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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS

DENISE DAMASCENO GUERREIRO

UTILIZAÇÃO DO PROCESSO BIOTECNOLÓGICO DO *OVÁRIO ARTIFICIAL* PARA AVALIAR A TOXICIDADE DO VITANOLIDO D E DA QUINOXALINA SOBRE OS FOLÍCULOS PRÉ-ANTRAIS CAPRINOS

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Orientador: Profa. Dra. Ana Paula Ribeiro Rodrigues

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Aprovado em: 16 de janeiro de 2019.

BANCA EXAMINADORA
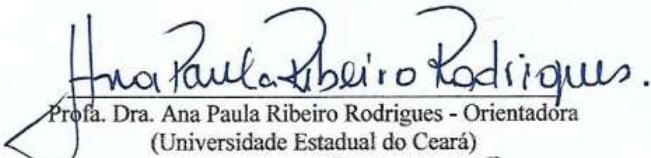
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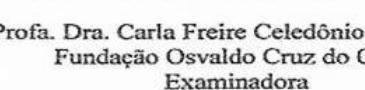
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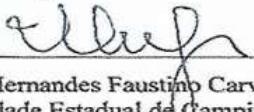
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Dedico ao meu amado filho, Johan Guerreiro.
Você me fez ver que mesmo diante de todas as
dificuldades é possível realizar um sonho.

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RESUMO

Embora o número de novos casos de câncer venha aumentando anualmente, o número de sobreviventes também vem crescendo ao longo dos anos, em função do diagnostico precoce e de tratamentos quimioterápicos mais efetivos. Porém, dentre os grandes efeitos indesejáveis da quimioterapia, destaca-se a resistência a multi-drogas (RMD) e a falha ovariana que leva à infertilidade. Portanto, a presente estudo teve dois principais objetivos: *1)* imunolocalizar membros da família de proteínas conhecidas como *Transportadores ABC* (ABCB1, ABCC2 e ABCG2) no tecido ovariano caprino (Fase 1) e *2)* avaliar a toxicidade de dois novos compostos com potencial anticâncer, ou seja, o *Vitanolido D* - VD (Fase 2) e a *Quinoxalina* - QX (Fase 3) sobre os folículos pré-antrais (primordiais, primários e secundários) cultivados *in vitro* no interior do tecido ovariano. Na Fase 1, ovários de cabras foram processados e analisados por imuno-histoquímica, qPCR e Western Blottingg para a identificação do ABCB1, ABCC2 e ABCG2. Nas Fases 2 e 3, fragmentos do córtex ovariano foram cultivadas *in vitro* por 2 e 6 dias em α-MEM$^+$ ou α-MEM$^+$ adicionado de paclitaxel (PTX – controle negativo) ou em diferentes concentrações de VD (1.5, 3.0 e 6.0 μM) ou QX (1.5, 3.0 e 6.0 μM), respectivamente. Nessas duas fases os folículos foram analisados antes (controle) e após o cultivo na presença dos compostos, considerando-se morfologia e ativação folicular; proliferação (Ki67) e apoptose (TUNEL) das células granulosa, a expressão proteica do ABCB1 e gênica dos fatores reguladores do ciclo celular *ciclinas* (A - CCNA, B1 - CCNB1, D1 - CCND1 e E1 - CCNE1) e *quinases dependentes de ciclina* (1 - CDK1; 2 - CDK2, 4 - CDK4 e 6 - CDK6)]. Os resultados mostraram que os três transportadores ABCs foram positivamente imunomarcadas em todas as categorias foliculares estudadas. No tocante aos compostos, a toxicidade do VD6.0 foi similar ao PTX e superior ($P <0,05$) às demais concentrações (1.5 e 3.0 μM), após 2 e 6 dias. A QX também apresentou alta toxicidade aos folículos pré-antrais, especialmente na mais alta concentração (6.0 μM), causando um *bournout* folicular e apoptose, superiores às concentrações de 1.5 e 3.0 μM. Ao contrário do VD, a QX inibiu a expressão do ABCB1 nos folículos pré-antrais. Esse trabalho mostrou pela primeira vez que três membros da família dos ABC transportadores foram identificados no ovário caprino. Apesar dos valiosos resultados obtidos nesse estudo, os dois compostos, isto é, o VD e a QX, necessitam ser melhor investigados, sob o ponto de vista da função ovariana, visando auxiliar na sua utilização como agentes quimioterápicos com menor risco para a fertilidade feminina.

Palavras-chave: Ovário artificial. Folículos pré-antrais. Função ovariana. Vitanolido D. Quinoxalina.

ABSTRACT

Although the number of new cases of cancer increases annually, the number of survivors has also increased over the years, due to the early diagnosis and more effective chemotherapy treatments. However, among the great undesirable effects of chemotherapy, the multidrug resistance (MDR) and the ovarian failure that lead to infertility. Therefore, the present thesis had two main objectives: 1) to immunolocalize members of the family of proteins known as ABC transporters (ABCB1, ABCC2 and ABCG2), responsible for the phenomenon of RMD in goat ovarian tissue (Phase 1) and 2) to evaluate the toxicity of two new compounds with anticancer potential, the Vitanolide D - VD (Phase 2) and Quinoxaline - QX (Phase 3) on the preantral follicles (primordial, primary and secondary) grown *in vitro* inside the ovarian tissue. In Phase 1, goat ovaries were processed and analyzed by immunohistochemistry, qPCR and Western Blotting for identification of ABCB1, ABCC2 and ABCG2. In Phases 2 and 3, fragments of the ovarian cortex were cultured in vitro for 2 or 6 days in α-MEM$^+$ or α-MEM$^+$ supplemented with paclitaxel (PTX - negative control) or at different RV concentrations (1.5, 3.0 and 6.0 μM) or QX (1.5, 3.0 and 6.0 μM), respectively. In these two phases the follicles were analyzed before (control) and after the culture in the presence of the compounds, considering morphology and follicular activation; proliferation (Ki67) and apoptosis (TUNEL) of the granulosa cells, the protein expression of ABCB1 and the gene of cyclin - cyclin regulatory factors (A - CCNA, B1 - CCNB1, D1 - CCND1 and E1 - CCNE1) and cyclin - dependent kinases 1-CDK1; 2-CDK2, 4-CDK4 and 6-CDK6)]. The results showed that the three ABCs transporters were positively immunolabelled in all follicular categories studied. Regarding the compounds, the toxicity of VD6.0 was similar to PTX and higher ($P < 0.05$) at the other concentrations (1.5 and 3.0 μM) after 2 and 6 days. QX also showed high toxicity to the preantral follicles, especially in the highest concentration (6.0 μM), causing a follicular burnout and apoptosis, higher than the concentrations of 1.5 and 3.0 μM. Unlike VD, QX inhibited the expression of ABCB1 in preantral follicles. This work showed for the first time that three members of the family of ABC transporters were identified in ovary goat. Despite the valuable results obtained in this study, the two compounds, i.e., RV and QX, need to be better investigated, from the point of view of ovarian function, aiming to aid in their use as chemotherapeutic agents with lower risk for female fertility.

Key words: Artificial ovary. Preantral follicle. Ovarian function. Whitanolido D. Quinoxaline.

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LISTA DE ABREVIATURAS E SIGLAS

°C	Graus Celsius
3Rs	Replacement, Reduction and Refinement
ABCs	ATP-binding cassetes
ATP	Adenosina Trifosfato
BSA	Bovine serum albumin (Albumina sérica bovina)
CDKs	Quinases dependentes de ciclinas
CGPs	Células germinativas primordiais
CO2	Dióxido de Carbono
DMSO	Dimethylsulfoxide (Dimetilsulfóxido)
DNA	Deoxyribonucleic acid (Ácido desoxirribonucleico)
Dr.	Doutor
Dra.	Doutora
EU	European Union (União Europeia)
Fig.	Figura
FOP	Falha ovariana prematura
FSH	Follicle-Stimulating Hormone (Hormônio Folículo Estimulante)
H2O2	Hydrogen peroxide (Peróxido de hidrogênio)
HCl	Ácido clorídrico
i.e.	Id est (isto é)
INCA	Instituto Nacional do Câncer
ITS	Insulin-transferrin-selenium (Insulina-transferrina-selênio)
IU	International unit (Unidade internacional)
L	Litro
LH	Luteinizing hormone (Hormônio Luteinizante)
LOE	Laboratório de Oncologia Experimental
M	Molar
MDR	Multidrug resistence
MEM	Minimum essential medium (Meio essencial mínimo)
Mg	Miligrama
mL	Mililitro
mm	Milímetro

mM	Milimolar
mm3	Milímetros cúbicos
mmol	Milimol
MOIFOPA	Manipulação de Oócitos Inclusos em Folículos Ovarianos Pré-antrais
mRNA	Messenger ribonucleic acid (Ácido ribonucleico mensageiro)
N	Núcleo do oócito
Ng	Nanograma
Nm	nanômetro
Nu	Nucleus (Núcleo)
O	Oocyte (Oócito)
O2	Oxigênio molecular
OMS	Organização Mundial de Saúde
P	Probabilidade de erro
PAS	Ácido Periódico-Schiff
PBS	Phosphate buffered saline (Tampão fosfato-salino)
PCR	Polimerase Chain Reaction (Reação em Cadeia da Polimerase)
pH	Potencial de hidrogênio
POF	Premature ovaraina failure
PPGCV	Programa de Pós-Graduação em Ciências Veterinárias
PTX	Paclitaxel
QX	Quinoxaline (Quinoxalina)
RMD	Resistência a múltiplas drogas
RT	Room temperature (Temperatura ambiente)
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SD	Standard deviation (Desvio padrão)
SEM	Standard error of mean (Erro padrão da média)
TdT	Deoxynucleotidyl transferase terminal
TUNEL	Terminal deoxynucleotidyl transferase-mediated biotinylated deoxyuridine
UECE	Universidade Estadual do Ceará
VD	Vitanolido D
VG	Vesícula germinativa
Vol	Volume
WD	Whitanolide D

SUMÁRIO

1 INTRODUÇÃO

Atualmente, uma alta taxa de crianças e mulheres jovens com câncer tem superado a doença devido aos avanços da medicina (BANN et al., 2015), sobretudo em relação ao diagnóstico precoce associado à aplicação de tratamentos mais efetivos (MEIROW et al., 2010). Em contrapartida, observa-se com muita frequência, a redução da fertilidade após a quimio/radioterapia, principalmente quando se usa agentes alquilantes, os quais podem provocar sequelas em longo prazo, influenciando assim na qualidade de vida dessas pacientes (STENSHEIM et al., 2011). Diante dessa situação, existe uma grande preocupação sobre o impacto da exposição a diferentes compostos (drogas, quimioterápicos), pois muitos produtos podem exercer efeitos sobre o sistema reprodutivo, podendo causar além da infertilidade, má formação fetal, dentre outros problemas (FAQI, 2012). De acordo com GOSWAMI; CONWAY (2005), os casos de infertilidade feminina, podem ser disparados pela falha prematura dos ovários (POF) ou menopausa precoce (amenorréia antes dos 40 anos de idade), devido à alta toxicidade das drogas. Desta forma, muitos esforços têm sido empregados visando o desenvolvimento de novos fármacos que apresentem menos efeitos colaterais, inclusive sobre a função reprodutiva.

Com intuito de minimizar as reações adversas, alguns laboratórios têm trabalhado no desenvolvimento de compostos sintéticos, como os derivados de Quinoxalina e substâncias naturais oriundas de plantas, como por exemplo, o Vitanolido D. No que concerne aos derivados da Quinoxalina, recentes estudos têm mostrado que esses compostos são potentes anticancerígenos, apresentando forte toxicidade contra uma variedade de células tumorais (BALDERAS et al., 2012; RODRIGUES et al., 2014; MARANHAO et al., 2016) No que se refere ao Vitanolido D, um estudo *in vitro* utilizando linhagens de células cancerígenas, demonstrou que esse composto atua como inibidor da ciclo-oxigenase-2 (JAYAPRAKASAM et al, 2003) e é capaz de induzir a apoptose através da ativação de caspases (SENTHIL et al., 2007). Apesar da constante investigação a respeito de drogas ou agentes quimioterápicos que tornem o tratamento mais efetivo e com menos efeitos adversos, outro problema presente é o fenômeno conhecido como resistência das células a multi-drogas. Um dos fatores mais conhecidos pela ocorrência desse fenômeno é a presença de uma família de proteínas transmembranárias, conhecidas como transportadoras ABC (*ATP-binding cassette*), as quais são capazes de expulsar as drogas para fora das células, reduzindo as chances de cura. Portanto, considerando a toxicidade dos agentes com propriedade antitumoral, aliada ao fenômeno da resistência a multi-drogas, testes toxicológicos são altamente requeridos, desde o processo de

desenvolvimento até o registro e comercialização de um novo agente quimioterápico (STOKES, 2002; MEYER, 2003).

É conhecido que a grande maioria dos testes toxicológicos são realizados *in vivo,* nos quais se utiliza um grande número de animais, são bastante onerosos e exigem normalmente um longo tempo para serem realizados. Além disso, os testes toxicológicos *in vivo*, envolvem questões éticas, pois algumas práticas utilizadas podem causar o sofrimento e a morte dos animais (CRUZ, 2003). Esses fatores têm contribuído de forma expressiva para o processo de reformulação, pelo qual, os testes de toxicidade vêm sendo submetidos, o que signifca que, parte desse processo envolve a substituição dos testes *in vivo* pelos testes *in vitro,* visando à redução do número de animais (RICHMOND, 2002). No que concerne à função reprodutiva feminina, é muito comum a administração de drogas aos animais para subsequente análise da função ovariana e folicular (NIE et al, 2018; HU et al., 2018). Essa exposição consequentemente acarreta danos ao indivíduo. Visando evitar esse sofrimento, nós acreditamos que a utilização do cultivo *in vitro* do tecido ovariano oriundo de animais de abatedouros, como os caprinos pode ser uma alternativa aos métodos de experimentação *in vivo*, para avaliar a toxidade de substâncias, novos compostos, quimioterápicos, sobre a função reprodutiva da fêmea, razão pela qual foi realizada a presente tese.

Portanto, para uma melhor compreensão do assunto como um todo, a revisão de literatura desta tese, aborda resumidamente, a organização estrutural e função da gônada feminina; o processo de formação e desenvolvimento dos folículos ovarianos; o conceito e importância do cultivo *in vitro* de folículos pré-antrais presentes no tecido ovariano; agentes quimioterapêuticos e os impactos sobre os folículos ovarianos; novas substâncias naturais ou sintéticas com potencial anticâncer e o fenômeno da resistência a múltiplas drogas. Essa tese também apresenta uma relevante contribuição para o campo da toxicologia reprodutiva, sob a forma de um artigo de revisão e três artigos técnicos científicos, envolvendo a toxicidade de dois novos compostos com potencial anticâncer, ou seja, o Vitanolido D e a Quinoxalina.

2 REVISÃO DE LITERATURA

2.1 ORGANIZAÇÃO ESTRUTURAL E FUNÇÃO DA GÔNADA FEMININA

O ovário é um órgão heterogêneo pertencente ao sistema reprodutivo das fêmeas, o qual pode ser dividido em duas regiões: a *medular* e a *cortical*. A região medular, na maioria das espécies, consiste na porção interna do órgão e é responsável pela sua sustentação e nutrição. Já a região cortical, localizada mais externamente, contém corpos lúteos, corpos albicans e folículos ovarianos em diferentes estágios de desenvolvimento (LEITÃO et. al., 2009). A morfologia desse órgão é dinâmica, com constante remodelação da vasculatura, padrões de inervações e transformações compartimentais, como por exemplo, a transformação do folículo primordial (folículo quiescente) até o estágio de folículo pré-ovulatório (MCGEE; HSUEH, 2000) e, além disso, varia de acordo com a idade e fase reprodutiva do inviduo (Figura 1).

Figura 1 - Imagem ilustrativa da organização e estrutura dos folículos presentes no ovário. Notar a sequência evolutiva desde os folículos primordiais até à formação da cavidade antral e ovulação.

Fonte: adaptado - http://www.futura-sciences.us/dico/d/biology-ovary-50002719/
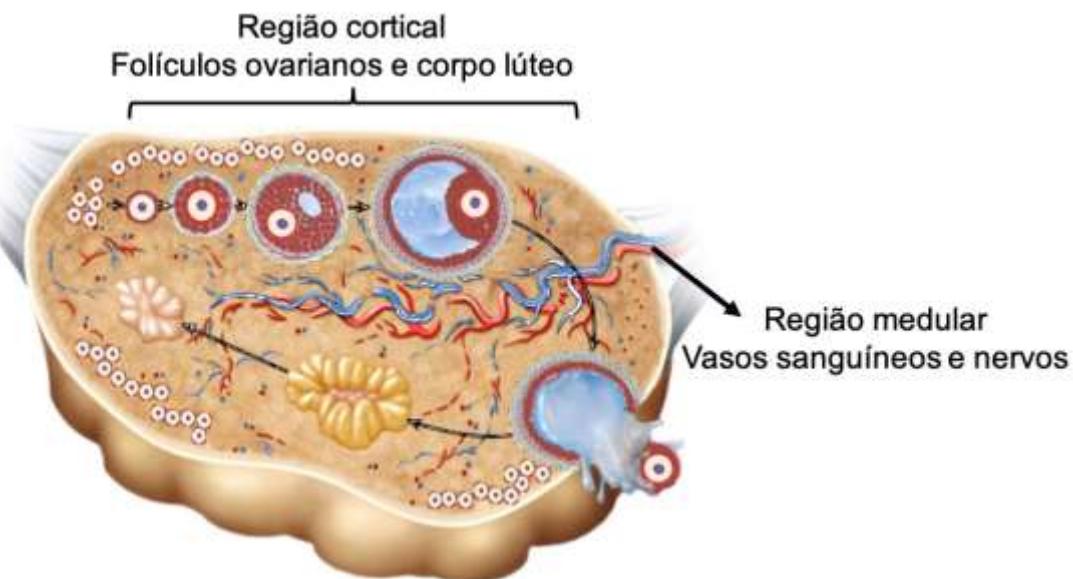
A função da gônada feminina não é apenas produzir e desenvolver oócitos fertilizáveis, para garantir a próxima geração, mas também é a glândula reprodutiva, responsável pela produção e liberação de hormônios esteroides e peptídeos, que controlam

vários aspectos do desenvolvimento e fisiologia da fêmea (PENG et al., 2010). A função ovariana é exercida pela interação de dois fenômenos que ocorrem no ovário, isto é, conhecidos como *oogênese* e a *foliculogênese* (SAUMANDE et al., 1991).

