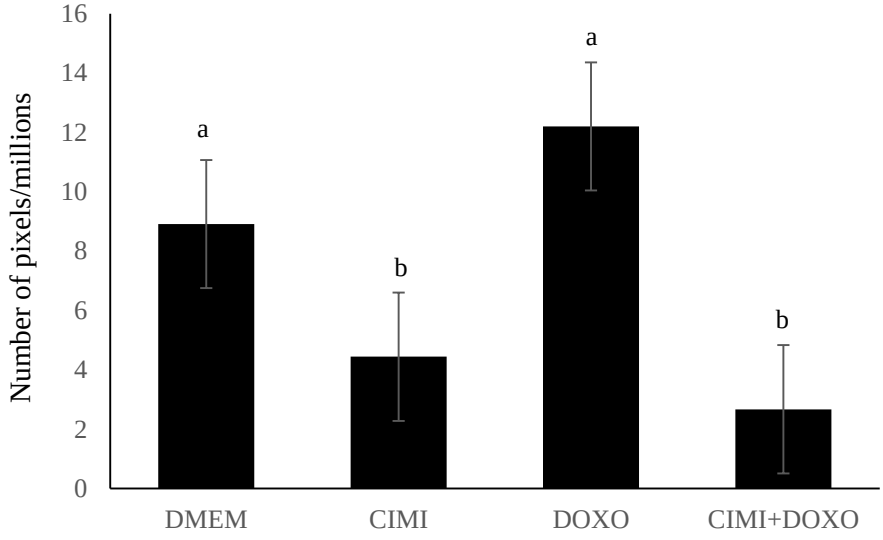
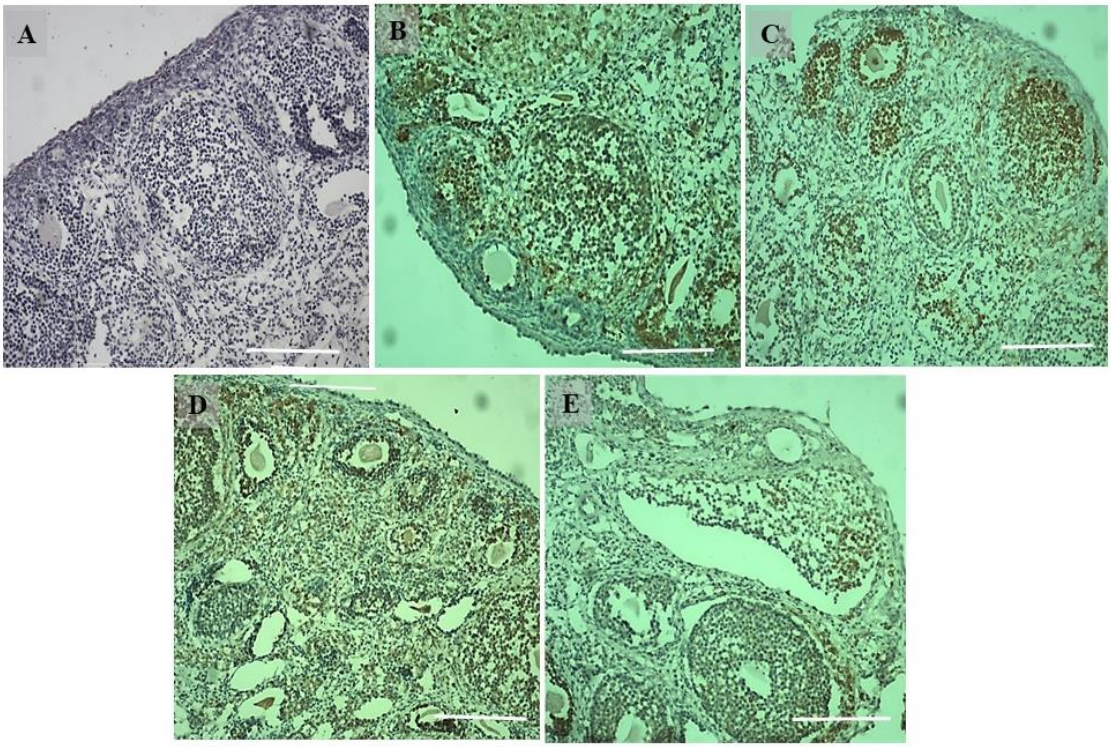


Figure 9. Localization of Immunohistochemical stain for CASPASE-3 (B-E) in mouse ovaries cultured in vitro for 6 days in the presence or absence of *Cimicifuga racemosa (L.) Nutt* or doxorubicin: (A) negative control; (B) control group ($DMEM^+$); (C) CIMI (5 ng/ml); (D) DOXO (0.3 μg/ml); (E) and CIMI + DOXO. (100x - Scale bar: 100 μm).



3. 5. Evaluation of the ovarian extracellular matrix after in vitro culture with Cimicifuga racemosa (L.) Nutt. and doxorubicin

After culture, no difference between treatments groups concerning to organization of collagen fibers in extracellular matrix was seen. Although ovaries cultured with 5 ng/ml of CIMI showed a numerical and visual tendency to present the highest percentage of collagen fibers than those cultured with DOXO (0.3 μg/ml), CIMI + DOXO (5 ng/ml + 0.3 μg/ml) or $DMEM^+$ ($p < 0.05$), no significant differences were observed (Fig. 10). Figure 11 shows regions containing collagen fibers after in vitro culture of ovarian from mice.

Figure 10. Collagen levels (mean ± SD) in the mouse ovary analyzed by the picro-sirius red kit. The ovaries were cultured for 6 days in DMEM^{+} (control group), DOXO (0.3 µg/ml), CIMI (5 ng/ml) and CIMI+DOXO (5 ng/ml + 0.3 µg/ml). a and b different lowercase letters indicate statistically significant differences between treatments ($p < 0.05$).

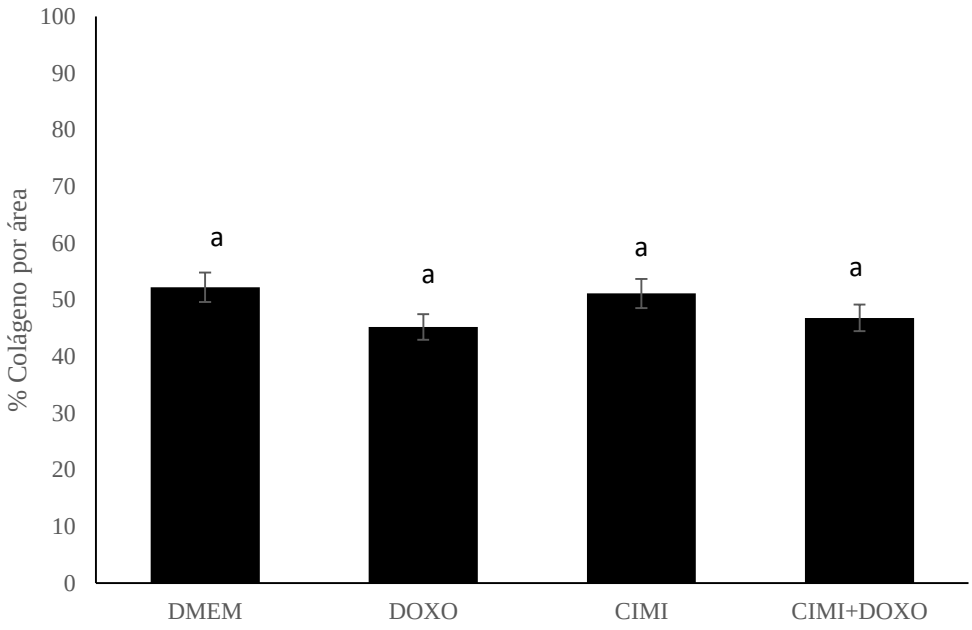
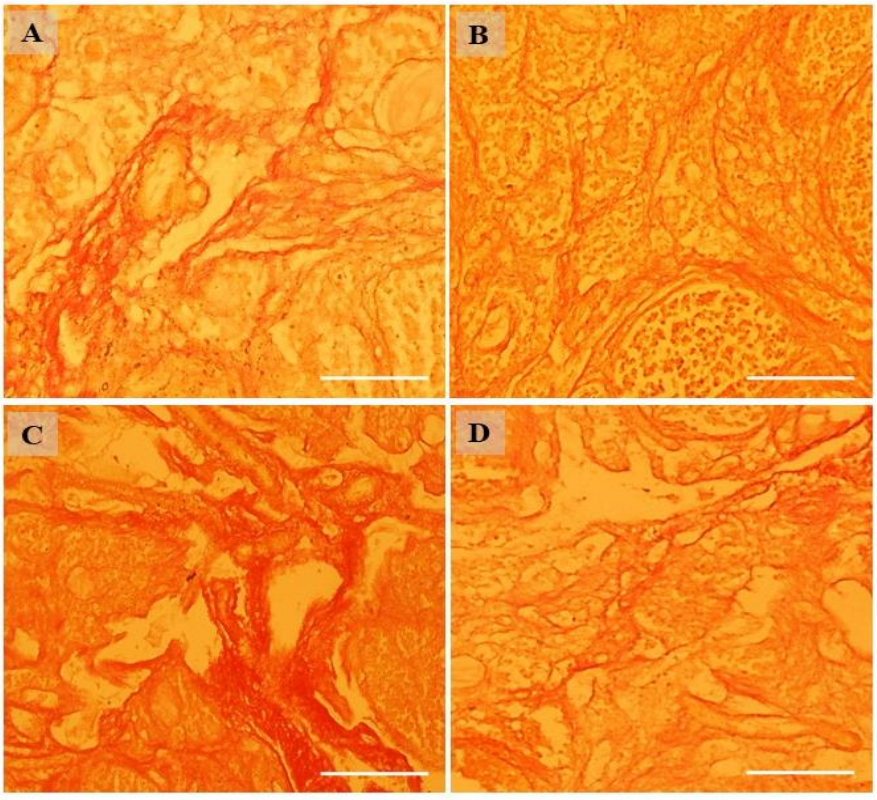


Figure 11. Representative images of collagen fibers labeled by picrosirius red spot: (A) control group (DMEM^{+}); (B) DOXO (0.3 µg/ml); (C) CIMI (5 ng/ml) and (D) CIMI (5 ng/ml) +DOXO (0.3 µg/ml). Scale bar: 100 µm (400x).