2.2 PROCESSOS DE OOGÊNESE E FOLICULOGÊNESE NO OVÁRIO MAMÍFERO

A oogênese é um processo complexo regulado por um grande número de fatores intra e extra-ovários (PESTY et al., 2007) e envolve diferentes eventos como: a geração de *células germinativas primordiais* (CGPs); a migração concomitante com um intenso período de proliferação e colonização da gônada ainda indiferenciada por essas células; diferenciação das CGPs em *oogônias*; proliferação das oogônias; parada da mitose das oogônia e início da meiose para dar origem ao *oócito primário* o qual, finalmente para no estágio de diplóteno da prófase I (KRYSKO et al., 2008) da primeira divisão meiótica até que esteja completamente desenvolvido (SÁNCHEZ; SMITZ, 2012). Pode-se dizer que a oogênese é caracterizada por uma *fase de crescimento* e uma *fase de maturação*. Durante a fase de crescimento, embora ocorra essa parada na meiose, o oócito continuam a crescer, sintetiza e acumula componentes (RNAm e proteínas) essenciais para os estágios iniciais do desenvolvimento embrionário. A fase de maturação da oogênese também pode ser entendida pela competência meiótica do oócito e significa a conclusão do ciclo celular meiótico que ocorre com a ovulação e após a fertilização. Nessa fase ocorrem eventos biossintéticos e secretórios que são compactados em questão de horas após a ovulação, induzida pela onda do hormônio luteinizante ou LH (MCGINNIS; LIMBACK; ALBERTINI, 2013). A competência meiótica e desenvolvimento dos oócitos é adquirida de forma gradual e sequencial e cresce interagindo com suas células somáticas que o circundam durante o processo de foliculogênese (MCLAUGHLIN; MCIVER, 2009).

A foliculogênese é definida como o processo de formação, crescimento e maturação folicular, iniciando-se com a formação do folículo primordial e culminando com o estádio de folículo pré-ovulatório, também chamado de folículo de Graaf ou folículo dominante. O folículo, é considerado a unidade morfológica e funcional do ovário dos mamíferos e é composto pelo oócito circundado por células somáticas (células da granulosa e tecais), que interagem entre si, promovendo a funcionalidade do folículo (FIGUEIREDO et al., 2008). Desta forma, o folículo mostra-se um elemento essencial na promoção de um ambiente ideal para a manutenção, o desenvolvimento e liberação de um oócito maturo no processo de ovulação (CORTVRINDT; SMITZ, 2001). De acordo com a presença ou ausência do antro, os folículos ovarianos podem ser classificados em *folículos pré-antrais* e *folículos antrais*,

respectivamente (SILVA, 2004). Os folículos pré-antrais podem ainda ser subdivididos em *primordiais*, de *transição*, *primários* e *secundários* (Figura 2) e estão detalhadamente descritos nos tópicos a seguir.

Figura 2 - Imagem ilustrativa do processo de foliculogênese. Notar a sequência evolutiva desde os folículos primordiais até a formação da cavidade antral, ovulação e fertilização.
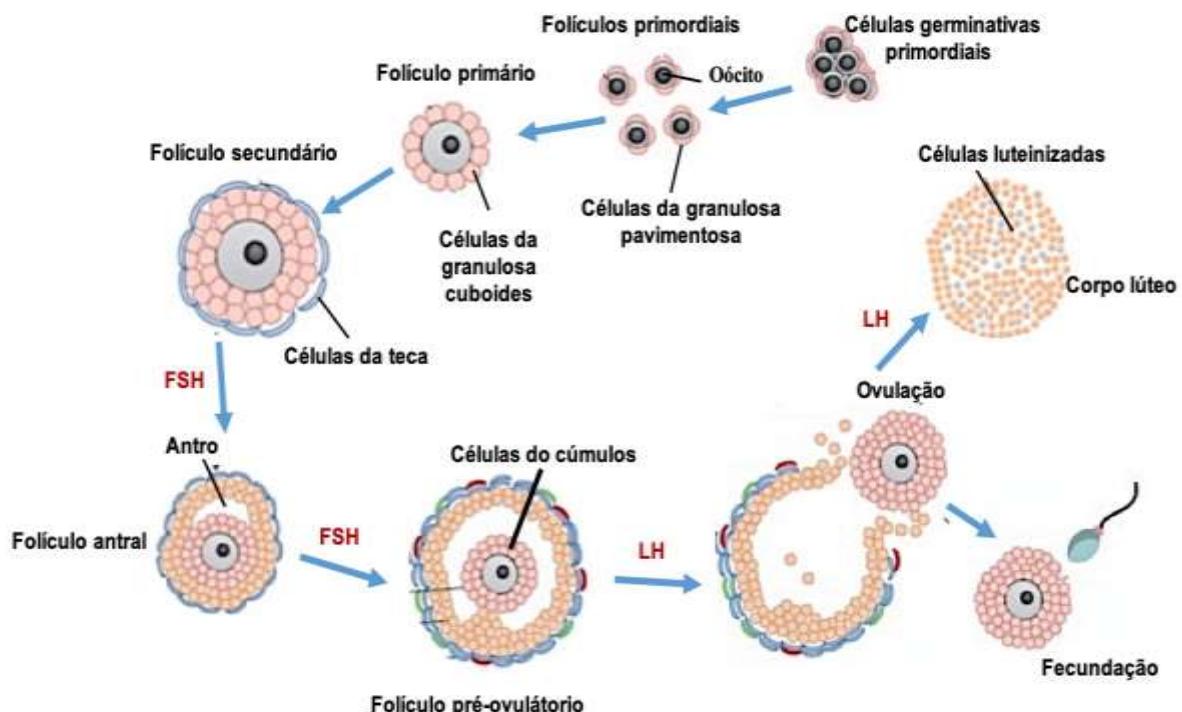
Fonte: Adaptada de GEORGES et al., 2014.

2.3 CARACTERIZAÇÃO DOS FOLÍCULOS OVARIANOS E MÉTODOS DE AVALIAÇÃO QUANTI E QUALITATIVA

Os folículos primordiais são os primeiros e os menores folículos encontrados no ovário; são quiescentes e compreendem 90 a 95% da população de folículos e constituem o pool de reserva folicular presente no ovário. Para que os folículos primordiais iniciem a fase de crescimento é necessário que sejam ativados. Morfologicamente, a ativação folicular é caracterizada pela mudança na morfologia das células da granulosa de pavimentosa para cúbica (Figura 2), proliferação dessas células e o início do crescimento oocitário (HIRSHFIELD, 1991). O mecanismo que estimula a ativação de apenas alguns folículos, enquanto o restante permanece quiescente, ainda é desconhecido. No entanto, é conhecida a participação de fatores endócrinos (gonadotrofinas) e fatores intraovarianos (fatores de crescimento e peptídeos) inibindo e estimulando esse processo (VAN DEN HURK; ZHAO, 2005). Após a ativação, os

folículos apresentam células da granulosa com ambos os formatos pavimentoso e cúbico, sendo este denominado de folículos de transição (SILVA et al., 2004a). Em seguida, quando todas as células que circundam o oócito tornam-se cúbicas, aumentando em número e volume, os folículos são chamados folículos primários (VAN DEN HURK; BEVERS; BECKERS, 1997).

A partir do estágio de folículo secundário observa-se a formação da zona pelúcida, que é claramente identificada ao redor do oócito. Além disso, nesse estádio, os precursores das células da teca são recrutados do estroma ovariano que irão constituir as camadas interna e externa de células da teca (PARROTT; SKINNER, 2000). Com o crescimento dos folículos secundários e a proliferação das células da granulosa originando várias camadas, ocorre entre estas a formação de uma cavidade repleta de líquido denominada antro (EDWARDS, 1974). A partir desse estádio, os folículos passam a ser denominados *antrais* e são caracterizados pelo aumento acentuado do diâmetro folicular ocasionado pelo crescimento do oócito, multiplicação das células da granulosa e da teca e acúmulo do fluido antral (DRIANCOURT, 2001). Tal fluido pode servir como uma importante fonte de substâncias reguladoras derivadas do sangue ou de secreções de células foliculares, como gonadotrofinas, esteróides, fatores de crescimento, enzimas, proteoglicanos e lipoproteínas (BRISTOL-GOULD; WOODRUFF, 2006) (Fig. 2).

Todas essas características acima mencionadas podem ser constatadas através da técnica de histologia clássica. A histologia clássica tem sido utilizada para a análise quantitativa e qualitativa de folículos inclusos no tecido ovariano, com a finalidade de verificar alterações na morfologia de todas as estruturas foliculares (membrana plasmática, oócito e suas células somáticas circundantes). Além de ser capaz de identificar alterações morfológicas, a histologia clássica também identifica o desenvolvimento folicular pela modificação do formato das células da granulosa de pavimentoso para cúbico; pelo aumento no número de células, bem como pela presença os folículos pré-antrais quanto ao seu estádio de desenvolvimento (primordial, intermediário, primário ou secundário), e ainda quanto às suas características morfológicas, em normais ou atrésicos (DEMIRCI et al., 2002), como mostra a figura 3.

Em cada fase do desenvolvimento folicular, vários fatores extracelulares de origem endócrina ou parácrina, como também alguns fatores intracelulares, atuam direta ou indiretamente nas células foliculares no sentido de orientar sua diferenciação, seja para o crescimento folicular ou atresia. Assim, o controle da atividade proliferativa e apoptótica no tecido ovariano é um processo complexo que envolve o equilíbrio entre diversas moléculas regulatórias e que podem ser alteradas por numerosos sinais e fatores externos (SALVETTI et al., 2009). Estima-se que 99,9% dos folículos ovarianos presentes ao nascimento não ovulam. O destino mais comum deles é a atresia, que geralmente ocorre por *apoptose*. A apoptose é um

processo fisiológico essencial para o desenvolvimento embrionário normal e para a homeostase dos tecidos adultos, sendo que sua ocorrência varia de acordo com o metabolismo do tecido. (MARKSTRÖM et al., 2002; ESCOBAR et al., 2008). A apoptose inicia-se por meio de um processo geneticamente determinado, ou seja, é regulada pela expressão de genes específicos (BARNETT et al., 2006), no qual, provavelmente, o desbalanço entre os fatores que promovem a sobrevivência e aqueles que induzem a apoptose irá determinar quais os folículos que continuarão o seu desenvolvimento ou sofrerão atresia (HSU; HSUEH, 2000). De acordo com o estímulo apoptótico inicial, a apoptose pode ser iniciada por duas vias principais: (1) via receptor extrínseco ou de morte e (2) via intrínseca ou mitocondrial. No entanto, há evidências de que as duas vias estão correlacionadas e que uma via pode influenciar a outra (IGNEY; KRAMMER, 2002). Independente do estímulo e do padrão apoptótico iniciado, tal processo é caracterizado por uma série de alterações bioquímicas e morfológicas, com a extensiva perda de volume celular, a condensação da cromatina, a fragmentação do DNA e a formação de corpos apoptóticos (RACHID; VASCONCELOS; NUNES, 2000).

Figura 3 - Ilustração histológica dos folículos ovarianos em diferentes estágios de desenvolvimento no córtex do ovário (A-D). Folículo primordial (A), folículo transição (B), folículo primário (C), folículo secundário (D), folículo antral (E), folículo degenerado, notar a desorganização das células da granulosa e retração do citoplasma do oócito (F). Proliferação das células da granulosa por Ki67 (G). Oócito e células da granulosa apóptoticas detectadas pelo teste de TUNEL (H). As setas pretas indicam marcação positiva para Ki67 e TUNEL. O, Oócito; N, Nucléo; CG, Células da granulosa; A, Antro
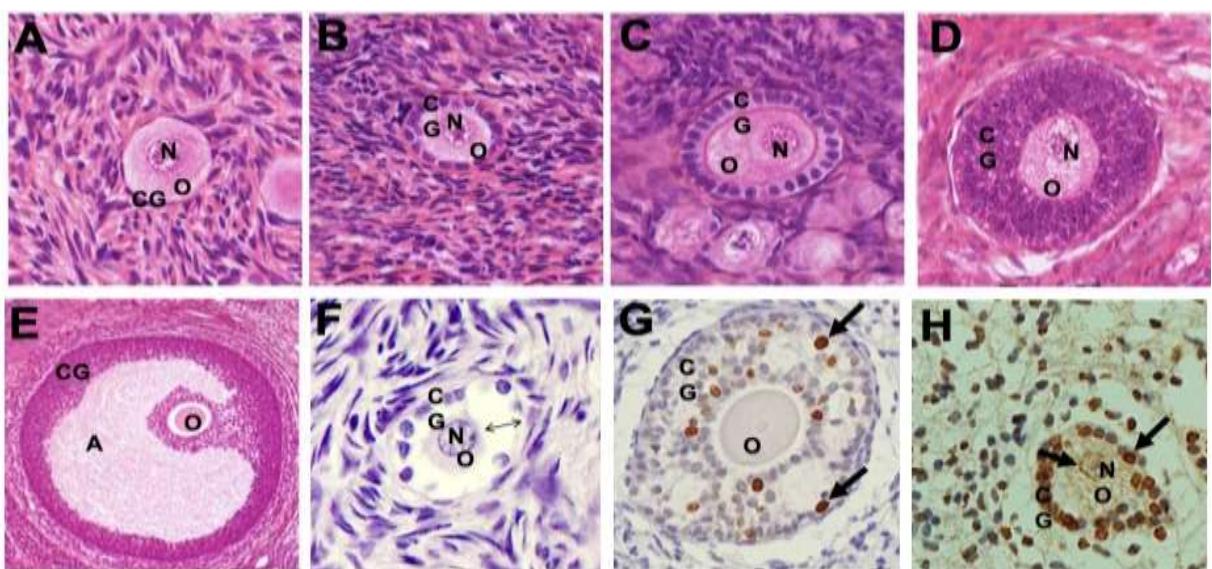
Fonte: Elaborado pelo próprio autor.

Para se avaliar a apoptose folicular, estudos tanto *in vivo* quanto *in vitro* tem utilizado a técnica de *deoxynucleotidil tranferase-mediated deoxyuridine triphosphate (dUTP) biotin nick end-labeling* (TUNEL). Esta técnica tem como princípio, a utilização de uma enzima *deoxynucleotidyl transferase* terminal (TdT) para adicionar nucleotídeos aos fragmentos das fitas de DNA quebradas na extremidade 3'-OH nas células apoptóticas (GAVRIELI; SHERMAN; BEN-SASSON, 1992). A incorporação da dUTP biotinilada às extremidades 3 'do DNA fragmentado é carreada numa reação contendo TdT. Dependendo das necessidades específicas da pesquisa e/ou equipamentos disponíveis, a dUTP biotinilada incorporada pode ser visualizados pela microscopia de fluorescência (GAVRIELI; SHERMAN; BEN-SASSON, 1992), utilizando fluoróforos específicos, ou microscopia de luz (ARENDS et al., 1990), após coloração com complexo avidina-biotina-peroxidase conjugado, gerando a coloração marrom no núcleo (Figura 3) das células apoptóticas, enquanto as células normais ficam com uma coloração mais clara (LIU et al., 2008). A técnica de TUNEL tem sido utilizada com sucesso em vários estudos para avaliar a ocorrência de apoptose em folículos pré-antrais (ROTI ROTI et al., 2012; MORGAN et al., 2013; CARVALHO et al., 2014).

Com relação à proliferação das células da granulosa, sabe-se que esse evento é influenciado por fatores mitóticos, como gonadotrofinas e fatores de crescimento (FIGUEIREDO et al, 2018), os quais estimulam a entrada dessas células no ciclo celular. O ciclo celular é dividido em quatro fases (G1, S, G2 e M) responsáveis pela sequência exata de eventos que levam à divisão celular, como mostra a figura 4. As fases G1, S e G2 constituem o que chamamos de intérfase e ocorrem nessa ordem. A fase S ou fase de síntese é a fase que requer mais tempo, pois é nela que ocorre a duplicação do material genético. Durante as fases G1 e G2 ocorre o crescimento celular, com multiplicação de organelas e intensa síntese de proteínas. Durante a fase M ou mitose ocorre a segregação do material genético, desde que as condições extras e intracelulares sejam consideradas favoráveis (ALBERTS et al., 2010). Pelo menos dois tipos de mecanismos de controle do ciclo celular são reconhecidos: uma cascata de fosforilações de proteínas que redirecionam uma célula de um estágio para o próximo e um conjunto de pontos de verificação que monitoram a conclusão de eventos críticos e atrasam a progressão para o próximo estágio, caso algum erro ocorra durante o processo. O primeiro tipo de controle envolve uma família de quinases altamente regulada (MORGAN, 1995). A ativação dessas enzimas geralmente requer associação com outras enzimas, transientemente expressas no período apropriado do ciclo celular, conhecidas como *ciclinas,* daí as primeiras serem conhecidas como *quinases dependentes de ciclinas* (CDKs). A associação das CDKs com as ciclinas resulta em um complexo ativo com especificidade de substrato única. A fosforilação e

a desfosforilação regulatórias ajustam a atividade dos complexos ciclina-CDKs, assegurando transições bem delineadas entre os estágios do ciclo celular (COLLINS et al., 2005). Atualmente, diferentes tipos de ciclinas (A, B, D e E). e CDKs (CDK1, CDK2, CDK4 e CDK6) que controlam positivamente o ciclo celular são bem conhecidas. Porém para a visualização de uma célula em proliferação (Figura 3), presente no tecido, vários estudos têm lançado mão da técnica de imuno-histoquímica, como por exemplo, a imunomarcação de proteínas como o Ki67 (SCALERCIO et al., 2015; VANACKER et al., 2013).

Figura 4 - Complexos Ciclina-Cdk durante o ciclo celular: Representação diagramática dos complexos bioquímicos que conduzem os eventos do ciclo celular.
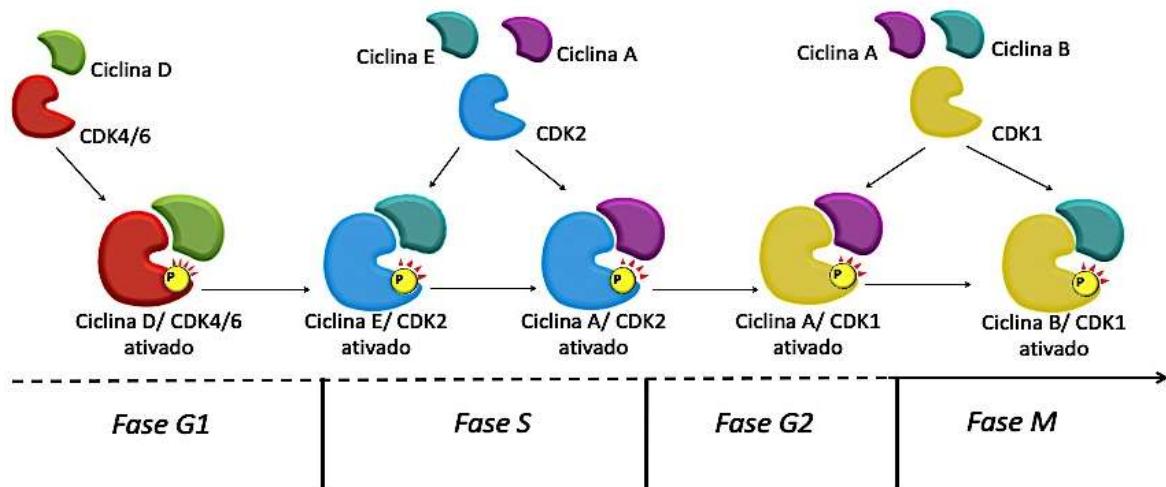
Fonte: Elaborado pelo próprio autor.

O antígeno para a proteína Ki67 está presente no núcleo das células em todas as fases do ciclo celular. Entretanto, células quiescentes ou em repouso na fase G0 não expressam o Ki67. Devido ao fato do Ki67 estar presente em todas as células em proliferação (células normais ou tumorais), essa proteína se tornou um excelente marcador para determinar a proliferação de uma determinada população celular (SCHOLZEN et al., 2000). A sobrevivência, o crescimento e a diferenciação celular podem também ser monitorados pela alteração dos padrões da expressão gênica utilizando-se a biologia molecular (MAZERBOURG; HSUEH, 2006). A reação em cadeia da polimerase (PCR) é uma técnica comum de laboratório usada para fazer muitas cópias (milhões ou bilhões) de uma região específica do DNA. Essa região do DNA pode ser qualquer gene em que o experimentador esteja interessado (BARTLETT; STIRLING, 2003). Por exemplo, pode ser a expressão de genes relacionados ao controle do ciclo celular, como as ciclinas e CDKs, descritas acima.

No entanto, para identificar a morte ou sobrevivência e, consequentemente o crescimento folicular, inclusive pela proliferação de suas células da granulosa é necessário que esses folículos sejam cultivados *in vitro* (GUERREIRO et al., 2016; SÁ et al., 2018*)*. O cultivo *in vitro* de folículos pré-antrais pode ser utilizado como uma *técnica de reprodução assistida*, visando, por exemplo, a obtenção de ocócitos fertilizáveis e produção *in vitro* de embriões ou pode ser utilizado como uma *metodologia alternativa para testes toxicológicos reprodutivos*. No presente estudo, será dada ênfase para essa segunda vertente.

2.4 CUILTIVO IN VITRO DE FOLÍCULOS NO TECIDO OVARIANO COMO MÉTODO ALTERNATIVO PARA TESTES DE TOXICIDADE REPRODUTIVA

A identificação do potencial efeito tóxico no sistema reprodutivo e seus mecanismos de ação é um grande desafio científico durante a avaliação da segurança de produtos químicos. A toxicidade reprodutiva é um dos mais complicados domínios da toxicologia, devido a múltiplos órgãos e tecidos envolvidos, diferentes modos de ação tóxica e dependência do sistema endócrino (LORENZETTI et al., 2011). De fato, algumas características essenciais da toxicologia reprodutiva são completamente diferentes de todas as outras áreas da toxicologia. Por exemplo, os efeitos adversos podem apenas ocorrer na próxima geração da prole e, quando ocorrem sobre as funções vitais de forma sistêmica, também podem induzir efeitos indiretos na reprodução (SPIELMANN, 2009). A maioria dos testes toxicológicos, além de muito onerosos e demorados, em geral, utiliza um grande número de animais vivos, envolvendo portanto, sérias questões éticas, principalmente porque algumas das práticas aplicadas podem causar o sofrimento e a morte de animais (CRUZ, 2003).

Apesar do uso de animais na experimentação ser uma prática bastante antiga, atualmente, o crescente movimento contrário ao uso de animais em pesquisa tem motivado a busca por métodos alternativos (HAJAR, 2011). Desenvolvidos e suportados pela União Européia (UE), os métodos alternativos devem atender aos princípios dos três "Rs" propostos por Russel e Burch em 1959, isto é*, Replacement, Reduction e Refinement* (Substituição, Redução e Refinamento) da experimentação animal, garantindo ao mesmo tempo, os mais altos padrões de segurança para os consumidores e pacientes, respeito ao meio ambiente e bem-estar animal. A *Substituição* é caracterizada pela não utilização de animais, substituindo-os pela aplicação de diferentes técnicas, como por exemplo, o cultivo in vitro de células e tecidos. A *Redução* pode ser entendida pela diminuição do número de animais em um único teste ou ao invés de utilizar animais em todas as fases experimentais, utilizam-se somente nas fases finais.

No tocante ao *Refinamento*, essa prática implica em implementar cuidados e tratamentos de forma a minimizar qualquer dor ou sofrimento aos animais que porventura necessitem ser usados (CAZARIN, et al., 2004).

Portanto, considerando a preocupação mundial atual do uso de animais na prática experimental, nós entendemos que o cultivo *in vitro* de tecido ovariano, inserido na técnica de Manipulação de Oócitos Inclusos em Folículos Ovarianos Pré-antrais (MOIFOPA) também conhecida como *Ovário Artificial*, pode ser uma excelente alternativa s ser utilizada em testes toxicológicos, especialmente, sob o ponto de vista, reprodutivo. Quando o tecido ovariano é cultivado in vitro na presença de substâncias ou compostos como os quimioterápicos, é possível avaliar os efeitos negativos causados sobre a função folicular e, consequentemente reprodutiva do indivíduo. Em outras palavras, através dessa técnica é possível predizer se determinado composto, em determinada concentração poderá influenciar negativamente, a fertilidade feminina, sem que haja a necessidade da utilização de animais vivos. Essa técnica poderá ter um grande valor para a área de Oncofertilidade, haja vista que a grande maioria, se não todos os agentes quimioterápicos utilizados na atualidade são gonadotóxicos e levam à falha ovariana prematura (FOP) e consequentemente à infertilidade, conforme destaca o tópico seguinte.

2.5 AGENTES QUIMIOTERAPÊUTICOS E OS IMPACTOS SOBRE OS FOLÍCULOS OVARIANOS

A administração de quimioterápicos, necessária para os tratamentos oncológicos pode resultar em efeitos colaterais, dentre eles danos devastadores aos ovários. Dessa forma, o desenvolvimento de terapias que possibilitem uma maior sobrevida e que resultem em menores efeitos colaterais aos pacientes é de extrema relevância e interesse para a clínica médica (MEIROW, 2000). Conforme mencionado, a grande parte dos tratamentos contra o câncer resulta em FOP ou na infertilidade (JENSEN & MORBECK, 2011), devido à alta toxicidade ovariana (MEIROW et al., 2010). Portanto, a impossibilidade de gestar um filho após tratamentos quiomioterápicos abala fortemente o equilíbrio emocional das mulheres submetidas a tratamentos oncológicos, podendo influenciar negativamente no tratamento e bem-estar das pacientes (MEIROW, 2000). Esse fato tem sido comprovado por meio de vários estudos pré-clínicos que descreveram padrões similares de falha ovariana após quimioterapia, principalmente após exposição a agentes alquilantes (OKTEM; OKTAY. 2007; GREEN et al., 2009; MEIROW et al., 2010). A gonadotoxicidade induzida pela quimioterapia envolve principalmente perda da reserva ovariana, ou seja, principalmente folículos primordiais

(DNISTRIAN et al., 1983). A falha ovariana induzida pela quimioterapia se manifesta pela perda rápida e permanente da menstruação em muitas pacientes. Isso indica que o efeito tóxico também atinge os folículos maduros (STEARNS et al., 2006). Esse fenômeno depende da idade da mulher, do regime quimioterapêutico e do número existente de folículos primordiais que constituem a reserva ovariana (MEIROW; NUGENT, 2001). Também tem sido observado um efeito direto sobre oócitos devido à capacidade do quimioterápico atravessar a barreira hematofolicular e alcançar os oócitos inclusos nos folículos ovarianos (PEREZ et al., 1999). Bar- Joseph et al. (2010) demonstraram a desintegração e dispersão mais rápida e pronunciada da cromatina nos oócitos em estágio de vesícula germinativa (VG). Embora não seja amplamente conhecido como os agentes antineoplásicos afetam os folículos ovarianos, pode-se admitir que possam agir de forma semelhantes às células tumorais.

Os fármacos antineoplásicos constituem um grupo heterogêneo de substâncias químicas capazes de inibir o crescimento e/ou os processos vitais das células tumorais com uma toxicidade tolerável sobre grande parte das células normais (MALUF; ERDTMANN, 2000). A maioria desses compostos afetam o DNA causando apoptose das células. Essas drogas podem ser classificadas em *agentes alquilantes*, *antraciclinas*, *antimetabólitos*, *compostos de platina*, *taxanos* e *agentes diversos* (CHUAI; XU; WANG, 2012)

Os agentes alquilantes são capazes de substituir em uma molécula um átomo de hidrogênio por um radical alqui (CHABNER; CALABRESI, 1995). Ligam-se ao DNA de modo a impedir a desespiralização da dupla hélice, fenômeno indispensável para a reprodução celular (ALMEIDA et al., 2005). Os agentes alquilantes agem em todas as fases do ciclo celular de modo inespecífico, inclusive em células que não estão em divisão ativa, como os oócitos e a população de folículos primordiais (BEN-AHARON et al., 2010).

As antraciclinas representam uma importante classe de antibióticos antitumorais (CULLINANE; CUTTS, 1994) e estão amplamente integradas numa variedade de tratamentos anticâncer. Dentre as drogas mais usadas nesse grupo destaca-se a *Doxorrubicina.* Atualmente, a doxorrubicina é usada para tratar cerca de 50% dos casos de câncer que ocorrem durante a pré-menopausa, incluindo cânceres de mama e pediátricos (CHOW; DONG; DEVESA 2010). A doxorrubicina transpassa a membrana celular, se acumula tanto no núcleo como nas mitocôndrias e induz o estresse oxidativo e a obliteração cromossômica por meio da inibição da topoisomerase II (MAILER; PETERING, 1976). Além disso, induz a toxicidade no ovário, que se manifesta pela redução na taxa de ovulação, acompanhada por uma redução no tamanho desse órgão, causadas provavelmente por um dano agudo (BEN-AHARON et al., 2010). Um estudo *in vitro* com tecido ovariano caprino, realizado por nossa equipe, demonstramos também

que a doxorrubicina, de forma dose dependente, reduziu o número de folículos primordiais e folículos em desenvolvimento, morfologicamente normais e, induziu a fosforilação da histona H2AX (indicação de quebra na dupla fita do DNA) acarretando na redução significativa da sobrevivência in vitro (GUERREIRO et al., 2016).

Os agentes metabólitos, exercem seus efeitos principalmente por bloquear bioquimicamente a síntese do DNA e, portanto, são restritos à fase S do ciclo celular (OLIVEIRA; ALVES, 2002). Muitos desses compostos são análogos de nucleosídeos que exercem a sua atividade por meio do bloqueio de vias do metabolismo dos nucleotídeos, substituem um dos blocos de construção dos ácidos nucleicos e atuam como antagonistas de DNA (PARKER; CHENG, 1990). Dentre os agentes antimetabólitos mais usados na quimioterapia, destaca-se o5-Fluorouracil (5-FU), o qual parece apresentar menor efeito tóxico sobre a função reprodutiva humana (MEISTRICH et al., 2001).

Os compostos de platina como a cisplatina e a carboplatina, alquilam o DNA (MACHADO, 2000; OLIVEIRA; ALVES, 2002). O mecanismo de ação desses compostos está relacionado com a inibição seletiva da síntese do DNA (CHABNER; CALABRESI, 1995). A cisplatina induz uma redução na taxa de ovulação e causa um declínio nos níveis de hormônio anti-mulleriano (AMH) e inibina-A em ratas (HIGDON et al., 1992; YEH et al., 2006; YEH et al., 2008). Em mulheres, estudos *in vitro* resultaram na destruição dos folículos primordiais, caracterizada por inchaço das células da granulosa, marcado pelo acúmulo de citoqueratina e inchaço nuclear. Foram perceptíveis, por meio de análise histológica, alterações na arquitetura dos folículos primordiais (MEIROW, 2000; GONFLONI et al., 2009). Contudo, o mecanismo de ação da toxicidade da cisplatina em ovários ainda não foi completamente explicado. CHIRINO; PEDRAZA-CHAVERRI (2009) sugeriram que tal toxicidade pode ser devida a um aumento da produção de espécies reativas de oxigênio e diminuição da produção de antioxidantes.

Já os taxanos (Paclitaxel, Docetaxel) são utilizados para reprimir e inibir o crescimento celular, a diferenciação e proliferação em várias linhas celulares de câncer. São comumente conhecidos como inibidores de mitose ou inibidores de microtúbulos. Em um ciclo celular normal, as células formam microtúbulos no início da divisão celular e estes são quebradas quando a célula para de se dividir. Os Taxanos estabilizam os microtúbulos, impedindo sua polimerização/despolimerização normal, fazendo com que as células cancerosas parem de se dividir durante a mitose, o que potencialmente retarda ou para o crescimento do tumor (MANFREDI; HORWITZ, 1984). Em ratas, o tratamento com paclitaxel (7,5mg/kg) resultou na redução no número de folículos primordiais (YUCEBILGIN et al., 2004) quando

comparado com animais não tratados, culminando com a perda de fertilidade. Outro estudo, realizado também em camundongas pré-púberes, mostrou que a administração de paclitaxel (2,5 -7,5 mg/kg) provocou o esgotamento da reserva de folículos primordiais de maneira dose-dependente (GÜCER et al., 2001). Resultados semelhantes foram observados por GUERREIRO et al. (2016), por meio do cultivo *in vitro* de tecido ovariano de cabras.

Como mostrado anteriormente, a maioria do quimioterápicos, independente do mecanismo de ação, podem causar danos irreversíveis nos ovários como a falha ovariana prematura e infertilidade (JENSEN; MORBECK, 2011). Esses resultados têm estimulado grandes esforços na tentativa de identificar novos compostos que, por ventura, possam minimizar esses efeitos indesejáveis.

2.6 INVESTIGAÇÃO SOBRE O USO DE NOVAS SUBSTÂNCIAS NATURAIS OU SINTÉTICAS COM POTENCIAL ANTICÂNCER

As plantas produzem uma grande variedade de metabólitos que estão ganhando importância devido às suas aplicações terapêuticas e biotecnológicas (SINGH; KAUR; SILAKARI; 2014). Tais metabólicos possuem grandes potencialidades como princípios ativos, podendo ser utilizados diretamente na terapia contra o câncer, como precursores na semi-síntese quimiofarmacêutica ou podem ser usados em modelos para síntese de novos princípios de uso terapêutico (VIEGAS; BOLZANI; BARREIRO, 2006). Dentre os quimioterápicos oriundos de plantas utilizados nas últimas décadas, destaca-se o paclitaxel, extraído do *Taxus brevifolia,* e os alcalóides vimblastina e vincristina, extraídos de *Catharanthus roseus (Apocynaceae).*

Com o avanço no conhecimento de mecanismos moleculares, da biologia celular e genômica, não apenas aumentou o número de alvos moleculares para doenças como o câncer, mas também levou a uma demanda cada vez mais curta para o desenvolvimento de novas substâncias (LAHLOU, 2013). O advento de novas tecnologias combinadas, como a química sintética e métodos computacionais, como *Docking* molecular, juntamente com a impossibilidade de realizar rápidas descobertas de novas substâncias naturais, levaram as indústrias farmacêuticas a olhar e investir mais nas bibliotecas de substâncias sintéticas (NARANG; DESAI, 2009; LAHLOU, 2013). Entre as vantagens do uso de drogas sintéticas estão o menor tempo de desenvolvimento, um custo menor e a aquisição sustentável. Muitos análogos de compostos naturais foram sintetizados com a intenção de melhorar seus perfis de eficácia e toxicidade. Dentre eles, estão a carboplatina, um análogo da cisplatina, responsável

por reduzir a toxicidade renal; a doxorrubicina, um análogo da daunorubicina, que reduz a cardiotoxicidade; e o topotecano, um análogo da camptotecina com perfil mais brando de toxicidade (NARANG; DESAI, 2009). Outros compostos sintéticos foram desenhados como novas estruturas por meio da parceria entre a química e a modelagem computacional de interações entre as drogas e seus alvos (NARANG; DESAI, 2009). Em vista da ocorrência de células resistentes às drogas existentes no mercado e a emergência de novas doenças, existe uma contínua necessidade no desenvolvimento de novos compostos naturais ou sintéticos como potenciais agentes terapêuticos (AJANI, 2014). Desta forma, serão descritas a seguir, dois novos compostos, sendo um de originado de plantas (Vitanolido D) e outro sintético (Quinoxalina), os quais têm demonstrado um grande potencial anticâncer.

2.6.1 Vitanolido D

O Vitanolido D (VD) é uma lactona esteroidal isolado da *Acnistus arborescens* (Figura 5)*,* planta pertencente à familia Solanaceae encontrada nas Américas do Sul e Central (BARATA et al., 1970). Vários compostos têm sido isolados desta espécie, como alcaloides, esteroides, flavonoides e terpenos (ALBUQUERQUE; VELAZQUEZ; VASCONCELOS-NETO, 2006). Dentro da classe dos esteroides destacam-se os vitanolidos (VERAS et al., 2004; MAIA et al., 2012; 2010). Os primeiros trabalhos sobre a atividade farmacológica de vitanolidos de *Acnistus arborescens* foram realizados sobre a sua utilização como inibidores de tumores. Em um desses trabalhos, utilizando o extrato alcoólico das folhas dessa planta, foi observada uma atividade biológica *in vitro* significativa contra células de carcinoma humano da nasofaringe e uma atividade *in vivo* contra sarcoma 180 em ratos, cuja ação farmacológica foi atribuída à vitaferina (GLOTTER, 1991). Recentemente, BATISTA et al. (2016) demonstraram o potencial quimioterápico do VD contra as linhagens de células tumorais como leucemia (HL-60), adenocarcinoma de cólon (HCT-116), glioblastoma (SF-268) e carcinoma de pâncreas (PANC-1). Os vitanolídeos têm sido isolados de plantas de vários gêneros, como por exemplo: *Acnistus* (KUPCHAN et al., 1965; CORDERO et al., 2009), *Jaborosa* (NICOTRA et al., 2003), *Physalis* (MALDONADO et al., 2011), *Withania* (CHOUDHARY et al., 2004), todos pertencentes à família Solanaceae, as quais despertam interesse por apresentarem diversas propriedades biológicas, como a atividade antitumoral (BARATA et al., 1970; MISICO et al., 2002).