3. 6. Evaluation of stromal cells density after in vitro culture of mice ovaries

Figure 13 expose histological sections of mouse ovaries in the control group (DMEM$^+$) and in the group contains CIMI (5 ng/ml) alone or in association with DOXO (0.3 µg/ml). Ovaries cultured in a CIMI (5 ng/ml) alone or in combination with DOXO (0.3 µg/ml) maintained a well-preserved ovarian structure similar to those cultured in control group (DMEM$^+$). However, ovaries cultured with DOXO (0.3 µg/ml) had a decrease in number of stromal cells, compared to the other groups (Fig. 12).

Figure 12. Stromal cell density on ovarian, after culturing in control group (DMEM$^+$), DOXO (0.3 µg/ml), CIMI (5 ng/ml) and CIMI+DOXO (5 ng/ml + 0.3 µg/ml).

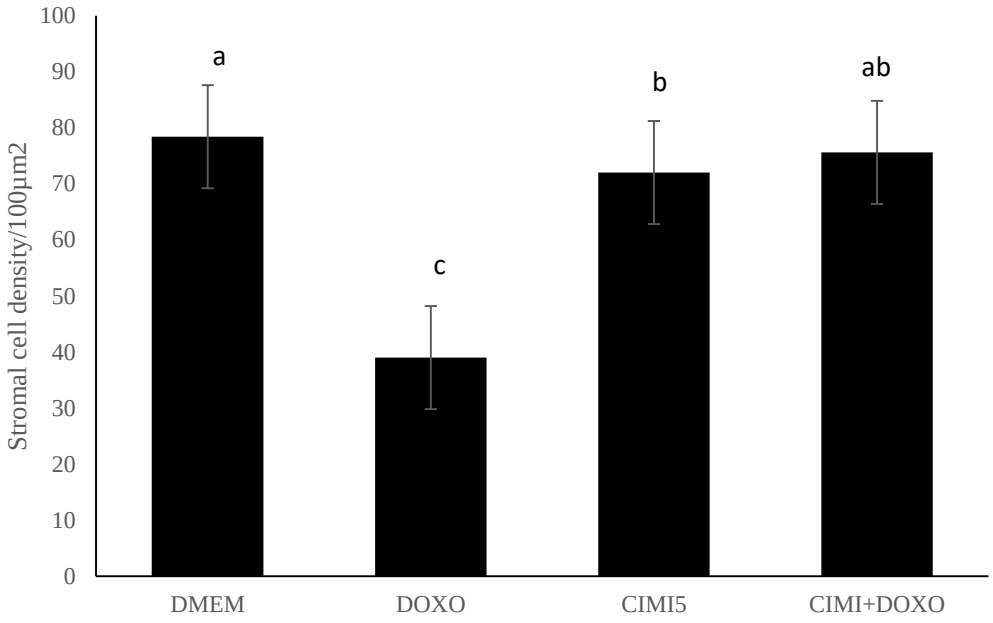
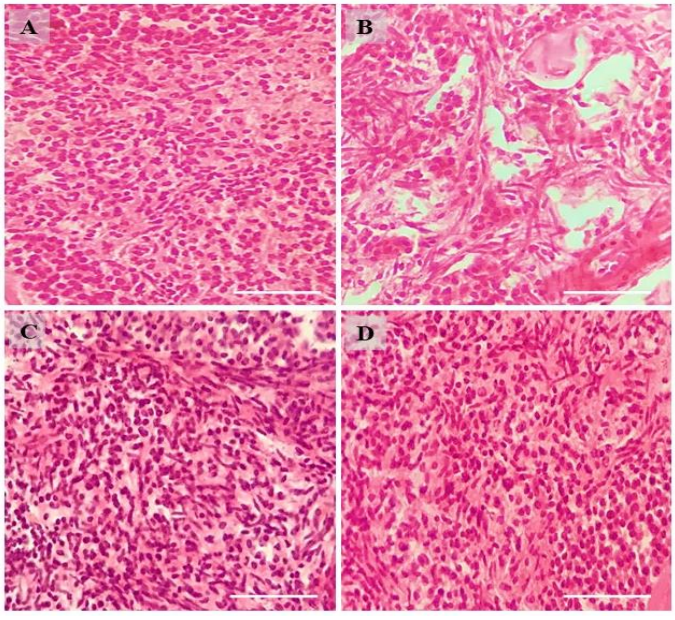


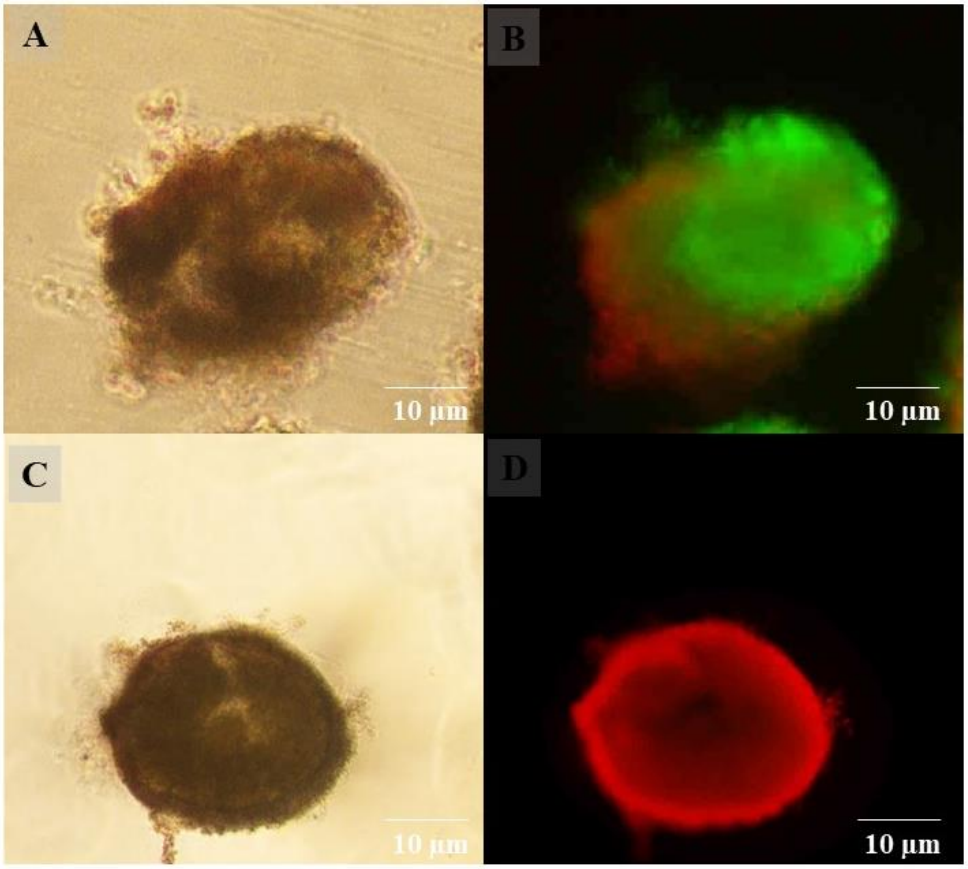
Figure 13. Representative images of ovarian stromal density after 6 days of culture: (A) control group (DMEM$^+$); (B) DOXO (0.3 µg/ml); (C) CIMI (5 ng/ml) and (D) CIMI (5 ng/ml) + DOXO (0.3 µg/ml). Scale bar: 100 µm (400x).



3. 7. Viability assessment of follicles after culture

The viability of morphologically normal follicles was confirmed by fluorescence microscopy. As shown in Fig. 14 (A-B), morphological normal follicles were stained positively for calcein-AM. On the other hand, atretic follicles were stained positively for ethidium homodimer-1 (Fig. 14 C-D). In this way, follicles from ovaries cultured in presence of CIMI alone or in combination with DOXO showed follicles stained positively by calcein-AM (green color-viable follicles), while follicles cultured in the presence of DOXO were positively stained for for ethidium homodimer (red color-degenerated follicles) (Fig. 14).

Figure 14. Evaluation of follicular viability by fluorescence microscopy after culture of mice ovarian. The follicles were staining with calcein-AM (green- viable follicle) and ethidium homodimer-1 (red- non-viable follicle). (A-B) preantral follicle cultured with *Cimicifuga racemose plus* doxorubicin (0,3 μg/ml) staining positively for calcein-AM; (C-D) non-viable preantral follicle cultured in presence of doxorubicin (0,3 μg/ml) staining with ethidium homodimer-1. Scale bars represent 10 μm.