Figura 5 - Acnistus arborescens (A) e vitanolido D composto isolado de A. arborescens (B).
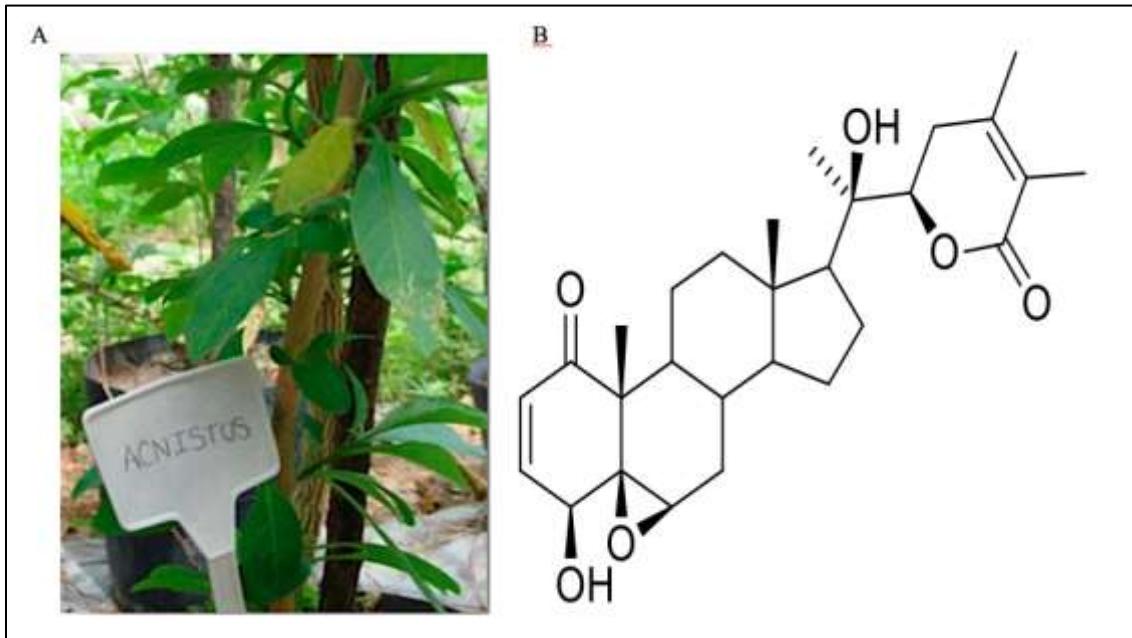
Fonte: BATISTA et al., 2016.

2.6.2 Quinoxalinas

Devido ao interesse na busca por novas substâncias que possam ser utilizadas no combate ao câncer, os pesquisadores devem enfatizar nos estudos de novas classes químicas que possam ser utilizadas com tal objetivo, como as quinoxalinas (ZARRANZ et al. 2004). Os derivados de quinoxalinas são uma importante classe de compostos heterocíclicos, nos quais um átomo de nitrogênio (N) substitui um ou mais átomos de carbono no anel de naftaleno, caracterizando uma família de benzodiazinas. Sua estrutura molecular é formada pela fusão de dois anéis aromáticos, benzeno e pirazina, sendo raro no estado natural, porém de fácil síntese (AJANI, 2014; PEREIRA et al., 2014). Mudanças na estrutura química, como a adição de novos grupamentos, podem alterar a atividade dessas moléculas (BALDERAS-RENTERIA et al., 2012). Nos últimos anos, o estudo de quinoxalinas e seus derivados têm despertado interesse da indústria farmacêutica devido à sua ampla variedade de atividades biológicas, assim como suas aplicações terapêuticas. Dentre suas atividades biológicas está o combate a fungos, bactérias, vírus, leishmania, tuberculose, malária, câncer, depressão, além de atividades anti-inflamatória e inibidora de quinase (AJANI, 2014; PEREIRA et al., 2014). Diversas quinoxalinas substituídas e seus derivados com suas variedades de grupos funcionais são importantes substâncias com atividades biológicas distintas que levam a uma significativa quantidade de pesquisas dirigidas a esta classe. O núcleo quinoxalina já demonstrou uma

potencial atividade anticâncer, fato qua as tornam visadas para o desenvolvimento de drogas. Devido a grande variedade de grupos funcionais possíveis para ligação ao núcleo, diversos alvos moleculares já foram descritos para a classe, dentre eles, inibidores de receptores de tirosinas quinases; agentes que geram dano de DNA; inibidores de topoisomerases; diminuição de fator indutor de hipóxia, entre outros (HUSAIN; MADHESIA, 2011; TRISTÁN-MANZANO et al., 2015). O potencial anticâncer exibido pelas quinoxalinas oferece uma base importante para drogas antineoplásicas, visto que algumas dessas moléculas sintéticas já mostraram atividade *in vitro* contra linhagens de câncer de mama, pulmão e sistema nervoso central (Figura 6) (PEREIRA et al., 2014; RODRIGUES et al., 2014).

Figura 6 - O composto PJOV 56 (E) -2- [2- (2-piridin-2-ilmetileno) hidrazinil] quinoxalina.

PJOV 56

(*E*)-2-[2-(2-pyridin-2-ylmethylene)hydrazinyl]quinoxaline

(Lead compound)
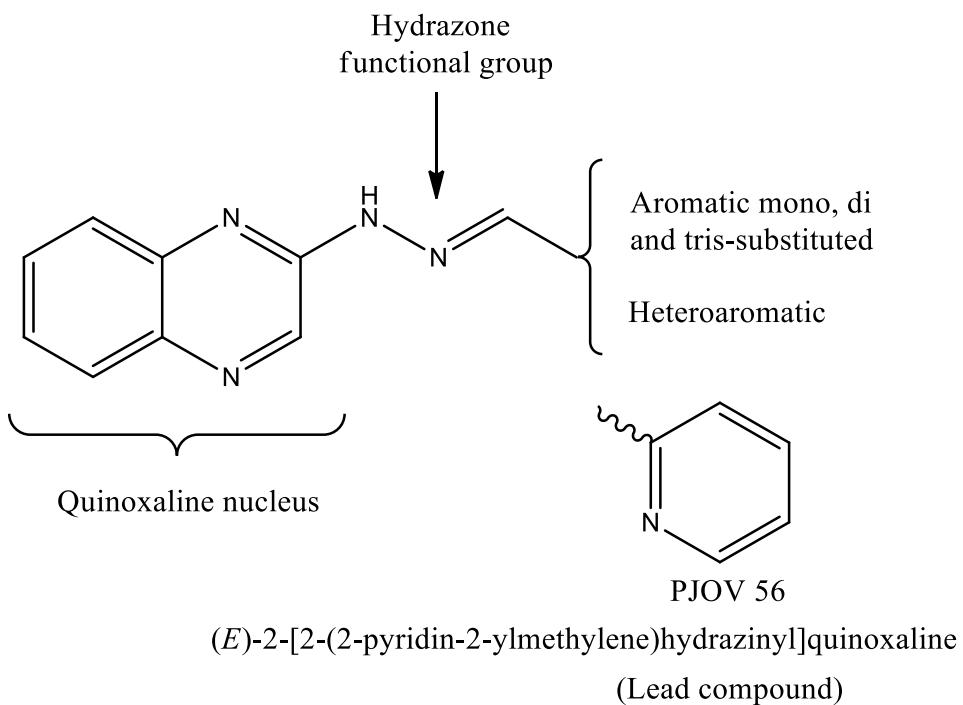
Fonte: RODRIGUES et al., 2014.

Embora a busca incansável por novos compostos com potencial anticâncer mais efetivos e com menos efeitos adversos seja uma demanda mundial dentro da oncologia (SHIN et al., 2018), não se pode esquecer outro grande problema que é o fenômeno da resistência a multi-drogas, cujo fator interefere na eficácia do tratamento (GILLET; GOTTESMAN, 2009). Portanto, esse assunto também foi aborado nesta tese.

2.7 FENÔMENO DA RESISTÊNCIA A MÚLTIPLAS DROGAS RELACIONADA AOS TRANSPORTADORES ABC

A eficiência das drogas quimioterápicas pode ser prejudicada pela má absorção de drogas administradas oralmente, aumento do metabolismo ou a excreção da droga, resultando em níveis mais baixos do fármaco no sangue e reduzindo assim a sua difusão para o tumor (PLUEN et al. 2001; JAIN 2001). Em muitos casos, se não a maioria deles, pacientes que respondem bem, inicialmente à quimioterapia, invariavelmente mostram uma perda de resposta mais tarde, resultando na reincidência de tumores (LUQMANI, 2005). Existem duas causas prováveis para este fato: ou as células tumorais podem ser intrinsecamente resistentes devido possivelmente a alguma característica genética ou essas células podem adquirir resistência após exposição à droga (HOLOHAN et al., 2013). Nos últimos anos, tem-se estudado intensivamente os mecanismos celulares de resistência a múltiplas drogas, utilizando principalmente modelos *in vitro*. As células cancerígenas em cultivo podem se tornar resistentes a uma única droga, ou a uma classe de drogas com mecanismos de ação semelhantes. Nesse caso, as drogas podem alterar o seu alvo celular ou ocasionar um aumento da reparação do dano ao DNA induzido pela quimioterapia (GOTTESMAN et al., 2002). Depois da seleção para a resistência a uma única droga, as células poderiam também mostrar resistência cruzada a outras drogas estruturalmente diferentes, fenômeno conhecido como *resistência a múltiplas drogas-RMD* (*multidrug resistence* – MDR - GREEN et al. 1999). Isso poderia explicar porque regimes de tratamento que combinam múltiplos agentes alvos diferentes não são mais eficazes (GOTTESMAN et al., 2002).

Provavelmente uma das formas mais significativas de resistência contra variedades de agentes antineoplásicos atualmente utilizados está relacionada à ação de um grupo de proteínas da membrana que podem forçar a passagem de moléculas citotóxicas, mantendo a concentração intracelular da droga inferior a um limiar capaz de eliminar as células tumorais (LUQMANI, 2005). Este é um dos mecanismos mais importantes de MDR, o qual resulta no aumento da taxa de efluxo de fármacos anticâncer a partir de células cancerosas por membros da família de transportadores de cassetes de ligação a ATP (ABC, do inglês *ATP-binding cassetes*) (DASSA; SCHNEIDER, 2001).

Os transportadores ABC são proteínas transmembranares que utilizam a energia resultante da hidrólise de ATP para transportar uma variedade de moléculas através de membranas biológicas (DEAN; ANNILO, 2005). Essas moléculas incluem aminoácidos, açúcares, peptídeos, lipídeos, íons e drogas quimioterapêuticas (HIGGINS, 1992). Os

transportadores ABC compreendem uma superfamília de proteínas conservadas evolutivamente, presentes desde as bactérias aos seres humanos (DASSA; BOUIGE, 2001). Até o momento, já foram identificados 48 genes ABC em humanos, os quais são divididos em seis subfamílias distintas (ABCA, ABCB, ABCC, ABCD, ABCE e ABCG) com base na sua sequência homóloga e na organização dos domínios (DEAN; ANNILO, 2001) como mostra a figura 7.

Figura 2- Representação esquemática da família de transportadores ABC. TMD- domínio transmembranar; NBD- domínio de ligação a nucleotídeos; Fe, ferro.
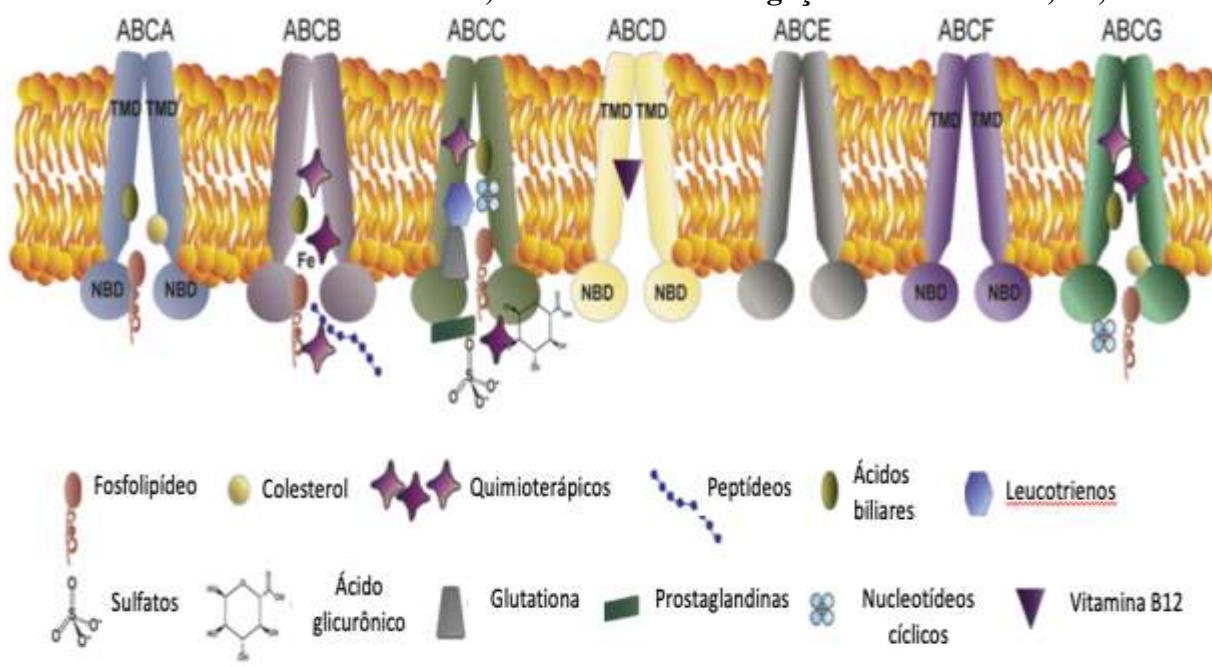
Fonte: DOMENICHINI et al., 2018- Adaptado

Essas proteínas podem atuar também em algumas funções reprodutivas, como moduladores da esteroidogênese, fertilização, implantação, transporte de nutrientes e respostas imunológicas. Funcionam como "*gatekeepers*" em vários locais de barreira (barreira de testículos de sangue e placenta) protegendo as células contra fatores xenobióticos potencialmente prejudiciais, incluindo drogas e toxinas ambientais A expressão RNAm e proteíca dos transportadores de ABC foi identificada no ovário ou linhagens de células ovarianas (BLOISE et al., 2016). No entanto, a atividade biológica desses transportadores e sua regulação permanecem em grande parte inexplorados. Dessa forma, compreender o papel dos transportadores ABC no ovário vai lançar luz sobre a regulação dos principais eventos reprodutivos, como a oogênese e a esteroidogênese ovariana. Dentre os transportadores ABC melhores descritos nos tecidos reprodutivos (principalmente na placenta), destaca-se a *p-*

glicoproteína transportadora de múltiplos fármacos ABCB1 (p-glicoproteína/ *Multi-Drug Resistance Gene-MDR1*), por ser encontrada apenas em mamíferos; e o ABCC2 (*Multidrug Resistance-Associated Protein 2 - MRP2*) , por transportar substâncias de importância fisiológica para a sobrevivência da célula; o ABCG2 (*Breast Cancer Resistance Protein - BCRP*) por apresentar multirresistência aos esteroides; como mostrado na figura 8 (DEAN; ANNILO, 2001). A identificação e localização dessas proteínas no tecido pode ser realizadas através de técnicas como imuno-histoquímica e imunofluorescência que permite reconhecer constituintes celulares, ou seja, antígenos, e assim identificar e classificar células específicas dentro de uma população celular morfologicamente heterogênea ou aparentemente homogênea. A visualização do complexo antígeno-anticorpo é possível pela adição de um fluorocromo (Alexa flúor, por exemplo) conjugado ao anticorpo (imunofluorescência) que pode então ser observado ao microscópio de fluorescência, ou, alternativamente, de uma enzima (peroxidase), como é o caso da imunohistoquímica cujo produto da reação pode ser visualizada por microscópios ópticos comuns (RAMOS-VARA, 2005).

Figura 3 - Participação dos transportadores ABC como importantes mediadores nos processos reprodutivos.

	Gametogêneses	Desenvolvimento embrionário	Endométrio	Transporte efluxo placental	Membrana fetal	Miométrio
Processo reprodutivo/ estrutura						
Transportador ABC	ABCA1 **ABCB1** ABCC4 ABCG1 **ABCG2**	ABCB1 ABCG2	ABCA1 **ABCB1** ABCC1 ABCC4 **ABCG2**	ABCA1 **ABCB1** ABCB4 ABCC1 **ABCC2** ABCC3 ABCC5 ABCG1 **ABCG2**	ABCA1 ABCC1 ABCC2 ABCC5 **ABCG2**	ABCA1 **ABCB1** ABCC1 ABCG1
Possível Função	esteriodogêneses e proteção dos gametas	proteção; competência embrionária	reconhecimento materno da gravidez; diferenciação e invasão trofoblástica; resposta imunológica	proteção placental e fetal; transporte de nutrientes e homeostase; resposta imunológica	proteção; sobrevivência; resposta imunológica	proteção; homeostase; resposta imunológica

Fonte: BLOISE et al., 2016- Adaptado

2.7.1 ABCB1

O primeiro transportador ABC identificado foi o MDR1 humano, também conhecido como proteína ABCB1 (JULIANO; LING, 1976). Consiste de uma glicoproteína de 170 kDa ligada à membrana, sendo codificada por um gene localizado no cromossomo 7p21 (BORST; SCHINKEL, 1997). O transportador ABCB1 está presente nos rins, intestino, placenta, fígado, glândulas suprarrenais e cérebro (principalmente no plexo coroide, astrócitos, microglia e capilares sanguíneos) (BORST; ELFERINK, 2002; SCHINKEL; JONKER, 2003). O ABCB1 tambem é expresso em embriões de camundongos (ELBLING et al., 1993) e bovinos (MORI et al., 2013), sugerindo que a atividade de efluxo do ABCB1 de substratos que envolvidos na seqüência de eventos que levam à progressão morfogênica de embriões (BLOISE et al., 2016). Outros estudos identificaram a preseça do ABCB1 em folículos pré-antrais e antrais (ratas: LEE; CROOP; ANDERSON, 1998), bem como em oócitos (camundongos: ELBLING et al., 1993; suínos: YOKOTA et al., 2006) e células granulosas (suínos: FUKUDA et al., 2006). Normalmente, o ABCB1 está envolvido na proteção celular por meio da extrusão de certos xenobióticos. Além disso, o transportador ABCB1 pode atuar no efluxo de drogas que não são usadas em quimioterapia contra o câncer (AMBUDKAR et al., 1999). Aller et al. (2009) demonstraram, através da cristalografia de raios X, que o transportador ABCB1 tem vários sítios que pode se ligar e ocasionar o efluxo de uma variedade de substratos para fora das células. Numerosos estudos *in vitro* têm mostrado que a expressão de ABCB1 em células tumorais reduz de forma significativa o acúmulo de uma ampla variedade de substratos quimioterapêuticos hidrófobicos neutros e catiônicos (DEELEY; WESTLAKE; COLE, 2006). Dentre esses agentes, inclui os taxanos (paclitaxel e docetaxel), epipodofilotoxinas (etoposido e teniposido), alcalóides da vinca (vinblastina e vincristina), antraciclinas (doxorrubicina e daunorrubicina) e antibióticos (dactinomicina e actinomicina) (GOTTESMAN et al., 2002; SZAKACS et al., 2006). Esse efluxo das substâncias citadas, resulta em resistência a múltiplos fármacos que é devido ao aumento da expressão do transportador ABCB1. Essa proteína funciona como uma bomba de efluxo de múltiplos fármacos de amplo espectro e possui 12 regiões transmembranares e dois sítios de ligação ao ATP, removendo eficientemente fármacos citotóxicos (CHEN et al., 1986).

2.7.2 ABCC2 (MPRP2)

Como nem todas as células resistentes a múltiplas drogas expressam o ABCB1, foi

iniciada uma busca por outras bombas de efluxo, levando à descoberta da proteína 2 associada a multirresistência a drogas (MRP2 ou ABCC2) (GOTTESMAN et al., 2002). O ABCC2 foi caracterizado como um transportador canalicular de ânions orgânicos multiespecífico (cMOAT) e possui em torno de 49% de aminoácidos idênticos ao ABCC1 (BORST; ZELCER; VAN HELVOORT, 2000). No fígado, possui o papel fisiológico de uma bomba de ânions orgânicos e desempenha um papel na desintoxicação de agentes alquilantes no epitélio apical do fígado e rim (BORST; SCHINKEL, 1997; SMITHERMAN et al., 2004). O ABCC2 tambem foi identificado na placenta e em mebranas fetais de humanos (ST-PIERRE et al., 2000; YOSHINO et al., 2010). Em geral, essa proteína transporta uma ampla gama de substratos, principalmente ânions orgânicos conjugados com ácido glicurônico, glutationa e sulfato, e sua expressão pode ser modulada por xenobióticos (TOCCHETTI et al., 2016). A superexpressão de ABCC2 tem sido observada em algumas células cancerosas e pode, por conseguinte, desempenhar um papel no surgimento de resistência a fármacos quimioterapêuticos, incluindo alguns agentes alquilantes (KONIG et al., 1999). Altos níveis de ABCC2 nucleares também foram observados em uma paciente com câncer ovariano recorrente que teve como tratamento adjuvante carboplatina + paclitaxel (TIAN et al., 2012).

2.7.3 ABCG2

O ABCG2 ou ABCG2/BCRP, o último membro da familia dos transportadores ABC a ser descoberto, foi identificado por três diferentes grupos de pesquisa, quase que simultaneamente (ALLIKMETS; SCHRIML; HUTCHINSON, 1998; DOYLE et al., 1998; MIYAKE et al., 1999). Subsequentemente, um número significativo de estudos foi publicado sobre este importante transportador de efluxo. Em humanos, ABCG2/BCRP é codificada pelo produto do gene ABCG2, localizado no cromossomo 4q22 (ALLIKMETS et al., 1998). Normalmente, o ABCG2 é expresso em vários tecidos, incluindo a placenta, cólon, membrana apical do epitélio nos intestinos delgado e grosso, ilhotas e células acinares do pâncreas, membrana canalicular do fígado e vesicula bíliar, pneumócitos, tecido mamário, endotélio venoso e capilar, glândulas suprarrenais, túbulos do córtex do rim, epitélio da próstata e na superfície luminal do endotélio de células de microvasos do cérebro humano (MALIEPAARD et al., 2001). O ABCG2 é um transportador de efluxo de alta capacidade com especificidade do substrato, reconhecendo moléculas de carga negativa ou positiva, aniões orgânicos e sulfato conjugados (STAUD; PAVEK (2005). Assim como os transportadores ABC anteriormente citados, o ABCG2 também é importante para farmacologia devido a eliminação de agentes

citotóxicos das células cancerígenas, tornando essas células resistentes à quimioterapia. Através do mesmo mecanismo de efluxo, o ABCG2 afeta os parâmentros farmacocinéticos das drogas, por outro lado também ajuda a proteger compartimentos do corpo contra xenobióticos potencialmente prejudiciais (STAUD; PAVEK (2005).

3 JUSTIFICATIVA

É conhecido, atualmente que o câncer é um grande problema de saúde pública em todo o mundo. Nos Estados Unidos, o *National Center for Health Statistics*, estimou um número assustador, ou seja, 1.735.350 novos casos até o final de 2018 (SIEGEL; MILLER; JEMAL, 2018). No Brasil, de acordo com o Instituto Nacional do Cãncer, 600 mil novos casos da doença foram estimados para o biênio 2018-2019 (INCA, 2018). Felizmente, o número de sobreviventes também tem aumentado, o que tem sido devido aos avanços na medicina, relacionados com o diagnóstico cada vez mais precoce e confiável, associado aos novos tratamentos mais efetivos (BEN-AHARON; SHALGI, 2012). No entanto, as terapias contra o câncer apresentam vários efeitos colaterais indesejáveis, alterando não somente o próprio estado de saúde do indivíduo como também afeta a qualidade de vida, trazendo sérios problemas permanentes, como por exemplo, a infertilidade, haja vista que são extremamente gonadotóxicos (MEIROW et al., 2010) e causam danos severos aos folículos ovarianos. Além disso, existe uma preocupação muito grande na introdução de novos agentes quimioterápicos anticâncer, em função dos problemas paralelos causados pelo fenômeno da *resistência a multi ou múltiplas drogas* (RMD). Portanto, baseada nessa grande problemática, a presente tese tem como foco, uma investigação sobre proteínas envolvidas no fenômeno da RMD, bem como no efeito causado por dois novos compostos com potencial anticâncer sobre a função exócrina do ovário. O ovário caprino foi escolhido nesse estudo, pois tem sido amplamente utilizado como modelo translacional para a espécie humana, seja para avaliar o efeito positivo (MBEMYA et al., 2017; SÁ et al., 2018) ou negativo (GUERREIRO et al., 2016; LEIVA-REVILLA et al., 2016) sobre a função folicular e ovariana.

No que se refere ao fenômeno de RMD, vários estudos têm demostrado o envolvimento de uma família de proteínas transmembranárias conhecidas como transportadores ativos *ATP-binding cassette* (ABC) (DASSA; BOUIGE, 2001). A superexpressão dessas proteínas nas células tumorais torna essas células insensíveis ou com baixa resposta a um amplo espectro de drogas (DEELEY et al., 2006; HIGGINS, 2007). Entretanto, a principal função dessas proteínas é o transporte de moléculas como açúcares, aminoácidos, nucleotídeos, peptídeos, lipídios, esteróides, sais biliares, toxinas e drogas quimioterápicas através de membranas biológicas utilizando a energia resultante da hidrólise do ATP (GOTTESMAN et al., 2002). Dentre os vários transportadores ABC, destacam-se o ABCB1 (glicoproteína P ou Gene de Resistência a Drogas Múltiplas - MDR1), ABCC2 (Proteína 2 Associada à Resistência a Múltiplos Medicamentos - MRP2) e ABCG2 (Proteína da Resistência ao Câncer de Mama -

BCRP). Essas proteínas desempenham um papel importante na manutenção da permeabilidade seletiva em vários tecidos, como a barreira hematoencefálica, barreira hemato-testicular e barreira materno-fetal ou barreira placentária (LESLIE et al., 2004).

No tocante à identificação de novos compostos com potencial anticâncer, é conhecido que não basta apenas que sejam capazes de eliminar as células tumorais, mas também devem exercer um mínimo de efeitos contra as células saudáveis. Desta forma, é absolutamente necessária, a realização de testes toxicológicos, inclusive testes toxicológicos reprodutivos, os quais, em sua maioria, envolve a utilização de muitos animais vivos, indo de encontro com o princípio dos 3Rs para a experimentação animal. Considerando, a relevância desse princípio, nós empregamos nesse estudo o modelo biotecnológico do *ovário artificial* (cultivo in vitro do ovário) caprino, para avaliar os folículos ovarianos. A utilização do cultivo in vitro do tecido ovariano caprino como método laboratorial para testes toxicológicos de drogas representa um grande impacto para o bem-estar animal, pois milhares de animais serão poupados de serem utilizados em experimentos *in vivo* (FIGUEIREDO et al., 2008). Além disso, esse modelo também permite identificar os efeitos causados por drogas já amplamente utilizadas ou novos compostos como o Vitanolido D e a Quinoxalina, sobre folículos ovarianos pré-antrais. O impacto positivo da aplicação desse modelo é o estabelecimento de tratamentos menos prejudiciais para a fertilidade feminina, especialmente na espécie humana.

Em relação às drogas escolhidas para esse estudo, sabe-se que muitos esforços têm sido realizados por vários laboratórios de pesquisa visando desenvolver e identificar novos compostos, sintéticos ou naturais que tenham propriedades anticâncer e, ao mesmo tempo, apresentem menos efeitos adversos para as células saudáveis. Dentre esses, destacam-se o Laboratório de Análise Fitoquímica de Plantas Medicinais (LAFIPLAN) e o Laboratório de Oncologia Experimental (LOE), ambos da Universidade Federal do Ceará, as quais têm investigado as propriedades anticâncer do Vitanolido D e da Quinoxalina, respectivamente. Embora, estudos já tenham demonstrado que, tanto o Vitanolido D (BATISTA et al., 2016), como a Quinoxalina (MARANHÃO et al., 2016) exercem efeitos contra diferentes linhagens tumorais, os efeitos de ambas as substâncias sobre a função ovariana ainda não foram avaliados, sendo portanto, uma necessidade imediata, a ser sanada pela presente tese.

4 HIPÓTESES CIENTÍFICAS

- As proteínas transportadoras ABCB1, ABCC2 e ABCG2 são expressas em folículos pré-antrais caprinos;
- O Vitanolido D e a Quinoxalina sintética (PJOV 56) afetam a função ovariana, demosntrada pela análise dos folículos pré-antrais caprinos presentes no ovário cultivado *in vitro*, na presença dessas substâncias.

5 OBJETIVOS

5.1 OBJETIVOS GERAIS

- Utilizar o modelo do *ovário artificial* para avaliar a toxicidade do Vitanolido D e da Quinoxalina sobre os folículos ovarianos;
- Imunolocalizar as proteínas transportadoras ABC nos folículos pré-antrais caprinos presentes no ovário.

5.2 OBJETIVOS ESPECÍFICOS

- Avaliar os efeitos tóxicos de diferentes concentrações do Vitanolido D (1.5, 3.0 e 6.0 μM) e da Quinoxalina (1.5, 3.0 e 6.0 μM) sobre a morfologia e desenvolvimento de folículos pré-antrais presentes no tecido ovariano cultivados in vitro por 2 e 6 dias, na presença dessas substâncias;
- Investigar a capacidade proliferativa, bem como a morte por apoptose das células da granulosa de folículos pré-antrais cultivados in vitro na presença de Vitanolido D ou Quinoxalina;
- Verificar a expressão gênica de fatores envolvidos no controle positivo do ciclo celular nas células dos folículos pré-antrais cultivados in vitro na presença de Vitanolido D ou Quinoxalina;
- Avaliar a expressão da proteína ABCB1 nos folículos pré-antrais cultivados in vitro na presença de Vitanolido D ou Quinoxalina PJO56.

6 CAPÍTULO 1:

Sistemas de cultivo *in vitro* como uma alternativa para o estudo de toxicologia reprodutiva feminina

"In vitro culture systems as an alternative for female reproductive toxicology studies*"*

Resumo

Estudos têm revelado que a exposição diária a diferentes produtos, sejam estes químicos ou naturais, pode causar danos irreversíveis à saúde reprodutiva feminina. Dessa forma, faz-se necessário a utilização de testes que avaliem os parâmetros de segurança e eficácia desses produtos. A maioria dos testes de toxicologia reprodutiva é realizada principalmente *in vivo*. No entanto, nos últimos anos, vários métodos de cultivo de células, inclusive células-tronco embrionárias e, tecidos têm sido desenvolvidos com o objetivo de reduzir a utilização de animais em testes toxicológicos. Um grande avanço para a área da toxicologia, uma vez que os sistemas de cultivo têm o potencial de se tornarem uma ferramenta a ser amplamente utilizada em oposição aos testes *in vivo*, rotineiramente utilizados na biologia reprodutiva e na toxicologia. A presente revisão tem, portanto, como objetivo descrever e destacar dados a cerca de processos de cultivo *in vitro* para avaliar a toxicidade reprodutiva, como alternativas aos métodos tradicionais utilizando testes *in vivo*.

Palavras-chave: toxicidade, função reprodutiva, métodos alternativos, função ovariana, gônada feminina

In vitro culture systems as an alternative for female reproductive toxicology studies

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Abstract

Studies have shown that daily exposure to different products, whether chemical or natural, can cause irreversible damage to women's reproductive health. Thus, it is necessary to use tests that evaluate the parameters of safety and efficacy of these products. Most reproductive toxicology tests are performed mainly *in vivo*. However, in recent years, various cell culture methods, including embryonic stem cells and tissues have been developed with the aim of reducing the use of animals in toxicological tests. A major advance in the area of toxicology, since farming systems have the potential to become a tool to be widely used as opposed to the *in vivo* tests routinely used in reproductive biology and toxicology. Therefore, the present review aims to describe and highlight data on *in vitro* culture processes used to evaluate the reproductive toxicity as alternatives to traditional methods using *in vivo* tests.

Keywords: toxicity, reproductive function, alternative methods, ovarian function, female gonad.

Introduction

Humans are exposed daily to a variety of chemicals or natural products, through their interaction with the environment and the use of pharmaceutical products, which included personal care products and aesthetic to pesticides. In this sense, toxicity tests should be employed as they ensure adequate protection of human health and the environment against the adverse effects of exposure to a variety of toxic products (Holmes and Creton, 2010). Toxicological tests are therefore an important part of the safety assessment to regulate these products. Therefore, whenever new products such as medicines, pesticides, food additives and others intended for human or animal use are to be introduced on the market, studies must be carried out to predict the toxicological risks of the new substance (Azevedo, 2010). Current methods used to report the toxicological risks of chemical substances depend in large part on the animal models used over the past 50-60 years. At the present time, approaches to *in vivo* toxicity tests depend mainly on the observation of adverse biological responses in homogeneous groups of animals exposed to high doses of a given agent. However, the relevance of animal

studies for risk assessment in heterogeneous human populations, even exposed to much lower concentrations has been questioned, because it is not yet known to what extent the results obtained can be extrapolated to humans. In addition, by using a large number of animals, these studies are expensive and time-consuming, as well as ethical issues involved with *in vivo* practices that should be discussed principally because some of these practices can cause suffering and death of animals (Cruz, 2003). In this way, the tests used in evaluating the efficacy and toxicity of drugs have undergone a process of reformulation, in which part of this process look at the replacement of *in vivo* tests by in vitro tests, aiming at the reduction of the number of animals (Richmond, 2002). To this purpose, several models such as the culture of cells, tissues and organs, are being developed and used to replace *in vivo* methods of experimentation.

In vitro tests are more economical; cells and tissues are easier to maintain in culture, require little physical space and can be used for various purposes such as: preparing antigens and antibodies; produce vaccines; isolating microorganisms, particularly different types of virus and assessing the toxicity of various products (Cruz, 2003).

Specifically, with regard to reproductive function, there is currently great concern about the impact of exposure to different compounds, since many products can have effects on the reproductive system, being able to cause infertility, fetal malformation, among others (Faqi, 2012). In the case of the female reproductive system, cases of infertility due to toxicity can be triggered by premature ovarian failure (POF) or early menopause, which refers to the development of amenorrhea due to a cessation of ovarian function before 40 years of age (Goswami and Conway, 2005). For this reason, toxicological tests are required in the investigative processes, since the development of products until its registration and commercialization (Stokes, 2002; Meyer, 2003).

Thus, considering the relevance of this theme for women's reproductive health, the purpose of this review is to present relevant informations on toxicological tests in general, with an emphasis on reproductive toxicology, and more specifically on alternative toxicological tests with a view to reducing the number of animals involved in the experimental research.

Toxicological tests

According to Corrêa et al. (2003), toxicology is the science that studies the adverse effects caused by the interaction between chemical substances and living organisms or biological systems. Toxicological tests have as their main objective the prediction of possible

adverse effects, which may occur when human or animal are exposed to a particular chemical. As a result of this exposure, the degree of toxicity varies from light, such as ocular irritation, to severe irritation, which can lead to permanent incapacitation of an organ (Stokes, 2002). There are two experimental models that can be applied to study the adverse effects of product exposure in human health, there are in vivo tests and in vitro tests. These tests allow a quantitative and qualitative evaluation of the impact of this exposure and are briefly described below.

In vivo tests

It is known that every year, millions of animals, which serve as experimental model, are used around the world. Various animals such as mice, rats, hamsters, rabbits, fish (zebra fish and trout), birds (mainly chickens), guinea pigs, amphibians (frogs), different species of primates, dogs, cats, among others are used in scientific research (Giridharan, 2000). In addition, these animals have been used as a tool to understand the effects of medical and surgical procedures; for the production of vaccines and antibiotics, as well as for the diagnosis and treatment of diseases (Hendriksen, 2007). The most stringent tests performed on animals are reserved for medicines and foods, in which a series of tests is performed, with a period of duration that can vary from days to years, to test, for example, general toxicity, eye and skin irritation, mutagenicity, carcinogenicity, and teratogenicity (Vandebriel & Van Loveren, 2010; Creton et al., 2010).