4. Discussion

This study demonstrated for the first time that *Cimicifuga racemosa* extract its capable to attenuate doxorubicin-induced damage in the mouse ovary after 6 days of culture. Consistently, the present study demonstrated that the antineoplastic doxorubicin reduced the number of morphological normal follicles and significantly affected follicular viability, density of the ovarian stroma and increased apoptosis, while the extract of *Cimicifuga racemosa* (5 ng/ml) alone or in co-treatment with doxorubicin (0.3 μg/ml) was capable to protect the stroma and the ovarian follicles. The extract of *Cimicifuga racemosa*, with its high antioxidant content, may protect the ovaries from reactive oxygen species (ROS) produced by doxorubicin (DAI et al., 2017; RUHLEN et al., 2008). In one study, rats with polycystic ovary syndrome (PCOS) after treatment with *Cimicifuga racemosa* extract had normalized ovarian MDA and GSH activities, and the authors report that it may be associated with their antioxidant mechanisms (ASMAA et al., 2020). Additionally, studies show that high doses of doxorubicin increase reactive oxidation, causing follicular cell apoptosis and changes in ovarian parenchyma (FORMELLI et al., 1987).

The follicular development process is strongly influenced by the ovarian stroma, which supporting tissue and play several important roles. Ovarian stromal cells are similar to fibroblasts or interstitial cells. In general, fibroblasts secrete proteins from the extracellular matrix (ECM), such as collagen, for cellular support, structure and repair. Besides, it promotes the formation of follicular capillaries, which provide nutrients for follicular growth and development (CUI et al., 2020). In the present study, it was possible to observe that the treatment with doxorubicin reduced the stroma density, and consequently causing a decrease in follicular support. The findings are consistent with reports from previous studies, in which doxorubicin causes damage to ovarian microvasculature and increases the production of ROS, triggering cell death by apoptosis which induces the loss of stromal cells (BEN-AHARON et al., 2010; WANG et al., 2019; LOPES et al., 2020). Thus, antioxidant compounds can reduce toxic effects, *Cimicifuga racemosa* can act as an antioxidant substance (WOBSER et al., 2021).

Ovaries cultured in presence of *Cimicifuga racemose plus* doxorubicin kept the stroma density similar to the control group, and treatment with *Cimicifuga racemosa* alone kept the ovarian stroma similar to the co-treatment group (CIMI *plus* DOXO). Antioxidant compounds present in CIMI extract such as methyl caffeine, ferulic acid, isoferulic acid, fucinolic acid, cimicifugic acid A, cimicifugic acid B, cimicifugic acid F, cimiracemate A, and cyimiracemate B, are able to protect the ROS in ovaries (JOANNA et al., 2002).

These authors believe that CIMI extract (5 ng / mL) can reduce oxidative stress and also prevent ovarian tissue from apoptotic processes. A study with the glycoside triterpene action, a compound found in the genus *Cimicifuga*, induced a positive response to stress and apoptosis in human breast cancer cells (EINBOND et al., 2008). Thus, it is believed that research with this compound may be useful for the prevention and treatment of human breast cancer. In another study, using antioxidants present in the extract of *Cimicifuga racemosa*, it was possible to observe a reduction in DNA damage induced by menadione in S30 breast cancer cells cultured *in vitro* (JOANNA et al., 2002). Also, recently Rabeenau et al. (2019), using neuronal and liver cells, showed that the extract of CIMI mediated a change from mitochondrial respiration to glycolysis, and this metabolic change was essential for the protective effects against oxidative damage.

In this study, it was observed that treatments with *Cimicifuga racemosa* alone or in combination with doxorubicin, showed less immunoreaction for caspase-3. This indicates that in these treatments there was less cell death due to apoptosis. Caspase-3 has already been shown to be a marker of apoptosis in mammalian cells and initiates the apoptotic cascade by activating other caspases (ELDUTAR et al., 2017). Compounds with antioxidant or anti-inflammatory properties can protect ovarian tissue by reducing the level of caspase-3 after chemotherapy-induced toxicity (KAYGUSUZOGLU et al., 2018). Corroborating the findings of this study, Wang et al. (2019) had already demonstrated the involvement of caspase-3 in doxorubicin-induced damage pathways in follicular and oocyte death. These findings imply that *Cimicifuga racemosa* can reduce apoptosis in the ovary.

Regarding ECM, the presence of *Cimicifuga racemosa* alone or in association with doxorubicin did not show any difference after 6 days of culture. However, it is believed that the damage caused to the ovarian structure only in the doxorubicin group has triggered healing by stimulating ECM. Several studies have shown that the presence of collagen fibers in the cortical stroma after chemotherapy, a characteristic sign of cell death and healing of ovarian tissue (OKTEM; OKTAY, 2007). The ovaries cultured with *Cimicifuga racemosa* alone and in association with DOXO did not differ between the groups, however, in the treatments with *Cimicifuga racemosa* the stroma cell density was similar to the control group, ruling out a possible stimulation of the ECM due to damage to the ovarian structure. Studies with *Cimicifuga racemosa* record its ability to inhibit collagenases and also reduce inflammatory processes (KUSANO et al., 2001; NISSLEIN, FREUDENSTEIN, 2003; and QIU et al., 2007). During inflammatory process or tissue damage conditions, the expression of matrix metalloproteinases (MMP) is increased by the action of proinflammatory cytokines, such as Tumor Necrosis Factor-Alpha (TNF-α) and Beta Growth Transformation Factor (TGF- β).

MMPs are responsible for the degradation of ECM macromolecules, including collagen, fibronectin, laminin and proteoglycans (McCARTHY et al., 2008). Besides, the compounds present in the rhizome of species of the genus *Cimicifuga* are able to prevent collagen degradation through the inhibition of collagen-like MMP, acting on wound healing (KUSANO et al., 2001).

Previous studies report that doxorubicin in mice ovaries accumulates in the developing follicles, with larger follicles (secondary, tertiary and antral) accumulating significantly more doxorubicin than smaller follicles (primordial and primary) (ROTI ROTI et al., 2012). Exposure to doxorubicin results in the loss of both primordial and growing follicles, mainly through effects on mitotically active granulosa cells (GCs), and leads to a reduction in ovulation rates (PEREZ et al., 1997; BEN-AHARON et al., 2010; MORGAN et al., 2013; ROTI ROTI et al., 2014).

In the present study, ovaries cultured in the presence of doxorubicin reduced the percentage of primordial and developing follicles compared to the control group, while the presence of *Cimicifuga racemosa* alone kept the percentage of primordial and developing follicles equal to the control group. However, there was no significant difference between groups concerning the percentage of primordial and developing follicles when compared between treatments. The presence of growing follicles inhibits the recruitment of primordial follicles, however, chemotherapy drugs have a cytotoxic effect on growing follicles and the increasing loss of this follicular population leads to an increase in the activation of primordial follicles, and thus, the loss of follicular reserve (WANG et al., 2019). In the present study, treatment with doxorubicin had the lowest percentage of normal follicles and a high rate of apoptosis, we suggest that this drug caused the activation of primordial quiescent follicles, an event that culminated in the development and, simultaneously, in the death of follicles due to apoptosis (SPEARS et al., 2019). The follicular development process is strongly influenced by the ovarian stroma, which is the supporting tissue, in the treatment with *Cimicifuga racemosa* alone and association there was no reduction of these cells, possibly due to the protection of the extract. Thus, important processes for folliculogenesis may have been preserved. We also highlight that the ovarian follicles from the co-treatment of Cimicifuga racemosa with doxorubicin showed viability after the cultivation period.

In conclusion, this study showed that the use of *Cimicifuga racemosa* extract at 5 ng/mL in the culture medium had protective effects on mice ovaries against doxorubicin-induced toxicity. The extract improved stroma density, and maintained the percentage of normal follicles, preventing damage caused by doxorubicin *in vitro*. Further studies are needed to assess

the therapeutic effects of *Cimicifuga racemosa* on female gametes, especially when they are exposed to substances harmful to reproductive health.

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Conflict of Interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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CAPÍTULO 3

Extract of *Cimicifuga racemosa* (L.) Nutt. protect ovarian follicles reserve against *in vitro* deleterious effects caused by dexamethasone in mice

Extract of *Cimicifuga racemosa* (L.) Nutt. protect ovarian follicles reserve against *in vitro* deleterious effects caused by dexamethasone in mice

Assis, E.I.T.[a,b]; Azevedo, V.A.N.[a,b]; De Lima Neto, M.F.[a,b]; Costa, F.C.[a,b]; Paulino, L.R.F.M.[a,b]; Barroso, P.A.A.[a,b]; Donato, M.A.M[c]; Peixoto, C.A.[c]; Do Monte, A.P.O.[d] ; Matos, M.H.T.[d]; Godinho, A.N.[b]; Freire, J.M.O.[b]; Souza A.L.P.[a,b]; Silva, J.R.V.[a,b]; Silva, A.W.B.[a,b]*

[a] *Laboratory of Biotechnology and Physiology of Reproduction -LABIREP - Nucleus of Biotechnology of Sobral – NUBIS, Federal University of Ceara, Sobral, CE, Brazil.*

[b] *Nucleus of Research in Animal Experimentation – NUPEX, Federal University of Ceara, Sobral, CE, Brazil.*

[c] *Laboratory of Ultrastructure, CNPqAM/FIOCRUZ, Federal University of Pernambuco, Recife, PE, Brazil*

[d] *Nucleus of Biotechnology Applied to Ovarian Follicle Development, Federal University of São Francisco Valley, Petrolina-PE, Brazil*

**Corresponding address (A.W.B., Silva): Nucleus of Biotechnology of Sobral - NUBIS, Federal University of Ceara, Av. Comandante Maurocélio Rocha Ponte 100, CEP 62041-040, Sobral, CE, Brazil. Phone / Fax: +55 88 36954744 [andersonweiny@sobral.ufc.br]*