For each chemical assay, for example, about 5,000 animals are used (Abbott,2005; Creton et al., 2010).

In the United States, for example, in 2004, the number of animals used in the scientific research was approximately 12 million. However, this number corresponds to an estimate that could reach up to 25 million (USDA Annual Report Animal Usage Statistics, 2014). On the other hand, in the European Union, in the same year, 3,87 million animals were used in researches, representing a decrease of 6% (254,000 fewer procedures) compared to the year 2013 (Annual statistics of scientific procedures on living animals Great Britain, 2014). This reduction is directly related to the strict measures that Europe has adopted in recent years regarding the use of animals in research.

In vivo tests, the whole animal or its organs and tissues are used. For this purpose, the animals are euthanized with the application of previously established protocols. Frequently, animals surviving after the pre-clinical test are sacrificed at the end of an experiment to avoid

their pain and discomfort (Rusche, 2003). In some cases, for example, in lethal dose analysis (LD 50), the animals die as a result of the experiment. The pain, anguish, and death experienced by animals during scientific experiments have been a matter of constant debate. The argument used by animal welfare advocates is that, in being alive, animals have the right not to feel pain or distress, and therefore their use for experimentation is unethical and must be reduced or even extinct (Rollin, 2003). In addition, the usefulness of in vivo toxicity tests has generated many controversies since many animal toxicity tests do not accurately reflect toxicity in humans, making the results false (Wilson-Sanders, 2011). An example of this can be observed in the experiments with thalidomide (drug used as sedative, anti-inflammatory) in rodents (Lenz, 1988; Saldanha, 1994). In these studies thalidomide was shown to be a low-risk drug for intoxication and side effects. However, it was responsible for the congenital malformation of thousands of children in the 1960 century. Likewise, there is a test that aims to test the ocular and cutaneous irritation of substances, especially cosmetics, in rabbits, called the Draize test, in which its low reproducibility highlights the concerns about the extrapolation of test results in animals for beings human rights (Quantin et al., 2015).

In view of the above facts, in recent years alternatives to animal experimentation have been sought through the development of toxicological tests based on the 3Rs principle. The 3Rs principle was postulated by Russell and Burch (1992) and published in *The Principles of Humane Experimental Technique*. This principle is thus called in function of the initials in English of its main objectives: 1) Reduction, 2) Refinement and 3) Replacement.

The 3Rs principle ensures at the same time the highest standards of safety for consumers and patients; respect for the environment and animal welfare (National research council (US) institute for laboratory animal research, 2004). The *reduction* can be understood by decreasing the number of animals in a single test or instead of using animals in all experimental phases, they would only be used in the final stages. *Refinement* involves implementing animal care and treatments to minimize any pain or suffering (Richmond, 2002; Doke & Dhawale, 2013). And finally, with regard to *substitution*, it is characterized by the non-use of animals. Different techniques are used for this practice, such as the *in vitro* culture of cells and tissues. Briefly, this principle is related to the reduction of the number of animals used in the research, the improvement in the conduct of the studies, in order to reduce animal suffering to the minimum possible, and the search for alternative methods that, in the end, replace the tests in alive Therefore, the main purpose of the 3Rs principle is to serve as a unifying concept, a challenge and an opportunity for scientific, economic and humanitarian benefits (Balls et al., 2000).

There is now a consensus that animal studies should be conducted only when 1) the objective is of justifiable importance; 2) there are no valid alternative methods; 3) all relevant reduction and refinement strategies have already been identified and implemented; 4) the design and conduct of the study minimizes the damage caused to animal welfare, not only in relation to the number of animals used, but also in relation to the pain and suffering caused, and 5) there is a maximum scientific benefit (Richmond, 2002). Thus, since the tests do not fit into any of the mentioned conditions, *in vitro* tests are suggested.

In vitro tests

The non-use of animals in toxicological tests, that is in vitro studies provide important tools for understanding the risks of chemicals, predicting possible effects on humans (Broadhead, & Combes, 2001). In the last decades many studies have been carried out to develop methodologies that use living tissues and cells of mammals, lower organisms and inert substrates in toxicological tests (Chamberlain & Parish, 1990).

A wide variety of human and animal cell lines from the most varied tissues and organs are stored in cell banks, facilitating the development of many studies (Castro,1978). The *American Type Culture Collection* (ATCC), founded in the 1960s was one of the pioneer banks in providing certified lineages (ATCC, 1994). Thus, cell culture techniques have been widely used as they are economical, relatively easy to maintain and require little physical space, with cell survival and / or proliferation being evaluated by counting the number of cells or by using vital dyes (Husoy et al., 1993).

Other *in vitro* assays used organs isolated from the animal, such as assays with rabbit isolated eye (IRE) (De Torres et al., 1997), isolated chicken eye (CEET) (Burton et al., 1981), as well as bovine corneal opacity and permeability (BCOP) (Burdick et al., 2002). All of these methods were designed to quantitatively evaluate the irritant potential of a product or a chemical after application, using parameters such as corneal opacity, permeability, and hydration, or corneal thickness (Chamberlain et al., 1997).

In addition to the use of live cells, computational systems can also be used, through the development of computerized databases and programs that evaluate the toxicity by determining structure-activity relationship, such as the Quantitation Structure-Activity Relationship

(QSAR), whose protocol relates the physicochemical structure of a component with its toxicity (Toropov et al., 2014).

In this way, several methods have been suggested to avoid the use of animals in experimentation. These methods provide an alternative means for testing drugs and chemicals that may affect different cells, tissue and / or organs, such as those related to the reproductive system. Unlike male reproductive physiology, which has a continuous formation of gametes, women are born with their limited number of germ cells. Thus, chemicals that destroy female gametes can lead to premature ovarian failure due to the destruction of the follicular reserve (Hoyer & Sipes, 1996). Considering the great relevance of preserving female fertility, many studies have been carried out on the reproductive toxicology of the female.

Female Reproductive Toxicology

Among the different areas in toxicology, the reproductive focus of this review studies the occurrence of adverse effects due to exposure to chemical or physical agents to the reproductive system (Eaton & Gilbert, 2013). This identification of the toxic potential in the reproductive system as well as the mechanisms of action is a major scientific challenge during the safety assessment of chemicals. In fact, reproductive toxicology is one of the most complicated domains of toxicology, due to multiple organs and tissues involved, different modes of toxic action and dependence of the endocrine system (Lorenzetti et al., 2011). In addition, some essential characteristics involving reproductive toxicology are completely different from all other areas of toxicology, such as the fact that some adverse effects can only be observed next in the generation after the parental crossing (Spielmann, 2009).

Most protocols for reproductive toxicology studies involve the use of live animals. However, over the past few years, a wide range of in vitro models have been developed to detect the teratogenic effects of chemicals, including tests that use dissociated cells from the brain and from buds of the embryo members of rats (Hartung et al., 2004; Hareng et al., 2005; Luciano et al., 2010) or the culture of whole rat embryos (Bremer & Hartung, 2004).

Alternative in vitro tests to evaluate female reproductive toxicity

Some in vitro systems can be used to evaluate reproductive toxicity in females: culture of isolated ovarian follicles, embryos, ovary (whole organ or only part of the tissue) embryonic stem cells, which will be described as follows.

Culture of embryos, oocytes, isolated ovarian follicles, follicular cells

The culture of isolated secondary ovarian follicles allows to evaluate the effect of toxic substances on the follicular development, so that the oocytes from these follicles can be matured and fertilized *in vitro*. Thus, the effect of substances on the characteristics related to oocyte maturation, such as meiotic spindle formation; chromatin damage; gene methylation; as well as on embryonic development can be evaluated (Zhang & Liu, 2015; Bouwmeester et al., 2016; Žalmanová, et al., 2017). The follicles can be isolated enzymatically or mechanically to obtain the oocyte / cell complex of the granulosa / theca cell intact (Cortvrindt et al., 1996). In vitro follicle culture systems differ in terms of the species used, the follicular stage in which the culture process is initiated, the type of culture system (96-well plates or droplets covered with mineral oil), the composition of the medium and final parameters evaluated (Rose et al.,1999). The characterization of this system is extremely important to enable comparison between the in vivo and in vitro systems (Smitz & Cortvrindt, 2002).

Siddique et al. (2014) evaluated the exposure of mouse secondary to the condensed cigarette smoke and benzo (a) pyrene and the results showed that both substances induced oxidative stress in the ovarian follicles. Another study evaluated the effect of cadime chloride on the development of secondary follicles of rats and found changes in follicular growth, differentiation and steroidogenesis (Wan et al., 2010).

Some authors have used only the oocytes to evaluate the effect of various substances such as cadmium (Leoni et al., 2002; Beker, et al., 2012), bisphenol A (Mlynarcikova et al., 2009) and nicotine (Vrsanska et al., 2003) on the maturation, fertilization and embryonic development of different species. In these studies, it was observed that the process of oocyte maturation is very sensitive to exposure of the mentioned drugs, so that lower maturation rates and, consequently, lower fertilization rates were obtained. In a recent study, Ferris et al. (2017) evaluated the exposure of bovine oocytes to 30 ng/mL bisphenol A during the oocyte maturation process. The authors observed a reduction in the rates of cleavage and formation of blastocysts, an increase in the rate of apoptosis and proportion of female blastocysts. Another study evaluated the effect of fluoride on fertilization and subsequent embryonic development of mice. After overexposure of oocytes to fluoride, a reduction in ATP production and mitochondrial membrane potential was observed, which influenced the rates of fertilization and embryonic development (Liang et al., 2016).

Studies on the direct effect of the exposure of embryos to different substances are mostly carried out using chick embryos (Gao et al., 2016; Strojny et al., 2016) and fish, mainly zebra fish (Zhang et al., 2016; Hu et al., 2017). Zhang et al. (2016) evaluated the effect of the 4-hydroxychlorothalonil metabolite from the chlorotalonil fungicide on zebra fish embryos and revealed that 4-hydroxychlorothalonil exhibited potent effects on endocrine disruption, causing negative impact on embryonic development.

In addition to the culture of oocytes and embryos, the *in vitro* culture system of granulosa cells or luteal cells has also been used, which allows evaluating how a specific substance can act specifically on these structures and thus determine the possible mechanism of action of the substance causing cellular toxicity (Sun et al., 2004). Twu et al. (2012) in a study with goats used corpus luteum cells to evaluate the effect of cantharidin and norcantaridine, natural toxins produced by Chinese beetles (*Mylabris phalerata* or *Mylabris cichorii*). According to these authors, these toxins are responsible for accidental intoxication in humans and animals, as well as being used in folk medicine to induce abortion. With the study, the authors observed that both substances inhibited the production of progesterone and that cantharidin inhibited steroidogenesis by reducing the expression of StAR protein, important for this process.

Culture of ovarian tissues or whole ovary

The culture of part (fragments) or whole ovary allows to evaluate different parameters in a controlled way and, therefore, they have the potential to allow a more complete evaluation of the studies of reproductive toxicity (Stefansdottir et al., 2014). This type of culture is already well established in different species mice (O'Brien et al.,2003), rats (Cain,et al.,1995), cows (Jimenez et al., 2016), sheep (Cavalcante et al., 2015), goats (Faustino eta al., 2011) to evaluate the positive effect of various substances on the development and oocyte and follicular function within the ovary itself. However, the number of studies using in vitro models is still low compared to the volume of in vivo studies. As we can see in table 1, different substances have already been tested using the ovarian tissue to evaluate the negative effect on the female reproductive function. Among these substances, we highlight 4-Vinylcyclohexene diepoxide (VCD), commercially used as an intermediate and chemical reactive diluent for diepoxide and epoxy resins (Huff, 2001); the 7.12, dimethylbenz (A) anthracene (DMBA), which is produced from the burning of organic compounds (cigarette smoke (Gelboin, 1980)); the monoethylhexyl phthalate (MEHP), which can be found in many products of daily use, such as PVC, plastic

bags, food packaging, cosmetics and industrial paints (Hauser et al., 2004; Heudorf et al., 2007); bisphenol A, which is present in several plastics to which humans are always in contact (Ikezuki et al., 2002). In addition to these substances, commercial chemotherapies such as docetaxel, doxorubicin (DXR), paclitaxel (PTX) (Guerreiro et al., 2016), and substances derived from plants with antitumor effects, such as *Oncocalyxone* A (Leiva-Revilla et al., 2016), the ethanolic fraction of *Auxemma oncocalyx* (Leiva-Revilla et al., 2016) and frutalin (Soares et al., 2017).

Table 1. Effects of different substances evaluated in vitro on ovary of different species.

Substances investigated	Concentrations	Species	Strategies	Main effects	References
4-Vinylcyclohexene Diepoxide	1, 3, 10, 30 e 100 µM	*Rattus norvegicus*	Culture of whole ovary for 15 days	Reduction in the number of primordial and primary follicles at concentrations of 30 and 100 µM after 8 days of culture.	Devine et al., 2002
7, 12, dimethylbenz (A) anthracene (DMBA)	12.5 nM-1µM	*Mus musculus*	Culture of whole ovary for 15 days	Reduction of the percentage of healthy preantral follicles.	Rajapaksa et al., 2007
7, 12, dimethylbenz (A) anthracene (DMBA) and metabolite DMBA-3,4-diol	12.5 nM-1µM	*Rattus norvegicus*	Culture of whole ovary for 15 days	Ovotoxicity after 15 days of culture occurred at lower concentrations of DMBA-3,4-diol (12.5 nM - primordial; 75 nM - primary) than DMBA (75 nM - primordial, 375 nM - primary).	Igawa et al., 2009
4-vinylcyclohexene diepoxide (VCD) and 7,12, dimethylbenz (A) anthracene (DMBA)	30 µM VCD 1 µM DMBA	*Rattus norvegicus*	Culture of whole ovary for 15 days	Reduction of primordial and primary follicles after 6 days of culture.	Keating et al., 2009
7, 12, dimethylbenz (A) anthracene (DMBA)	1 µM	*Rattus norvegicus*	Culture of whole ovary for 6 days	Increased mRNA expression for glutathione S-transferase (GST) in treated ovaries.	Bhattacharya & Keating, 2012
Mono-ethylhexyl phthalate (MEHP)	100 µM	*Homo sapiens*	Culture of fetal ovarian fragment for 3 days	Increased mRNA levels for LXRα and SREBP resulting in deregulation of lipid synthesis in the fetal gonads.	Muczynsk et al., 2012
Docetaxel	0.1, 1 e 10µM	*Mus musculus*	Culture of whole ovary for 6 days	Damage to the granulosa cells of developing follicles after 6 days of culture.	Lopes et al., 2014
7, 12, dimethylbenz (A) anthracene (DMBA)	12.5-75 nM	*Rattus norvegicus*	Culture of whole ovary for 8 days	Reduction in the number of primary and secondary follicles after 8 days of culture.	Madden et al., 2014
Dexrazoxane (Dexra) and Doxorubicin (DXR)	20 µM Dexra 50 nM DXR	*Callithrix jacchus*	Culture of ovarian fragments for 24 hours	DXR increased the percentage of antral follicles containing granulosa cells positive for γH2AX.	Salih et al., 2015
Bisphenol A (BPA)	440 µM	*Rattus norvegicus*	Culture of whole ovary for 8 days	Reduction in the number of primary and secondary follicles after 2 days of culture and primordial follicles after 4 days.	Ganesan & Keating, 2016
Oncocalyxone A and ethanolic fraction of *Auxemma oncocalyx*	1,2, 12 e 34 µg/ml A. oncocalyx 1, 10 e 30 µg/ml onco A	*Capra hircus*	Culture of ovarian fragment for 7 days	*Oncocalyxone* A and *A. oncocalyx* reduced the number of normal morphological follicles in a concentration-dependent manner	Leiva-Revilla e al., 2016
Doxorubicin (DXR) and Paclitaxel (PTX)	0.003, 0.03 e 0.3µg/mL DXR 0.001; 0.01 e 0.1µg/mL PTX	*Capra hircus*	Culture of ovarian fragment for 7 days	DXR and PTX reduced the percentage of preantral follicles after 1 and 7 days of culture, with PTX being more toxic than DXR.	Guerreiro et al., 2016
Frutalin	1-200 µg/mL	*Capra hircus*	Culture of ovarian fragment for 6 days	Reduction of follicular survival after 6 days of culture	Soares et al., 2017

As can be seen, most studies on toxicology, using this type of strategy, focus on the use of mouse and rat females as models to assess the adverse effects of substances on reproductive function and fertility. Because they require a large number of animals for reproductive tests, these species have been used as models, due to the ease of handling and the reduced space demand, besides being very well characterized, be it anatomical, physiological or genetically and have short life cycles (gestation, breastfeeding, and puberty) (Harkness & Wagner, 1993; Santos, 2002). However, rodent and human reproductive physiology differ in many respects, such as ovarian size and consistency, follicular distribution, oocyte diameter, luteal body signaling (Betteridge & Rieger, 1993; Ménézo & Hérubel, 2002), as well as duration of folliculogenesis (Smitz & Cortvrindt, 2002; Ménézo & Hérubel, 2002). On the other hand, it is believed that domestic animals such as the goat can be an excellent experimental model for the human species, since it presents similarities in the ovary (size and consistency) and in other aspects such as follicular diameter and the process itself of folliculogenesis (6-7 months) with the ovaries of women (Smitz & Cortvrindt, 2002; Baldassarre, 2008). Besides the great similarity between the ovaries of both species, the ovaries can easily be obtained in slaughterhouses. From the point of view of animal welfare, this would be extremely desirable since the lives of thousands of laboratory animals will be spared, since a large number of such animals will no longer be used in in vivo experiments (Figueiredo et al., 2008). In view of this possibility, goat ovarian tissue has been used to evaluate the in vitro toxicity of different drugs, as demonstrated by Guerreiro et al. (2016). In this study, the authors evaluated the toxicity of two antineoplastic drugs widely used in the treatment of different types of cancer, DXR and PTX during the in vitro culture of goat ovarian tissue (data presented in Table 1). The authors observed deleterious effects of drugs such as oocyte retraction, nuclear pyknosis germ cell and large disorganization of granulosa cells. In addition, it has also been found that PTX is more toxic to preantral follicles than DXR. In another study, Leiva-Revilla et al. (2016) also used the in vitro culture of goat ovarian tissue as a strategy to evaluate the toxicity of two substances with antiproliferative effects, that is, *oncocalyxone* A and the ethanolic fraction of *Auxemma oncocalyx*. According to the study, both substances adversely affected follicular development in a dose-dependent manner.