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Abstract

The goal of the present study are to investigate if *Cimicifuga racemose* extract (CIMI) reduces the deleterious effects of dexamethasone (DEXA) on mouse ovaries after 6 days of in vitro culture. Thus, mice ovaries (n = 32) were collected and cultured in (i) $DMEM^+$ alone (control group); (ii) $DMEM^+$ supplemented with 5 ng/ml of CIMI; (iii) $DMEM^+$ added of DEXA (4 ng/ml) or (iv) $DMEM^+$ containing 5 ng/ml CIMI *plus* 4 ng/ml of DEXA. The culture was conducted at 37.5°C, in 5% CO2, for 6 days. After culture, the ovaries were destined to

histological (survival, activation and follicular growth), immunohistochemical, as well as extracellular matrix configuration (ECM) and stroma density analyses. The results show that the presence of DEXA (4 ng/ml) reduces the percentage of morphologically normal follicles when compared to cultured control group, while the presence of CIMI (5 ng/ml) can maintain the percentage of normal follicles similar to cultured control group, preventing the deleterious effects caused by DEXA. Regarding follicular activation and development, the ovaries cultured in the presence of DEXA (4 ng/ml) reduced the percentage of primordial follicles compared to cultured control group, while the presence of CIMI (5 ng/ml) alone, maintained the percentage of primordial follicles equal to cultured control group. Regarding follicular development and ECM, the presence of CIMI (5 ng/ml) alone or in association with DEXA (4 ng/ml) did not influence the growth of the follicles and the configuration of ECM. In contrast, the presence of DEXA (4 ng/ml) negatively affected the stromal density in the ovary, while CIMI (5 ng/ml) was able to prevent this reduction. In addition, immunohistochemistry analyses showed absent to moderate immunoreaction for caspase-3 in ovaries cultured in the presence of CIMI (5 ng/ml). Thus, we can conclude that the extract of *Cimicífuga racemosa* was able to prevent the deleterious effects caused by dexamethasone in mice ovaries after 6 days of culture.
Keywords: folliculogenesis; toxicity; mice; phytomedication; glucocorticoid; in vitro culture.

1. Introduction

Glucocorticoids (GCs) are steroid hormones widely used in intensive care due to their anti-inflammatory and immunosuppressive effects. The choice of GC has valuable implications (HILL et al., 2021) and it is estimated that 1-2% of the world population is on long-term therapy with GC (LI et al., 2021). Among exogenous glucocorticoids, hydrocortisone is identical to endogenous cortisol, while prednisone, prednisolone, dexamethasone and others are synthetic and vary in potency and affinity for their receptor. Among glucocorticoids, dexamethasone is a potent drug that has been widely used by general population, including women in reproductive age. Interestingly, recent studies shows satisfactory results of dexamethasone in in the treatment of acute respiratory distress syndrome (ARDS) caused by SARS-CoV-2 (COVID-19) infection (VILLAR et al., 2020; HORBY et al., 202; JOHNSON et al., 2021). An in vitro study, dexamethasone (4 ng/mL) showed effective suppression of IL-6 production in normal human bronchial epithelial (NHBE) cells stimulated with rh-TNF-α (Ho et al., 2004).

Although GCs are effective, the prolonged treatment can be accompanied by two central problems: the first is the harmful side effects mediated by GCs, such as osteoporosis, thinning/streaking of skin and muscle atrophy, and the second is the emergence of resistance to GC (CLARISSE et al., 2020). It is also known that exposure to GCs leads to numerous changes in several biological systems, including the reproductive system (HUŁAS-STASIAK et al., 2016). Glucocorticoid receptors (GR) are present inside cells and have already been detected in most tissues (ADCOCK, 2000), including in mouse ovarian tissue (ILLERA et al., 2005). Thus, steroids such as GCs can act to regulate the development of ovarian follicles. In this regard, several studies have shown that GCs regulate reproductive function through the direct or indirect suppression of follicular functions (YANG et al., 2019). A recent study conducted by Dare et al. (2018) revealed that high doses of dexamethasone (12 mg/kg and 10 mg/kg) cause multiples changes in histological characteristics of ovary and uterus. Studies have also shown that dexamethasone plays an important role in the mouse's ovarian function, in the development and pre-implantation of the embryo through the GR and makes it difficult to differentiate the follicle and oocyte maturation by activating steroidogenesis pathways (VAN MERRI et al., 2006). In addition, dexamethasone acts by suppressing the expression induced by luteinizing hormone (LH) of the protein StAR and the production of progesterone by a mechanism mediated by GR (HUANG et al., 2001). In another study using mice, it was observed that maternal exposure to dexamethasone led to a significant reduction in the pool of healthy primordial follicles in the offspring and increased follicular atresia (HUŁAS-STASIAK et al., 2016). Thus, conducting studies that investigate the potential of products, especially those of plant origin; capable of attenuate the deleterious effects of GCs on ovarian function in order to preserve fertility are extremely necessary. To develop these protective treatments, the mechanisms behind the harmful effect of GCs in the ovary and follicle need to be better understood (ALASMARI et al., 2014; CHO et al., Et al 2019; YUAN et al., 2014).

Cimicifuga racemosa (L.) Nutt. (Black cohosh; *Cimicifuga racemosa*) is a widely used plant and it is effective in relieving the symptoms of menopause. It is a member of *Ranunculaceae* family, native to eastern North America (NIU et al., 2021). Phytochemical studies have shown that constituents of *Cimicifuga racemosa* stand out, including triterpene glycosides, phenolic constituents and formonectin (an isoflavone). Among the biological activities associated with *Cimicifuga racemosa*, its antioxidant, anti-inflammatory, antidiabetic, antiviral, antiangiogenic, vasodilatory and immunosuppressive effects stand out (GUO et al., 2017). The ethanolic and isopropanolic extracts were equipotent to the action of estrogenic drugs and dopaminergic drugs (MAHADY, 2005). A study using ethanolic extract of *Cimicifuga racemosa* showed that its bioactive compounds inhibited the aromatization of

androgens in rats with letrozole-induced polycystic ovary syndrome (PCOS), compensating for the oxidative ovarian stress, which may be involved in the pathogenesis of PCOS (ASMAA et al., 2020). However, it is still unknown whether the *Cimicifuga racemosa* extract has a protective effect against dexamethasone -induced damage in mouse ovary.

This study aimed to investigate the protective action of *Cimicifuga racemosa* extract against the deleterious effects of dexamethasone on morphology, activation, growth, and organization of extracellular matrix (ECM), as well as on density of cells in ovarian stroma and on the rate of apoptosis in mice ovary cultured *in vitro.*

2. Material and methods

2.1. Animals

For this study, Swiss mice (*Mus musculus*) were kept in polyethylene boxes lined with wood shavings (6 animals/box), with free access to filtered water and feed. The animals were kept at an average temperature of 22±2°C, following 12h light-dark cycles. The animals were used according with the guidelines and normative resolutions of the National Council for Control in Animal Experimentation (CONCEA). This study was approved by the Institution's Ethics Committee on the Use of Animals (CEUA), approved under protocol Nº. 05/18.

2.2. Evaluation of the estrous cycle

All females (18g and/or 2 months of age) had their estrous cycle evaluated once a day, for 15 days by a single evaluator, as established by Marcondes, 2002. According to the observed cells, it was determined whether the female was in proestrus, estrus, metestrus or diestrus. Only females with a regular cycle, that is, lasting 4 to 5 days, were used to carry out the experiment.

2.3. Chemicals

Dexamethasone (CAS number 50-02-2), *Cimicifuga racemosa* extract (CAS number: 8477626-1), the culture medium and other reagents used in the present study were purchased from Sigma (St. Louis, USA) unless than otherwise indicated in the text. The stock concentration of dexamethasone was prepared in ethanol as directed by the manufacturer. The working concentrations were prepared by serial dilutions in base medium-DMEM. The extract

of *Cimicifuga racemosa* was dissolved in 60% (v/v) ethanol for all experiments according to manufacturer's guidelines.

2.4. Experimental design

The ovaries were cultured according to the protocol described by O'Brien et al. (2003). Ovaries (n = 32) were collected from euthanized mice and cultured individually in 24-well plate containing 1 ml DMEM/HAMS F12 supplemented with ascorbic acid (10 μg/ml), penicillin G (75 μg/ml), *Insulin-Transferrin-Selenium* (ITS) (10 μg/ml insulin, 5.5 μg/ml transferrin, and 5 ng/ml selenium) and *Bovine Serum Albumin* (BSA) (10 μg/ml). This base medium was called $DMEM^+$. Then, the ovaries were distributed in the following treatments: (i) $DMEM^+$ alone (control); (ii) $DMEM^+$ added with 5 ng/ml of CIMI (CIMI group); (iii) $DMEM^+$ supplemented with 4 ng/ml of dexamethasone (DEXA group); or (iv) $DMEM^+$ enriched with CIMI 5 ng/ml *plus* DEXA 4 ng/ml (CIMI + DEXA group). The culture was performed at 37.5°C, in 5% CO2, for 6 days. Approximately half of the culture medium was replaced every 2 days. After culture, the ovaries were destined for analysis of survival, activation, follicular growth, ECM configuration, stromal cell density and immunohistochemistry. The concentration of CIMI used in this research was identified in previous experiments by our group, while the concentration of dexamethasone (4 ng/mL) was defined based in previous research conducted in vitro (LAMOTE et al., 2004; HO et al., 2004).