Ovarian tissue culture has the advantages of evaluating the effect of substances on the pool of primordial follicles, which consist of the ovarian reserve. However, this strategy is limited in regard to the duration of culture time, since short periods may not be sufficient to

ensure follicular development. On the other hand, cell viability can be reduced with very a long period of culture (Alves et al., 2013).

Validation of alternative methods

Although it has existed since 1991, the European Union (EU) Reference Laboratory for Alternatives to Animal Testing (EURL-ECVAM) was formally created only in 2011. Due to the increasing need for animal replacement through new methods developed and proposed by the EU, the creation of this laboratory occurred after large investments of time and resources to coordinate and promote the development, validation and use of alternative methods (Adler et al., 2011). In Brazil, the National Network of Alternative Methods for the Use of Animals (RENAMA) was recently created through Ordinance No. 491, of February 3, 2012, whose objective is the development of methods capable of reducing, refining or replacing of animal use as experimental models, based on the principles of Russell & Burch (1992).

Validation can be defined as the process by which the relevance and reliability of a particular approach, method, process, or assessment are established for a definite purpose (OECD, 2005). The relevance of a procedure refers to the scientific value and practical utility of the results obtained, whereas reliability is related to the reproducibility of these results within and between laboratories over time, in relation to a clearly defined and specific purpose (Balls et al.,1990; Balls & Karcher, 1995). The initial focus on validation was the performance of alternative methods as assessed in multi-laboratory laboratory studies, which usually involve the analysis of chemical substances.[100]

Validation consists only of one of the steps in the progress of a test, from its design to its regulatory application (Balls et al., 1995). In fact, the validation process of alternative methods is aimed at verifying the optimization, transferability, reproducibility, and relevance of the proposed method with the objective of being submitted to the regulatory agency. Once approved, the method becomes officially available for the toxicological evaluation of raw material. The worldwide availability of validated methods occurs through the Organization for Economic Cooperation and Development (OECD) and pharmacopeias (ICCVAM) .

Considering the information mentioned above, for a new method to be accepted, it must be evaluated in order to establish its relevance for implementation and reliability.7 For this to be possible, it is necessary to harmonize validation processes, through international committees (Schechtman, 2002). Thus, scientific validation of alternatives for assessing in vitro toxicity as a laboratory method for drug testing and its subsequent acceptance by government bodies

responsible for drug release is critical and will have important positive consequences for animal welfare, since thousands of animals will be spared *in vivo* tests.

Final considerations

Reproductive toxicology tests are extremely important because animals, including humans, are exposed daily to a large number of chemicals that may have toxic effects on reproductive function. In the case of the female, these effects may cause premature ovarian failure, infertility or even become hereditary, and may result in serious consequences, such as congenital malformation and deleterious effects on the offspring. However, most toxicity trials involve the use of live animals, which has generated ethical problems in the scientific community. Thus, it is necessary to invest in the improvement of *in vitro* culture techniques, especially ovarian tissue or even follicles isolated from the ovary, that allows to quickly and safely trace the effect of chemical compounds on ovarian function and fertility.

Conflicts of interest

The authors declare no conflicts of interest.

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7 CAPÍTULO 2:

Transportadores ABC (ATP-binding cassette) em folículos pré-antrais de caprinos: expressão de genes e proteínas

″ATP-Binding Cassette (ABC) Transporters in Caprine preantral follicles: Gene and Protein Expression″

Resumo

As proteínas de resistência a múltiplas drogas ABCB1, ABCC2 e ABCG2 são uma bomba de efluxo dependente de energia que funciona em processos de desintoxicação sistêmica. Essas proteínas são fisiologicamente expressas em uma variedade de tecidos, mais abundantemente no fígado e epitélio intestinal, placenta, barreira hematoencefálica e várias células-tronco, até o momento, essas bombas não foram identificadas no tecido ovariano caprino. Portanto, o objetivo deste estudo foi analisar a expressão das proteínas ABCB1, ABCC2 e ABCG2 e expressão protéica em folículos pré-antrais de caprinos. Fragmentos (3 x 3 x 1 mm) de 5 pares de ovários (n = 10) obtidos de 5 cabras foram coletados e imediatamente submetidos a testes de qPCR, Western blot e imunofluorescência para detecção de mRNA e identificação e localização dos transportadores ABC, respectivamente. RNAm para ABCB1, ABCC2 e ABCG2 e a presença de suas proteínas foram observadas em amostras de tecido ovariano. Marcações positivas foram observadas para as três proteínas de transporte em todas as categorias foliculares estudadas. No entanto, as marcas foram localizadas principalmente no oócito das categorias primordial, transição e folículos primários. Em conclusão, o tecido ovariano de cabra expressa RNAm para os transportadores ABCB1, ABCC2 e ABCG2 e a expressão destas proteínas nos folículos pré-antrais é estágio dependente de folículos.

Palavras-chave: ABCB1, ABCC2, ABCG2, folículos pré-antrais, resistência múltiplas drogas

ATP-Binding Cassette (ABC) Transporters in Caprine preantral follicles: Gene and Protein Expression

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ABSTRACT

The multidrug resistance proteins ABCB1, ABCC2 and ABCG2 are an energy-dependent efflux pump that functions in systemic detoxification processes. Physiologically expressed in a variety of tissues, most abundantly in the liver and intestinal epithelia, placenta, blood-brain barrier, and various stem cells, until now, these pumps were not identified in goat ovarian tissue. Therefore, the aim of this study was to analyze ABCB1, ABCC2 and ABCG2 mRNA and protein expression in goat preantral follicles. Fragments (3 x 3 x 1 mm) from 5 pairs of ovary (n = 10) obtained from 5 goats were collected and immediately submitted to qPCR, Western blot and immunofluorescence assay for mRNA detection and identification and localization of the ABC transporters, respectively. mRNA for *ABCB1*, *ABCC2* and *ABCG2* and the presence of their proteins were observed on ovarian tissue samples. Positive marks were observed for the three transport proteins in all follicular categories studied. However, the marks were primarily localized in the oocyte of primordial, transition and primary follicles categories. In conclusion, goat ovarian tissue expresses mRNA for the ABCB1, ABCC2 and ABCG2 transporters and the expression of these proteins in the preantral follicles is a follicle-dependent stage.

Keywords: ABCB1, ABCC2, ABCG2, preantral follicles, multidrug resistance

INTRODUCTION

The superfamily of active transporters ATP-binding cassette (ABC) is one of the largest protein families in biological systems (Dassa and Bouige, 2001). These ABC transporters are composed by 50 evolutionarily conserved functionally transmembrane proteins and are present both in prokaryotes and eukaryotes (Sharom, 2008). The main function of these proteins is the transport of molecules such as sugars, amino acids, nucleotides, peptides, lipids, steroids, bile salts, toxins and chemotherapeutic drugs through biological membranes using the energy resulting from the hydrolysis of ATP (Gottesman et al., 2002). In this way, they are of great importance for the cellular protection system, through the efflux of toxic xenobiotics, endogenous metabolites, amphipathic compounds, including various drugs used clinically. Therefore, they can profoundly affect drug therapy leading to the multidrug resistance (MDR) by several chemotherapeutic drugs (Sharom, 2008).

Among the several ABC proteins, only three are known to have a multi drug resistance effect; ABCB1 (P-glycoprotein or Multi-Drug Resistance Gene - MDR1), ABCC2 (Multidrug Resistance-Associated Protein 2 - MRP2) and ABCG2 (Breast Cancer Resistance Protein - BCRP). An overexpression of these proteins in tumor cells is correlated with the phenomenon of multidrug resistance, a phenomenon that makes these cells insensitive or low responders to a broad spectrum of drugs (Deeley et al., 2006; Higgins, 2007). Thus, these proteins play an important role in the maintenance of selective permeability in several tissues, such as the blood-brain barrier, spinal-blood-brain barrier, blood-testis barrier and maternal-fetal barrier or placental barrier (Leslie et al., 2004).

In general, these transporters are expressed in various organs such as, liver, kidneys, testis, placenta and uterus of pregnant women (Leslie et al., 2005). As regards to the ovarian structure, ABCB1 has already been identified in preantral and antral follicles (rats: Lee et al., 1998), as well as in oocytes (mice: Elbling et al., 1993; swine: Yokota et al., 2006) and granulosa cells (swine: Fukuda et al., 2006). In addition, some authors have also identified this protein in mouse (Elbling et al., 1993), swine (Schoevers et al., 2016) and bovine (Mori et al., 2013) embryos. According to Bloise et al. (2015), ABCB1 is highly responsive to progesterone and luteinizing hormone (LH) and according to these authors an imbalance of this protein in granulosa cells has the potential to impair the folliculogenesis and steroidogenesis.

As regards to ABCG2, the expression of this protein has been reported in mouse ovarian tissue (Dankers et al., 2012), as well as in murine (Sawicki et al., 2006) and bovine embryos (Mori et al., 2013). At this time, the presence of ABCC2 in ovarian follicles has not been

reported, but has already been identified in placental tissues (apical region of trophoblast), suggesting an important role in the efflux of substances from the maternal compartment (St-Pierre et al., 2000, Azzaroli et al., 2007).

Although little information is available about these proteins in the reproductive system, studies in human indicate that ABC transporters play a role during gametogenesis, as well as embryonic and fetal development. In general, some studies have shown that ABC transporters may be involved in efflux of steroids, which affect ovarian follicular development (Ueda et al., 1992; Van Kalken 1993, Wang et al., 2006). However, the understanding of the expression pattern of ABC transporters in ovaries of domestic animal, such as goats, has not yet been reported in the literature. Our laboratory has devoted many resources and efforts to demonstrate that goat ovary can be used as a model for the elucidation of preantral folliculogenesis in vitro in humans. Therefore, in molecular point of view, the presence of these proteins in the caprine preantral follicles would be a strong indicator that the ovary of the goat can be an excellent model for the human gonadal function. We hypothesized that the gene expression and protein distribution ABCB1, ABCC2, and ABCG2 transporters would differ with stage of development in caprine preantral follicles. Therefore, the objective of the present study was to analyze the gene expression and protein distribution of the ABCB1, ABCC2 and ABCG2 transporters in the preantral follicles of goats.

MATERIAL AND METHODS

Source of ovaries and experimental protocol

This study was approved and performed under the guidelines of Ethics Committee for Animal Use of the State University of Ceará nº 6475219/2015. Unless mentioned otherwise, all the chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA) and antibodies were purchased from ABCAM (Inc. Abcam, Cambridge, MA, EUA), except actin antibody which was purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

Ovaries were obtained from four adult cross-bred goats (*Capra hircus*) at a local slaughterhouse. Immediately postmortem, ovaries were washed in 70% alcohol followed by two rinses in minimum essential medium (MEM) supplemented with 100 µg/mL penicillin and 100 µg/mL streptomycin plus 25 mM HEPES. Ovaries were transported within 1 h to the laboratory into tubes containing 15 mL of MEM-HEPES at 4 °C (Chaves et al., 2008).

In the laboratory, three ovarian cortex fragments (3 x 3 x 1 mm) of each ovarian pair (n = 4) were obtained with a sterile scalpel. The fragments were randomly divided to quantitative real-time polymerase chain reaction (qPCR), western blot and immunohistochemistry.

RNA extraction and real-time PCR (qPCR)

One fragment of each animal (n = 4) were stored in micro centrifuge tubes (1.5 mL) containing 100 μL of trizol in ice and stored at -80 °C until RNA extraction. Total RNA was isolated with Trizol® Plus RNA Purification Kit (Invitrogen, São Paulo, SP, Brazil). The isolated RNA preparations were treated with DNase I and Pure Link™ RNA Mini Kit (Invitrogen, São Paulo, SP, Brazil). Complementary DNA (cDNA) was synthesized from the isolated RNA using SuperscriptTM II RNase H-Reverse Transcriptase (Invitrogen, São Paulo, Brazil). The qPCR reactions had a final volume of 20 μL and contained the following components: 1 μL cDNA as a template in 7.5 μL of SYBR Green Master Mix (PE Applied Biosystems, Foster City, CA, USA), 5.5 μL of ultra-pure water and 0.5 μL of each primer. The primers were designed to perform the amplification of ABCB1, ABCC2 and ABCG2 mRNA levels (Table 1). Two candidate reference genes, glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and peptidylprolyl isomerase A (PPIA), were selected as endogenous controls for normalization and to study the expression and stability of gene expression in all samples. Primer specificity and amplification efficiency were verified for each gene. The expression stability of these genes was analyzed using BestKeeper software. BestKeeper highlighted PPIA as the reference gene with the least overall variation. The thermal cycling profile for the first round of RT-PCR was as follows: initial denaturation and activation of the polymerase for 15 min at 94 °C, followed by 40 cycles of 15 s at 94 °C, 30 s at 60 °C and 45 s at 72 °C. The final extension was for 10 min at 72 °C. All reactions were performed in a real-time PCR Mastercycler (Eppendorf, Germany, Hamburg, Germany, USA). The delta-delta-CT method (Livak and Schmittgen, 2001) was used to transform threshold cycle values (Ct) into normalized relative expression levels of mRNA (Silva et al., 2011).

Table 1. Oligonucleotide primers used for polymerase chain reaction analysis.

Target gene	Primer sequence (5'→3')	Sense Anti-sense[a]	Genbank accession nos.
ABCB1	CAGCTGTTGTCTTTGGTGCC	S	GI: 926730158
	TCTGGTCGAGTCGGGTAGTT	AS	(*Capra hircus*)
ABCC2	GCTGCGGTGGATCTAGAGAC	S	GI: 926724898
	AGCAGTTCTTCAGGGCTGTC	AS	(*Capra hircus*)
ABCG2	CGGCATTCCAGAGACAACCT	S	GI: 114384163
	GGCCAGGTTTCATGACTCCA	AS	(*Capra hircus*)
GAPDH	ATGCCTCCTGCACCACCA	S	GI: 443005
	AGTCCCTCCCACGATGCCA	AS	(*Ovis aries*)
PPIA	TCATTTGCACTGCCAAGACTG	S	GI:548463626
	TCATGCCCTCTTTCACTTTGC	AS	(*Capra hircus*)

[a]S, sense; AS, anti-sense.

Western blot analysis

Ovarian fragments (n = 4) were lysed in buffer [Tris-HCl 0,5 M, EDTA 20 mM, NaCl 10 mM, 0.1% sodium dodecyl sulfate (SDS) and Protease Inhibitor Cocktail 10x concentrated], and the cell lysates were centrifuged at $10,000 \times g$ at 4 °C for 10 min to extract the proteins. Protein concentration was determined using the Qubit 2.0 (Thermo Fisher Scientific, Waltham, MA, USA). The ovarian proteins (12 µg) were separated according to their molecular weight by SDS-PAGE in 12 % gradient polyacrylamide gels. Proteins were transferred from the gels to PVDF membranes (Hybond-P, GE Healthcare Life Sciences, Pittsburgh, PA, USA) using a semi-dry transfer unit (TE 70, GE Healthcare Life Sciences, USA) and allowed to air-dry. Membranes were blocked after which the membrane was blocked with blocking buffer (5 % nonfat dry milk in Tris-buffered saline) for 1 h at room temperature, with mild agitation, followed by incubation with respective antibodies (Table 2). Membranes were then washed three times in TBS-T and incubated with horseradish peroxidase-linked anti-mouse and anti-rabbit antibody (NA931; NA934, GE Healthcare, Helsinki, Finland) for another 2 h, washed again three times in TBS-T, and rinsed twice with Tris–HCl (50 mM; pH 7.4). Immunoreaction was visualized by exposing the membranes to a solution containing BCIP® (5-bromo-4-chloro-3-indolyl phosphate 0.15 mg/mL), NBT (nitroblue tetrazolium 0.30 mg/mL), Tris (100 mM) and MgCl2 (5 mM), pH 9.5. The reaction was stopped by washing the membranes several times

with ultrapure water. The membranes not exposed to the primary antibody were used as negative controls (data not shown).

Table 2. Antibodies used for western blot and immunofluorescence.

Antibody	Concentration		Reference
	WB	IF	
Mouse monoclonal to P Glycoprotein – ABCB1	1:1000	1:300	ab 3083
Rabbit monoclonal [EPR10998] to MRP2-ABCC2	1:1000	1:250	ab 172630
Mouse monoclonal [1H2] to BCRP/ABCG2	1:1000	1:300	ab 130244
Goat polyclonal Secondary Antibody to Mouse IgG - H&L (Alexa Fluor® 488)	-	1:200	ab 150113
Donkey polyclonal Secondary Antibody to Rabbit IgG - H&L (Alexa Fluor® 488)	-	1:200	ab 150073
Actin Antibody (H-196) is a rabbit polyclonal IgG	1:2500	-	sc-7210
Actin Antibody (C-2) is a mouse monoclonal IgG$_1$	1:2500	-	sc-8432

Immunofluorescence detection of ABC transporters

For this analysis, the ovarian fragments were fixed with 4 % (w/v) paraformaldehyde in phosphate-buffered saline (PBS; pH 7.2) and subsequently dehydrated and embedded in paraffin wax. Briefly, tissue sections (5 μm) were mounted on Superfrost Plus slides (KnittelGlass, Bielefeld, Germany), deparaffinized with Citrisolve (Fisher Scientific, Ottawa, Canada) and rehydrated in a graded ethanol series. Antigen retrieval was performed by incubating tissue sections in 0.01 M sodium citrate buffer (pH 6) for 5 min in a pressure cooker. After cooling until 37 ºC, tissue sections were washed in PBS and blocked for 1 h at room temperature using PBS containing 1 % (w/v) BSA. Follow antigen retrieval, slides were incubated overnight at 4 °C with primary antibodies (ABCB1, ABCC2 and ABCG2; Table 2). Then, slides were incubated with the secondary antibody Alexa Fluor® 488 for 1 h at room temperature, stained with Evans blue (1: 10.000) and mounted with Vectashield Mounting Medium® (Vector Laboratories, Inc., Burlingame, CA, USA). Immunostaining of each protein was evaluated in approximately 200 follicles. For treatment and positive staining classified in weak, moderate and strong (Figure. 1) using a confocal laser scanning microscope (LSM 710, Zeiss, Oberkochen, Germany). All the analyses were performed with the software ZEN 2008.

Alexa Fluor® 488 with an excitation and emission wave length of 495-551 nm was used. The negative control was obtained by omitting the primary antibodies.