2.5. Morphological evaluation and assessment of in vitro follicular activation and growth

The cultured ovaries were fixed in paraformaldehyde (4% in phosphate buffered saline - PBS, pH 7.4) for 24 h and destinated to histological analyses. Thus, the ovaries were dehydrated in gradual series of ethanol, clarified with xylol and soaked in paraffin wax. For each ovary, 7 μm sections were mounted on slides and stained using the Hematoxylin-Eosin method. The histological analysis was carried out by an experienced researcher who is unaware of the treatments of each group under analysis. The ovaries were examined by the microscope (Nikon) at 400x magnification. The follicle development stages were classified as primordial (1 layer of flattened or flattened granular cells around the oocyte) or growing follicles (primary, 1 layer of cuboidal granular cells; secondary, 2 or more layers of cells of the cuboidal granulosa around the oocyte). These follicles were individually classified as histologically normal when an intact oocyte was surrounded by granulosa cells well organized in 1 or more layers, and which had no picnotic nucleus. Degenerated follicles were defined as those with retracted

oocyte, with pycnotic nucleus and/or surrounded by disorganized granulosa cells, detached from the basement membrane (GOUVEIA et al., 2021). The evaluation of follicular activation and growth was performed by classifying the follicles by primordial, primary and secondary follicles and by measuring the follicular and oocyte diameter before and at the end of each culture period, respectively.

2.6. Immunohistochemistry

Immunohistochemistry was performed as previously described by Barberino et al. (2020). The sections (5 µm thick) of each block were cut using a microtome (EasyPath, São Paulo, Brazil) and mounted on Starfrost glass slides (Knittel, Braunschweig, Germany). The tissue sections were incubated in 0.01 M citrate buffer (pH 6), heated in the microwave (20 min, 90 W, 96ºC) and cooled for 20 min (RT) to recover the antigenicity of activated caspase-3. Sections were washed twice for 5 min with 0.05 M Tris buffered saline (TBS) (pH 7.4). Subsequently, endogenous peroxidase activity was depleted by incubating the sections in 3% (v/v) H_2O_2 in TBS (10 min, RT). Non-specific staining was blocked with normal goat serum (NGS) (1/4, Dako, Glostrup, Denmark) diluted in TBS, enriched with 0.3% Triton-X 100 (v/v) (Tx - Sigma) and 1% (w/v) BSA (Sigma) for 30 min at room temperature. Subsequently, the sections were incubated overnight (RT) with a primary antibody directed against activated caspase-3 (host: rabbit, polyclonal, Sigma) diluted in TBS, enriched with 0.3% (v/v) Tx and 5 % (w/v) BSA (1/225). The following day, following two washing steps of 5 min each with Dako Wash Buffer (Dako), the sections were incubated for 30 min in RT with anti-rabbit Envision (Dako). Following another two washing steps for 5 min each with TBS and one step with distilled water, a positive immunostaining was visualized by incubating the sections for 10 min (RT) with the chromogen 3-amino-9-ethylcarbazole (Dako). The sections were contrasted with Carazzi's hematoxylin (Klinipath, Olen, Belgium), rinsed and mounted with Medium Faramount (Dako). Using an optical microscope (Nikon) connected to a computer equipped with Image-Pro Plus® software (Media Cybernetics) five photos were taken at the same magnification (100x) and analyzed qualitatively.

2.7. Analysis of the extracellular matrix and evaluation of the density of the ovarian stroma

To assess the collagen fibers of extracellular matrix in ovarian cortex, staining with Picrosirius Red (Abcam Kit) was performed following the methodology described by Vanié (2017) with modifications. Ovarian sections of 7 µm were dewaxed in xylol and incubated in

Sirius Red solution (0.1%) for 1 hour at room temperature. Then, the excess dye will be removed with acetic acid solution (0.5%) and the sections were dehydrated and subjected to slide assembly with subsequent observation under an optical microscope (Nikon, Eclipse, TS 100, Japan) under magnification 400x. For each treatment, the percentage of the area occupied by collagen fibers in ten different fields was measured with the aid of a DS Cooled DS DS-Ri1 camera attached to a microscope (Nikon, Eclipse, TS 100, Japan), the microscope and the images were analyzed by Image J Software (Version 1.51p, 2017) with 400x magnification. Only collagen fibers were marked in red with the picrosirius color, while the follicles remained colorless (white). The analyzer software automatically excludes the circumference of unstained follicles from the total area marked in red. The Image J Software was used to quantify the percentage of collagen fiber in uncultured and cultured tissues. The staining intensity of collagen fibers was determined by measuring the average pixel intensity of the total area imaged after background subtraction.

The evaluation of cell density of ovarian stroma was performed after 6 days of culture in the different treatments: (i) control group ($DMEM^{+}$); (ii) DEXA (4 ng/ml), (iii) CIMI (5 ng/ml); (iv) CIMI (5 ng/ml) + DEXA (4 ng/ml). The number of stromal cells was calculated in an area of 100 μm^2. For each treatment, ten fields from different sections of histological preparations were evaluated. The average number of stromal cells per field was calculated as previously described (CAVALCANTE et al., 2019). All evaluations and measurements were performed by a single operator.

2.8. Statistical analysis

Statistical analysis was performed using the computer program GraphPad Prisma (5.0). Data that did not show normal distribution, selected by the Shapiro-Wilk test, underwent logarithmic transformation. The parametric variables of the average percentage of normal follicles and follicular activation in each treatment were evaluated by Fisher's exact test and the comparison between treatments using Kruskal-Wallis. For the analysis of collagen fibers, the data were analyzed by the Kruskal-Wallis test, followed by the Dunn comparison. The results were expressed as mean and standard error (mean ± S.E.M). Differences were considered significant when $p < 0.05$.

3. Results

3.1 - Evaluation of the protective effect of Cimicifuga racemosa in mouse ovaries exposed to dexamethasone for 6 days of in vitro culture: follicular survival, activation and development

The ovaries cultured in presence of dexamethasone (4 ng/mL), showed a damage of ovarian structure and promoted a reduction in the number of normal follicles compared to ovaries cultured in the presence of CIMI (5 ng/mL) alone. The association of CIMI (5 ng/mL) and DEXA (4 ng/mL) promotes an increase in the percentage of morphologically normal follicles compared to DEXA (4 ng/mL) group. However, no significant differences (P>0.05) were observed when compared all treatments to the cultured control group ($DMEM^{+}$) (Fig.1). The morphology of follicles from mice ovarian follicles cultured in different treatments are showed in figure 2.

Figure 1. Percentage (mean ± SE) of normal follicles evaluated at the end of 6 days of culture by classical histology, hematoxylin and eosin staining, the ovaries were divided between the control group ($DMEM^{+}$) and subjected to treatments with DEXA (4 ng/ml), CIMI (5 ng/ml) and CIMI+DEXA (5 ng/ml + 4 ng/ml). a and b different lowercase letters indicate statistically significant differences between treatments (P <0.05).

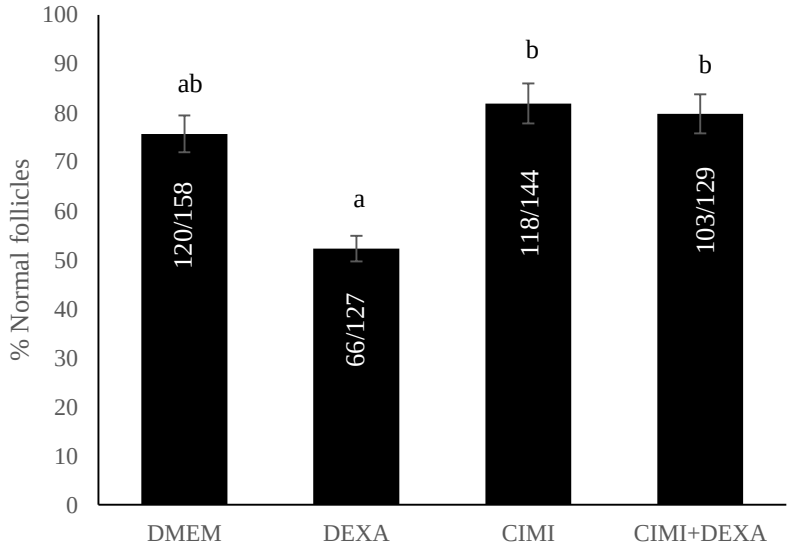
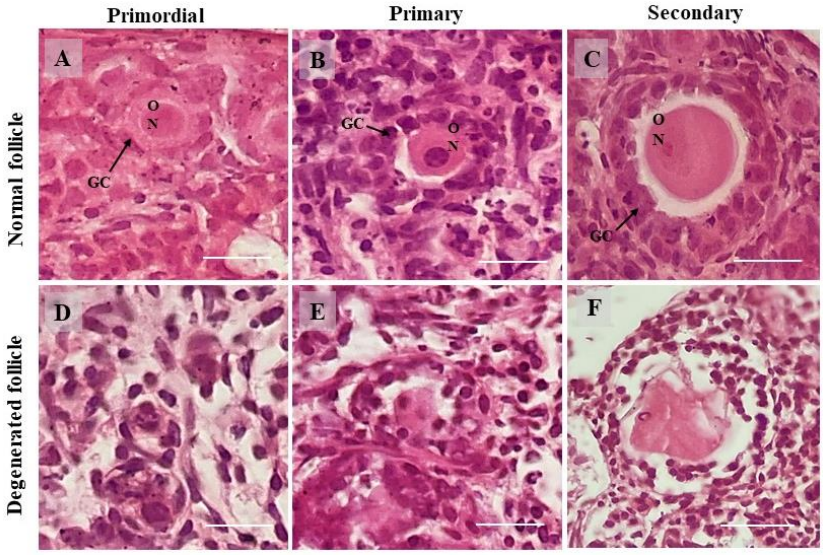
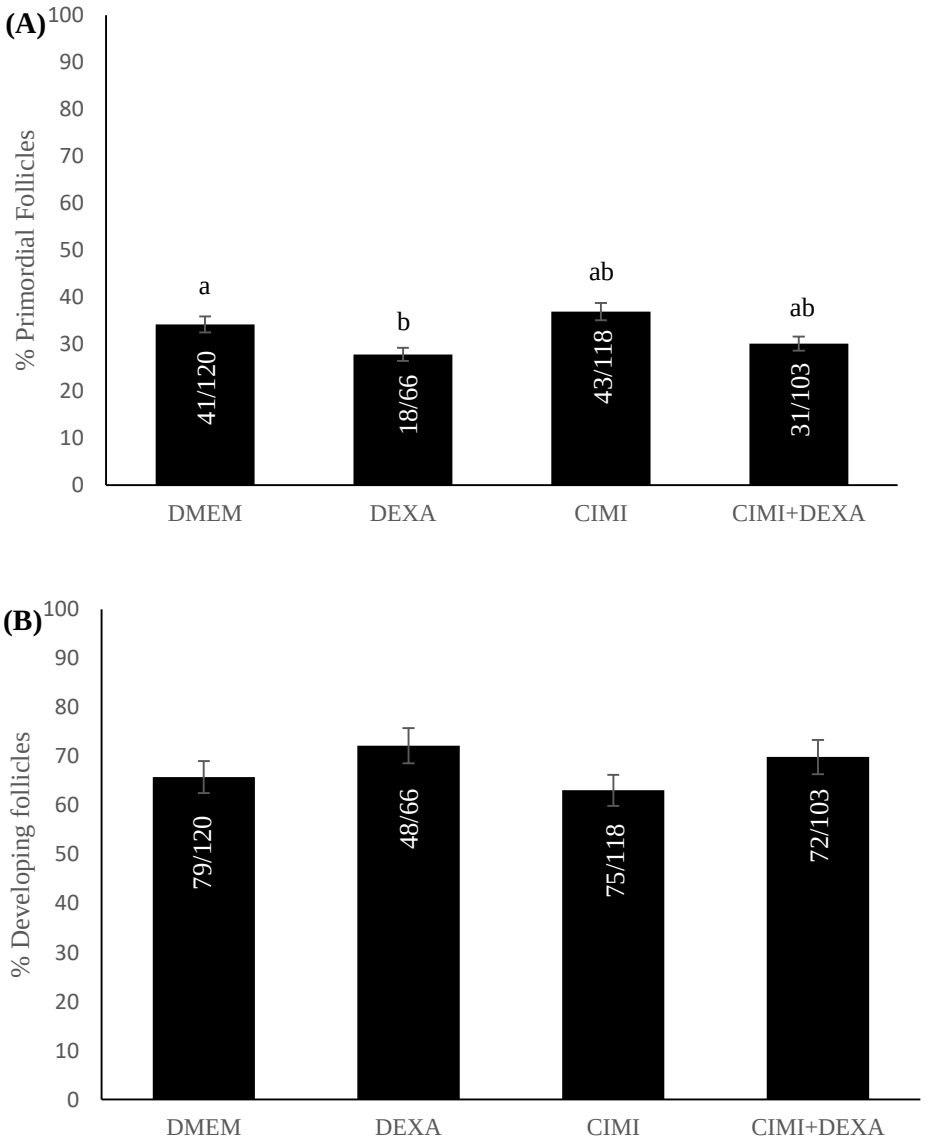


Figure 2. Representative images of cultured mice ovary showing morphologically normal (a-c) and atretic (d-f) follicles from different categories stained with hematoxylin and eosin. (A) Normal and (D) atresic primordial follicle; (B) Normal and (E) atretic primary follicle; (C) Normal and (F) atretic secondary follicle. CG: Granulosa cells ; O: Oocyte; N: Oocyte nucleus. Scale bar: 100 μm (400x).



After *in vitro* culture, there was a reduction ($P<0.05$) in the percentage of primordial follicles (Fig. 3A) in treatment containg DEXA (4ng/mL) compared to cultured control ($DMEM^{+}$). The presence of CIMI (5 mg/mL) in the culture medium maintains the percentual of primordial follicles similar to control group ($DMEM^{+}$). However, no significant difference was observed between treatment groups (Fig. 3A). The percentage of developing follicles do not showed difference ($P>0.05$) between cultured groups (3B).

Figure 3. Percentage (mean ± SE) of (a) primordial and (b) developing follicles evaluated at the end of 6 days of culture by classical histology, hematoxylin and eosin staining. The ovaries were cultured in the (i) control group ($DMEM^+$); (ii) $DMEM^+$ supplemented with CIMI (5 ng/ml) or (ii) $DMEM^+$ added CIMI(5 ng/ml) +DEXA (4 ng/ml). a and b different lowercase letters indicate statistically significant differences between treatments ($P < 0.05$).



3.4. Immunohistochemistry expression of activated caspase-3 in cultured mice ovary: Cimicifuga racemose reduce the immunohistochemical staining for caspase-3

The intensity of immunoreaction for caspase-3 throughout the ovarian structure is presented in Table 1. Immunohistochemical analysis showed that Caspase-3 was expressed differently in all treatments (Table 1). The immunoreaction was considered absent, weak or moderate for caspase-3 in primordial and primary follicles in different treatments groups. In secondary follicles, a strong reaction to caspase-3 was observed in the control group ($DMEM^+$), in the treatment with DEXA (4 ng/ml) isolated, and in the treatment with CIMI (5 ng/ml) alone.

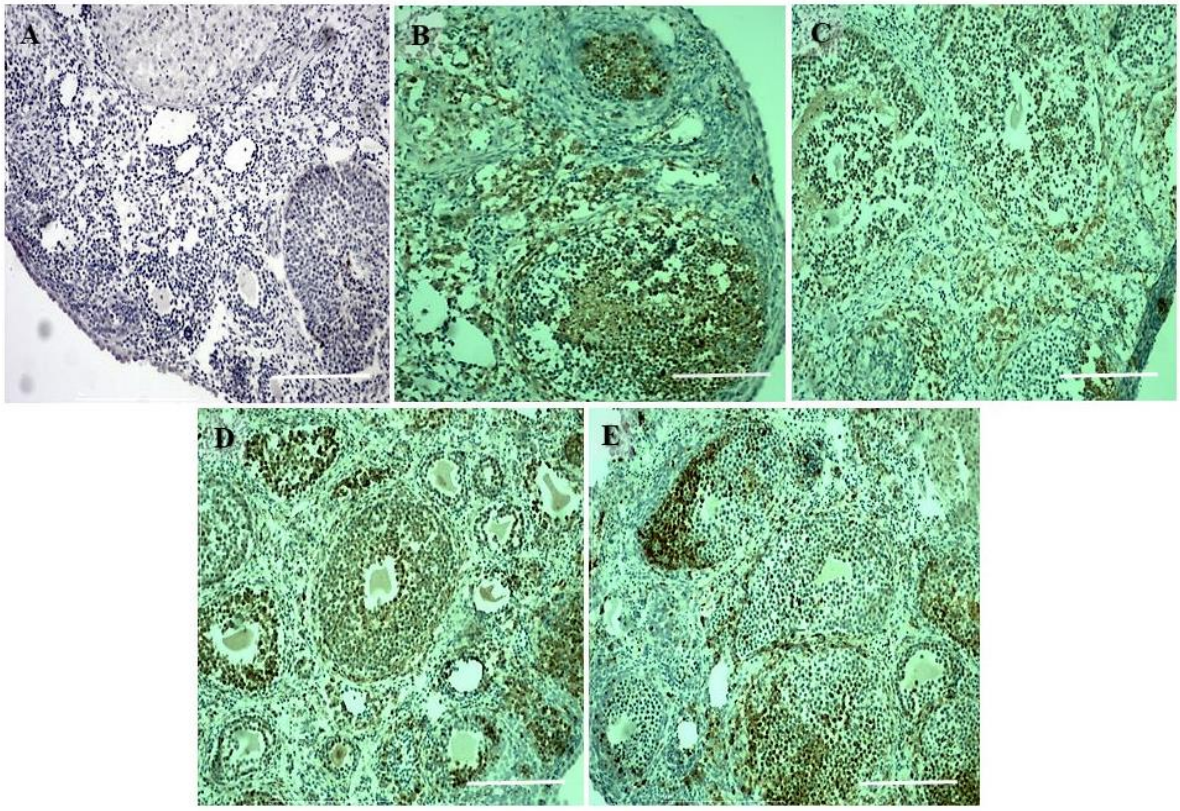
In co-treatment (CIMI+DEXA) the reaction was moderate. Moreover, the stromal ovarian cells showed a weak reaction (CIMI and CIMI + DEXA), moderate ($DMEM^{+}$), or strong for (DEXA 4 ng/ml) (Fig. 4).

Table 1. Relative intensity of the immunohistochemical staining for caspase-3 in mice ovary.

	Caspase-3			
Treatment	$D\text{-}MEM^{+}$	CIMI 5	DEXA 4	CIMI+DEXA
Structure				
Primordial follicle	+	+	++	-
Primary follicle	++	+	++	+
Secondary follicle	+++	++	+++	++
Stromal cells	++	+	+++	+

(−) absent; (+) weak; (++) moderate; (+++) strong immunoreaction.

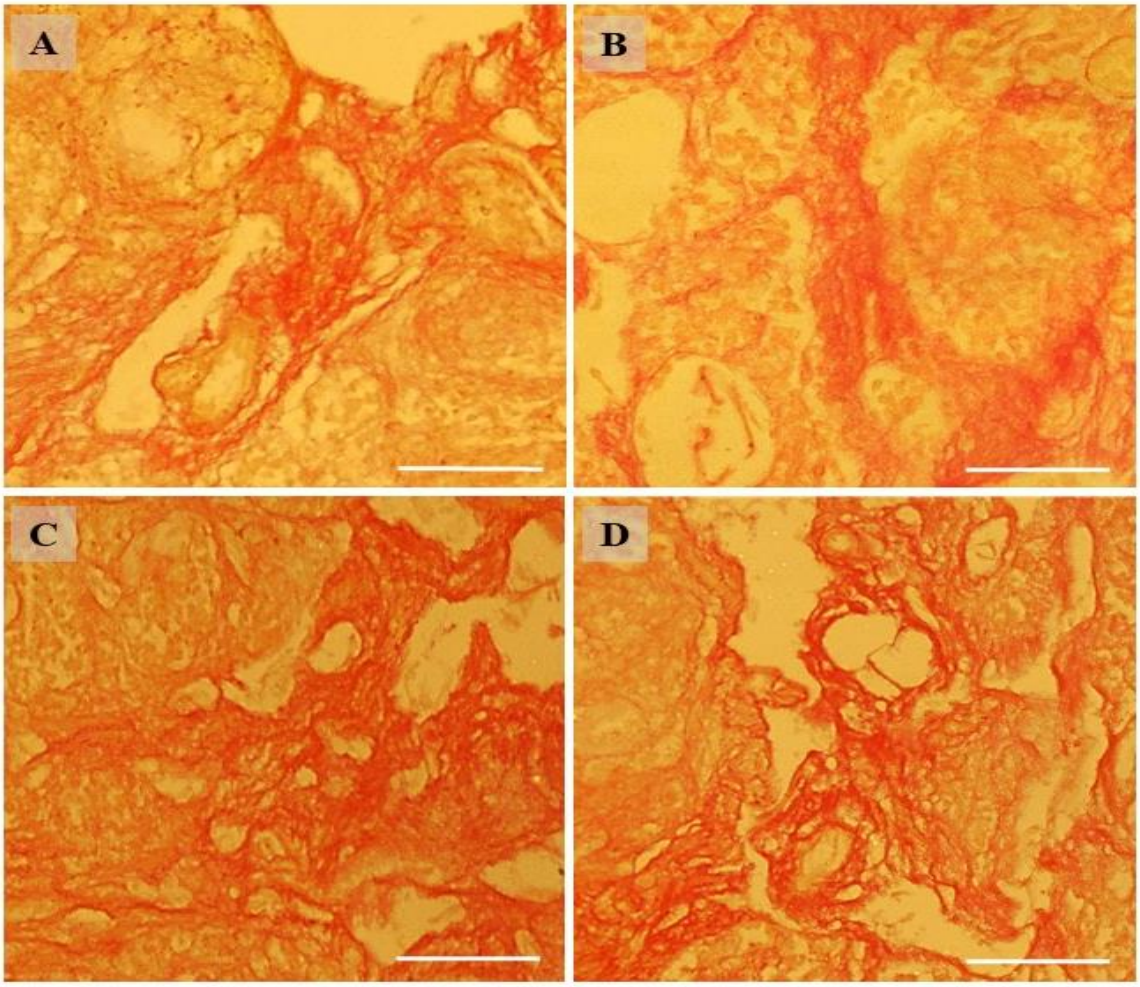
Figure 4. Immunohistochemical location of caspase-3 (B-E) in mice ovaries after culture: (A) negative control; (B) cultured control group ($DMEM^{+}$); (C) CIMI (5 ng/ml); (D) DEXA (4 ng/ml); and (E) CIMI + DEXA. Scale bar: 100 µm (100x).



3.5. Evaluation of extracellular matrix after ovarian culture in presence of Cimicifuga racemose alone or in association with Dexametasone

Picrosirius red analysisshowed no statistical difference in the percentage of collagen fibers between ovaries cultured in the different treatments. Therefore, there was no significant damage to the ECM, independent of treatment. After evaluating the ovaries stained with picrosirius red, it was possible to verify the regions containing collagen fibers in all treatments (Fig. 5).

Figure 5. Representative images of collagen fibers labeled by picrosirius red spot: (A) control group ($DMEM^{+}$); (B) DEXA (4 ng/ml); (C) CIMI (5 ng/ml) and (D) CIMI+DEXA (5 ng/mL + 4 ng/ml). Scale bar: 100 μm (400x).



3.6. Evaluation of stromal cell density after in vitro culture

The presence of dexamethasone in culture medium reduces significantly the stromal cell density in the ovary after 6 days of culture compared to treatment containing CIMI (5 ng/mL) alone and control group (DMEM^{+}). The presence of CIMI (5 ng/mL) associated to DEXA (4 ng/mL) in the culture medium was capable to protect the stromal cell density against dexamethasone-induced damage (Fig. 6). However, when all treatment groups were compared with control medium (DMEM^{+}), it was possible to see a reduction in the maintenance of stromal cell density. Figure 7 shows histological sections of the control group (DMEM^{+}), and those cultured in medium (DMEM^{+}) supplemented with CIMI (5 ng/ml) and DEXA (4 ng/ml) separated and in association.

Figure 6. Stromal cell density on ovarian after culturing in: Control group (DMEM^{+}), DEXA (4 ng/ml), CIMI (5 ng/ml) and CIMI+DEXA (5 ng/mL + 4 ng/ml).

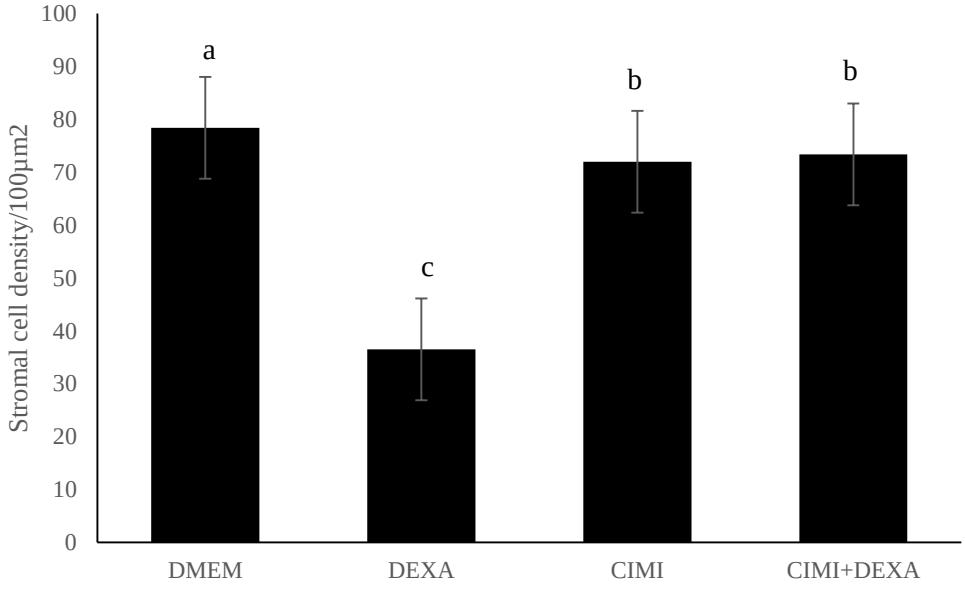
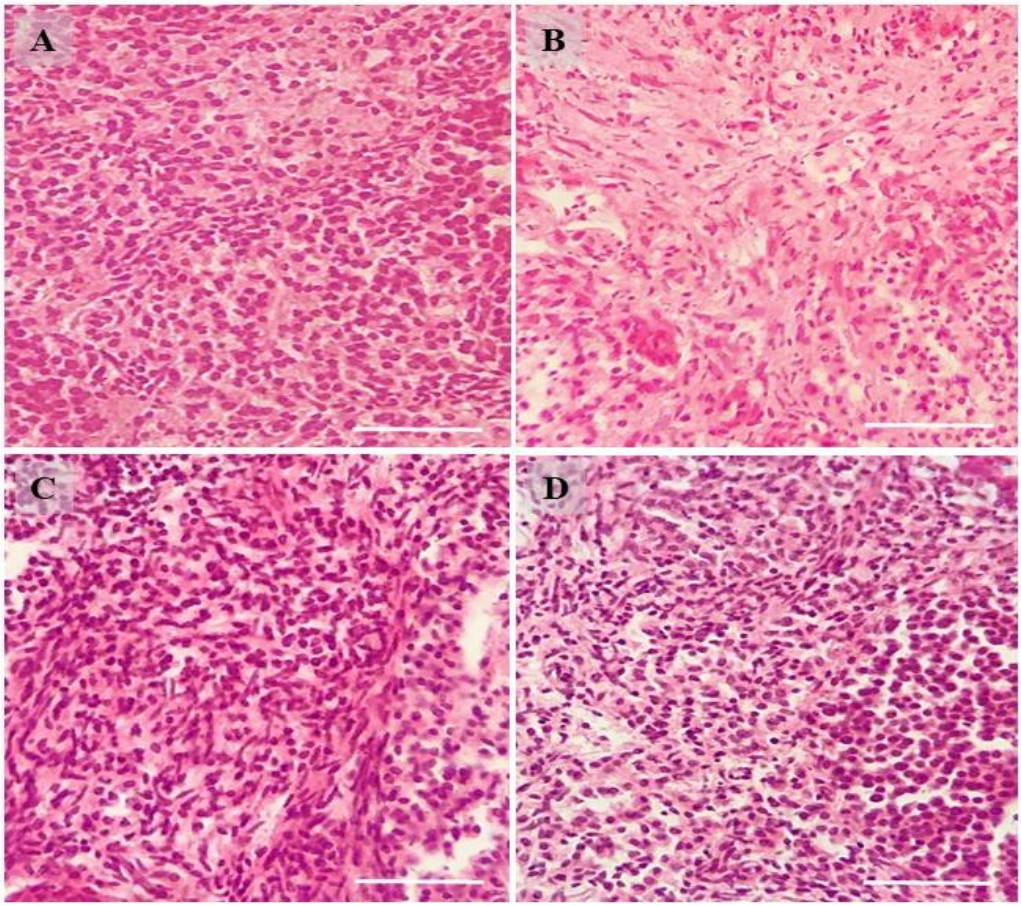


Figure 7. Representative images of the density of the ovarian stroma after culture: (A) control group ($DMEM^+$); (B) DEXA (4 ng/ml); (C) CIMI (5 ng/ml) and (D) CIMI+DEXA (5 ng/ml + 4 ng/ml). Scale bar: 100 μm (400x).



4. Discussion

The present study shows for the first time that *Cimicifuga racemosa* extract can attenuate the damage induced by dexamethasone in mice ovaries cultured *in vitro*. Extract of *Cimicifuga racemosa* is used globally as a natural drug to relieve symptoms of menopause (CAMPOS et al., 2012). However, the exact mechanism of action of *Cimicifuga racemosa* has not yet been fully elucidated (BURDETTE et al. 2013). In our study, the culture medium containing *Cimicifuga racemosa* (5 ng/ml) promoted better rates of morphologically normal follicles and prevented apoptosis (reducing expression of caspase-3), in addition to protecting the ovarian stromal cells against dexamethasone-induced citotoxity.

Cimicifuga racemosa maintained a greater number of normal follicles in medium that contain dexamethasone. This compound was able to reduce tissue injuries induced by dexamethasone and to maintain the adequate follicular structure. Moreover, it did not cause damage to the cells of the ovarian stroma and maintaining its normal density. This response possibly occurred due to the phytochemical properties and biological activities from this plant

as an antioxidant, anti-inflammatory, and antineoplastic activity, among other properties (BOLLE et al., 2007). The damage caused in ovarian structure when cultured with dexamethasone, may be occurred by the overproduction of reactive oxygen species (ROS), due an imbalance in physiological processes (MATSUWAKI et al., 2004). *In vitro* studies have shown that the addition of antioxidants, such as N-acetylcysteine, to the culture medium, inhibits apoptosis and/or promotes the growth of preantral follicles in rats and humans (TILLY and TILLY, 1995; OTALA et al., 2002). Thus, we believe that *Cimicifuga racemosa* can maintain follicular survival by inhibiting oxidative stress.

Our results showed that presence of dexamethasone in culture medium negatively affected the stromal cell density, which is in accordance with studies carried out on human fetal ovaries in which dexamethasone decreased the stroma density (POULAIN et al., 2012). Additionally, dexamethasone is a potent inhibitor of mouse hippocampal progenitor cell proliferation (KIM et al., 2004). In the present study, it is important to highlight that the presence of *Cimicifuga racemosa* alone or associated with dexamethasone in the culture medium was able to prevent damage, keeping the stroma density like the control group. It is important to note that in mice that containing an abnormal ovarian stroma, the proliferation of granulosa cells from secondary follicles is impaired. A study with the extract of *Cimicifuga racemosa* with and without vitamin C increased the expression of Ki-67, an indicator of cell proliferation, in the granulosa cell layer (ASMAA et al., 2020). This influence on cell proliferation may have helped in our study not only in granulosa cells but in all ovarian stroma. *Cimicifuga racemosa* was important for the maintenance of the ovarian stroma, which gives full support to the follicle, directly influencing follicular survival (KINNEAR et al., 2020).

Glucocorticoids have been widely used in clinical application for anti-inflammatory and immunosuppressive function. Previous study reported that glucocorticoids adversely affect the reproductive system and can directly act on ovary (WHIRLEDGE et al., 2010). Conflicting findings are reported for dexamethasone in literature. In bovine model, oocytes from isolated secondary follicles cultured in vitro with dexamethasone showed signs of degeneration and poor ultrastructure was maintained in these cells (BARROSO et al., 2020), while tested in ovarian tissue from bovine, the presence of dexamethasone maintains ultrastructure from preantral follicles in cultured tissue (SILVA et al., 2017).

An in vitro study, dexamethasone (4 ng/mL) do not show significant effects on the number of viable cultured polymorphonuclear leukocytes (PMN) when compared to control group (LAMOTE et al., 2004). In addition, dexamethasone in the same concentration (4 ng/mL) showed effective suppression of IL-6 production in normal human bronchial epithelial (NHBE) cells stimulated with rh-TNF-α in vitro (Ho et al., 2004), whereas in this research,

dexamethasone (4 ng/mL) showed a strong immunoreaction for caspase-3 in secondary follicles and ovarian stroma.

We believe that the DEXA can act directly on the ovary by inhibiting the production of steroid hormones and inducing apoptosis. Studies show that dexamethasone stimulated the activation of caspase-3, which resulted in apoptosis in granulosa cells of rat from preantral follicles (YUAN et al., 2014). Hughes and Gobe (2007) report that caspase-3 was considered a specific immunohistochemical marker for apoptosis. The balance between apoptosis and cell proliferation during reproductive age is crucial for establishing fertility (AITKEN et al., 2011). In this study, the use of *Cimicifuga racemosa* extract alone and in combination with dexamethasone did not show strong immunoreaction for caspase-3, indicating that CIMI can prevent apoptosis on *in vitro* culture of mice ovary. High concentrations of ROS, deplete the antioxidant capacity of cells, causing irreversible cellular injuries, triggering a signaling program that leads to senescence, apoptosis, or necrosis (HALLIWELL, 2007). Our results demonstrated that *Cimicifuga racemosa* had protective effects on ovarian follicles and stroma, supposedly through its antioxidant activity.

In the *in vitro* culture of ovarian tissue, there is evidence that ROS induces mitochondrial apoptosis pathways through the activation of cell death receptors on the membrane (CHANDRA et al., 2000). The GC-induced mitochondrial apoptotic pathway disrupts the potential of the mitochondrial membrane and releases key apoptosis-inducing factors, such as cytochrome C (JOHNSON et al., 1999). One study showed that the extract of *Cimicifuga racemosa* inhibited the dissipation of the mitochondrial membrane potential and the oxidation of cardiolipin and decreased the release of reactive oxygen species and 3-nitrotyrosine, in sublingual glands of rats (DA et al., 2017).

Regarding follicular activation, the ovaries when cultured in the presence of dexamethasone reduced the percentage of primordial follicles compared to the control group, while the presence of *Cimicifuga racemosa* alone, kept the percentage of primordial follicles equal to the control group. However, there was no significant difference between groups when compared to treatments. Concerning to development, the present study showed that there was no difference between treatments. Inflammation is connected to several biological processes showed that positive regulation and an increase in circulating inflammatory mediators altered follicle populations in ovaries of neonatal rats (Fuller et al., 2017). We believe that, possibly, the cytokine regulation by dexamethasone (Hua-Lin Chen et al., 1993), which influences the activation and follicular development, also happened in the treatments with *Cimicifuga racemosa*, since studies show that the administration of compounds present in the extract of *Cimicifuga racemosa* leads to a decrease in the concentration of TNF-α in human blood

(SCHMID et al., 2009). Besides, other studies have shown that components of the extract of *Cimicifuga racemosa* can inhibit the translocation of the nuclear transcription factor kappa B (NF-κB) and subsequent production and release of TNF-α (SHIN et al., 2004; QIU et al., 2007).

We can be concluded that extract of *Cimicifuga racemosa* was able to protect ovarian tissue against the deleterious effects caused by dexamethasone in the maintenance of follicular morphology and apoptosis at the end of 6 days of culture. Moreover *Cimicifuga racemosa* can exerce a protective effect on the ovarian stroma against the damage caused by dexamethasone in the cultured mouse ovaries.

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Conflict of Interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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9 CONCLUSÕES GERAIS

Diante do exposto, conclui-se que o extrato de *Cimicifuga racemosa* (L.) Nutt. (CIMI) é capaz de prevenir os efeitos deletérios causados pela doxorrubicina ou dexametasona na manutenção da sobrevivência folicular, além de apresentar um efeito protetor no estroma ovariano e reduzir o índice de apoptose em ovários de camundogas cultivados in vitro por 6 dias. Esses achados mostram que a CIMI possui propriedades funcionais na foliculogênese e este composto representa uma ferramenta biotecnológica promissora em estudos pré-clínicos *in vitro* para compreensão de mecanismos responsáveis pela manutenção da fertilidade. Assim, a continuidade das investigações *in vivo* poderá fornecer elementos que venham a esclarecer os efeitos da CIMI quando associada a DEXA ou DOXO a fim de verificar se há a potencialização ou redução dos efeitos identificados.

10 PERSPECTIVAS

Os resultados obtidos nesta dissertação poderão auxiliar na compreensão dos efeitos do extrato de *Cimicifuga racemosa* (L.) Nutt. na função ovariana e crescimento folicular *in vivo*, fornecendo uma base científica sólida para futuros testes da associação da CIMI com a DOXO ou DEXA em camundongas. Além disso, medicamentos que tenham como base a *Cimicifuga racemosa* poderão ser desenvolvidos para preservar a função ovariana durante a quimioterapia. Entretanto, ressalta-se que os estudos desenvolvidos neste trabalho foram realizados com animais saudáveis e *in vitro*. Desta forma, mais estudos devem ser realizados para avaliar como os compostos da *Cimicifuga racemosa (L.) Nutt.* atuariam em animais induzidos a doenças e posteriormente tratados com os fármacos testados neste trabalho, de modo a avaliar se a o tratamento iria responder de forma positiva reduzindo os efeitos adversos causados por doxorrubicina na quimioterapia ou por dexametasona em tratamentos clínicos antiinflamatórios.

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