Statistical analysis

All statistical analyses were performed using Sigma Plot 11 (Systat Software Inc., USA). The immunofluorescence staining of ABC transporters among follicular classes and follicular compartments were analyzed by Fisher's exact test. A linear regression analysis was performed to evaluate the association between staining intensity and follicular classes. Data are presented as mean ($\pm$ SEM) and percentage and the results were considered different when $P < 0.05$.

RESULTS

mRNA and protein expression of ABCB1, ABCC2 and ABCG2

The mRNA and protein expression data for the ABCB1, ABCC2 and ABCG2 transporters in goat ovarian tissue are shown in figure 1. All of eight ovaries evaluated presented gene (Fig. 1A) and protein (Fig. 1B) expression for the three ABC transporters investigated. mRNA levels were higher for ABCB1 and ABCC2 ($P < 0.05$) compared to ABCG2. The western blot analysis revealed bands of 141, 150 and 140 kDa, which are compatible with the molecular weight of the ABCB1, ABCC2 and ABCG2 proteins, respectively (Fig. 1B).
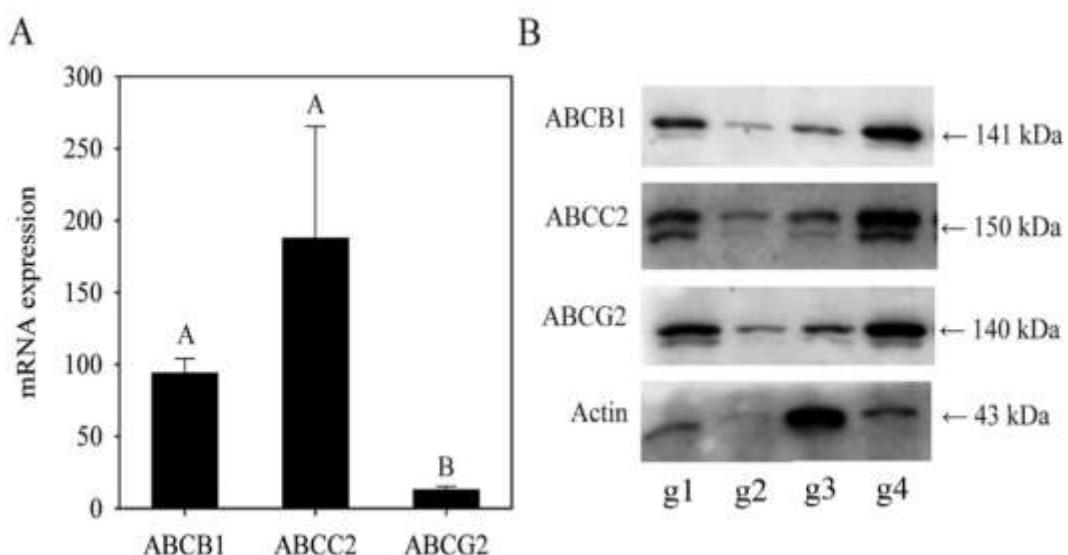
Figure 1. Gene expression (A) and immunodetection (B) of ABC transporters in goat ovarian tissue. The expression of mRNA (mean ± SD) was normalized to GAPDH and PPIA. [A,B] differ significantly among the ABC transporters gene expression (P < 0.05). The g1–g4 on Fig. 1B refer to samples from four different goats, using actin as Western blot reference.

Immunofluorescence detection of ABCB1, ABCC2 and ABCG2

A total of 150 ovarian follicles were evaluated for the immunolocalization of each protein within the different follicular categories (primordial, transition, primary and secondary) and structures (oocyte, granulosa and oocyte/granulosa cells) (Fig 2). Figure 2 shows the immunostaining of ABC transporters (ABCB1, ABCC2 and ABCG2) in different follicular categories. Positive staining was observed for all three proteins in all follicular categories, mainly in the oocytes of the primordial, transitional and primary follicles. In each follicle category, the staining was classified as moderate to strong for all the proteins studied (Figures 2A-C, F-H and L-N). In the secondary follicles, the labeling was strong on granulosa cells and oocytes for ABCB1 and ABCC2 (Fig. 2 D and I), and was considered weak for ABCG2 (Fig. 2O).
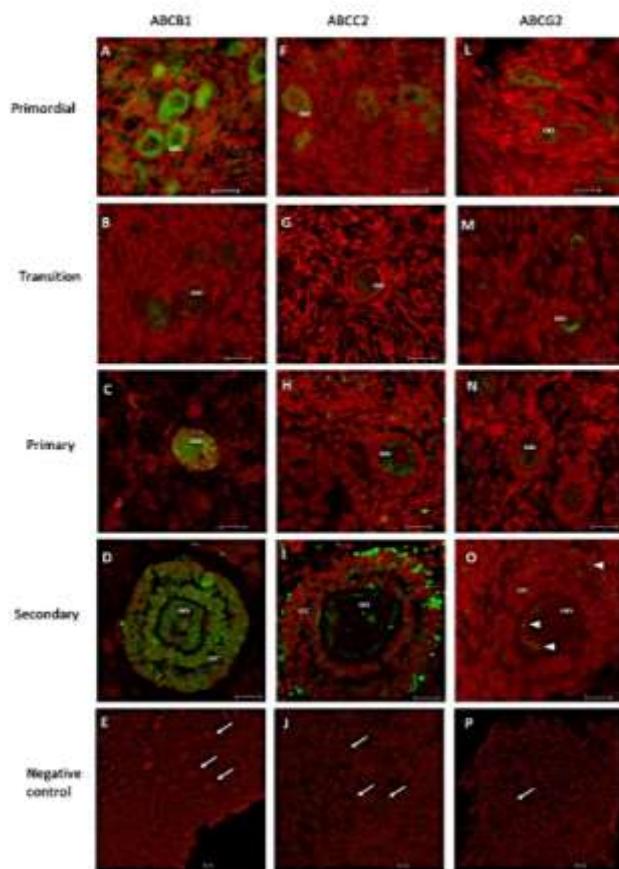
Figure 2. Immunodetection of ABC transporters (green bright fluorescent spots) in goat ovarian tissue. ABCB1 (A-D), ABCC2 (F-I) and ABCG2 (L-O) protein immununodetection in primordial, transition, primary and secondary follicle. Negative control (E, J and P). Ovarian tissues were counterstained with Evans blue (red contrast). The white arrowheads in O indicate the green bright fluorescent spots in secondary follicle and the arrow in E, J and P indicates ovarian follicles. GC – granulosa cells, OO – oocyte. Scale bars = 20 μm.

Regarding to the percentage of positive-stained follicles for ABCB1, no significant difference was observed among the different categories of follicles (figure 3A). However, considering the follicular structures, separately, this protein was more frequently found in oocytes of primordial follicles (p <0.05) than oocytes of developing follicles (transition, primary and secondary) as shown in Fig. 3B. On the other hand, the percentage of developing follicles with concomitant staining in the oocyte and granulosa cells was higher than that of primordial follicles. Except for transitional follicles, the preantral follicles did not show immunostaining for ABCB1, only in granulosa cells.

The correlation analysis (Fig. 3C) showed that there was no association between the intensity of staining for ABCB1 and the follicular category (r = 0.06; P = 0.48).
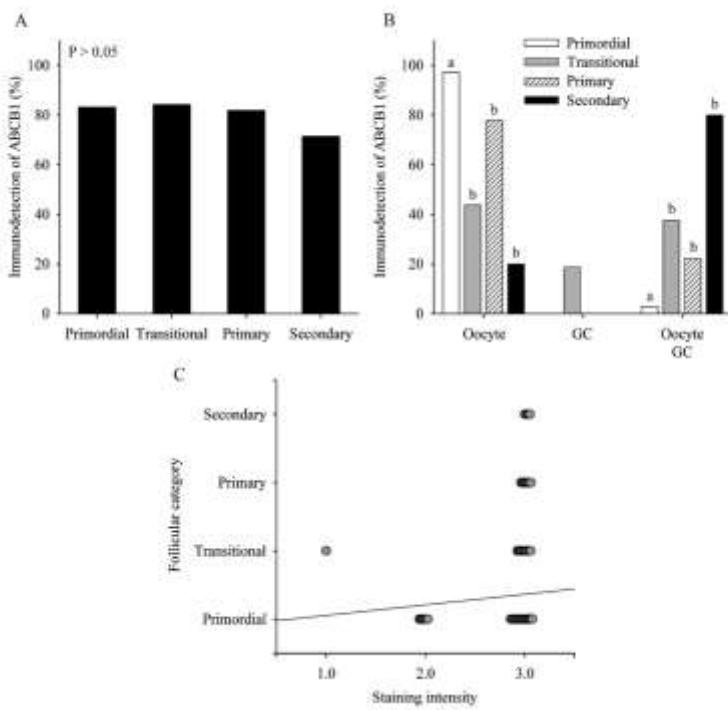
Figure 3. Percentage of positive stained follicles for ABCB1 in the different follicle categories (A) e follicular compartments (B). [a,b] indicate differences among follicle categories within the same follicular compartment. (p<0.05). GC: granulosa cells. Relationship between staining intensity to ABCB1 and follicular category (C). Each circle of the graph is a follicle recorded (n = 163). The staining intensity were defined by the values (weak = 1; moderate = 2; strong = 3) to dependent variable, while the follicular categories were determined as primordial = 1; transitional = 2; primary = 3; and secondary = 4. A linear regression is represented by the equation and the black line [staining intensity = 2.962 - (0.101 × follicular class); r = 0.06; P > 0.05].

With regard to ABCC2, it was observed that the percentage of positive stained primary follicles was lower than the percentage of primordial and transitional follicles (figure 4A). In these three preantral follicle categories, the immunostaining was observed only in the oocyte. In the primary follicles, immunolocalization was predominantly observed in the oocyte, though, in some follicles it was also detected a concomitant staining for ABCC2 in the oocyte and granulosa cells. On the other hand, in secondary follicles, the presence of this protein was detected in all compartments (oocyte, granulosa and theca cells). However, in this follicular

category, the immunostaining in the oocyte was significantly lower when compared to the other categories (figure 4B).

Interestingly, a negative correlation was observed between the follicular development category and the immunostaining (figure 4C), showing that the intensity of staining for ABCC2 reduces with follicular development (r = -0.15; P < 0.05).
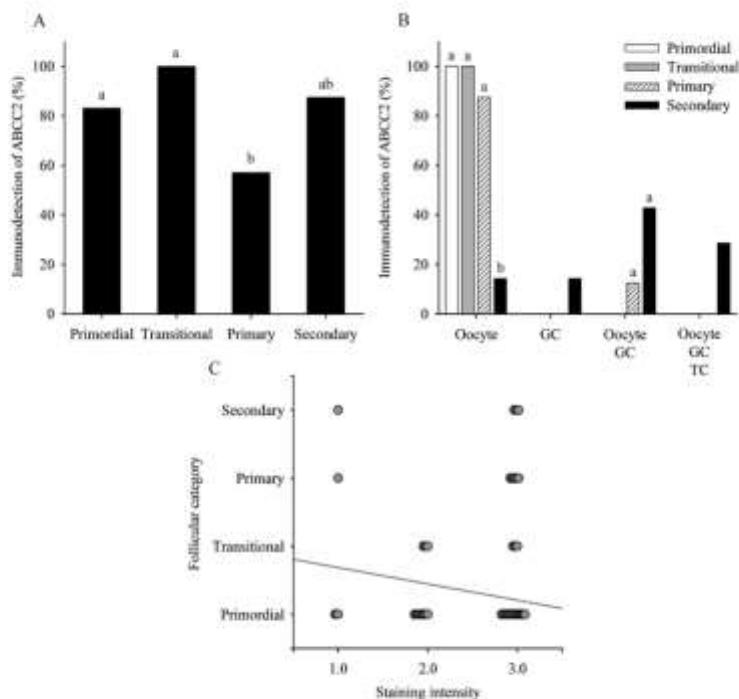
Figure 4 Percentage of positive stained follicles for ABCC2 in the different follicle categories (A) e follicular compartiments (B). [a,b] indicate differences among follicle categories within the same follicular compartment.(p < 0.05). Relationship between staining intensity to ABCC2 and follicular category (C). Each circle of the graph is a follicle recorded (n = 159). The staining intensity were defined by the values (weak = 1; moderate = 2; strong = 3) to dependent variable, while the follicular categories were determined as primordial = 1; transitional = 2; primary = 3; and secondary = 4. A linear regression is represented by the equation and the black line [staining intensity = 2.962 – (0.101 × follicular class); $R^2 = 0.02$; r = –0.15; P < 0.05].

Regarding ABCG2, this study revealed that there was no significant difference in the immunostaining of this protein among the different follicular categories studied (Fig. 5A). With regard to follicular structures, it was observed that the percentage of primordial and primary follicles with oocyte staining was higher than that of transitional follicles (P < 0.05). The

percentage of transitional and secondary follicles showing staining only in granulosa cells was higher than that observed for primordial and primary follicles (figure 5B). The transitional follicle was the only follicular category that showed immunostaining simultaneously in the oocyte and granulosa cells for this protein.

As reported for ABCC2 immunostaining, a negative correlation between follicular category and staining intensity was observed (Fig. 5C) meaning that in the more advanced stages the protein immunostaining for ABCG2 is lower than the initial categories.
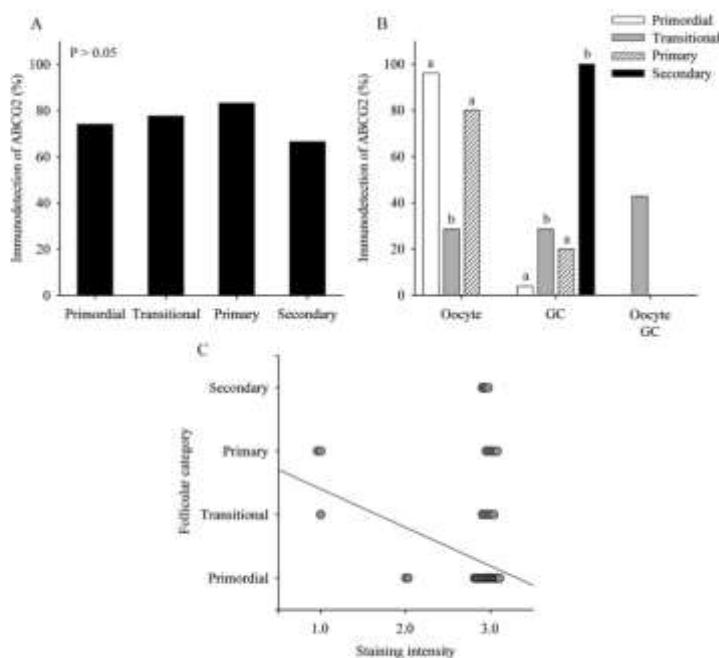
Figure 5. Percentage of follicles staining for ABCG2 in different categories (A) and follicular compartments (B). [a,b] difference within the same follicular compartments (P < 0.05). Relationship between staining intensity to ABCG2 and follicular categories (C). Each circle of the graph is a follicle recorded (n = 181). The staining intensity were defined by the values (weak = 1; moderate = 2; strong = 3) to dependent variable, while the follicular categories were determined as primordial = 1; transitional = 2; primary = 3; and secondary = 4. A linear regression is represented by the equation and the black line [staining intensity = 3.091 – (0.111 × follicular class); R^2 = 0.06; r = –0.26; P < 0.001].

Discussion

The ATP-dependent transport proteins investigated in the present study (ABCB1, ABCC2 and ABCG2) are known mainly for conferring chemoresistance to tumor cells (Sharom, 2008), although they are also present in healthy cell membranes, (Ruiz et al., 2013). ABC transporters play an important role in the detoxification of various tissues, i.e. they are responsible for modulating the transport of different substrates from the cytosol to the extracellular compartment (George and Jones, 2012; Tarling et al., 2013), avoiding the accumulation of endogenous substrates and metabolites in the intracellular compartment (Bellamy, 1996). Many of these proteins are related directly or indirectly to major reproductive processes (Bloise et al., 2016), through the efflux of androgens (testosterone, dihydrotestosterone, dehydroepiandrosterone sulfate: Klein et al., 2014); estrogens (17-estradiol, estrone, estriol: Wang et al., 2006); glucocorticoids (cortisol, dexamethasone: Pavek et al., 2007) and progestogens (pregnenolone, 17 hydroxyprogesterone: Wang et al., 2006). However, to our knowledge there are no investigations regarding to the role of these proteins in ovarian follicular development, especially early preantral follicles. Therefore, this is a pioneering work regarding the gene and protein expression of these ABC transporters in the goat preantral follicles.

Our data revealed gene and protein expression for ABCB1, ABCC2 and ABCG2 in the preantral follicles present in ovarian goat tissue. Previous studies have also revealed the presence of ABCB1 in rat (Lee et al., 1998), mice (Elbling et al., 1993), cows (Mori et al., 2013) and sows (Fukuda et al., 2006, Yokota et al., 2011) antral follicles. In swine, the exposure of granulosa cells from antral follicles to gonadotropins and steroid hormones increased mRNA levels for ABCB1 (Fukuda et al., 2006; Yokota et al., 2011). ABCG2 has also been identified in the ovary (Dankers et al., 2012) and mouse embryos (Sawicki et al., 2006) and bovine embryos (Mori et al., 2013). However, at the present moment there are no reports of the presence of ABCC2 in ovarian follicles, being detected the expression of this protein only in the placenta and fetal membranes of humans as demonstrated by Yoshino et al. (2011). These authors demonstrated an increase in mRNA levels for these proteins in cells of the amnion and chorion after arsenic exposure, suggesting that ABCC2 may be involved in controlling the accumulation of this compound in cells. Our results showed high levels of ABCB1 and ABCC2 in the ovary, suggesting that this protein may be important for the development and follicular homeostasis through the transport of steroid hormones, prostaglandins and glutathione (Liu et

al., 2006; Bakos and Homolya, 2007). A study realized by Koenig et al. (2003) showed that glutathione (GSH) is one of the main substrates of ABCC2. Previously, Johnson et al. (2002) suggested that the role of this protein is related to cellular defense against potentially harmful electrophiles, preventing cell damage due to free radicals and alkylating agents, since GSH can be conjugated to electrophilic xenobiotics through glutathione S-transferases, generating chemical soluble products less harmful.

Regarding protein expression at the different follicular stages, it was found that both ABCB1 and ABCG2 expression are maintained throughout preantral folliculogenesis. These proteins are likely necessary for the transport of substances required throughout the progress of the initial folliculogenesis. Although the immunostaining for ABCC2 in the primary follicles was lower than that observed in the previous stages (primordial and transition), it was similar to that observed in the secondary follicles. The evaluation of ABCs transporter expression along folliculogenesis, especially of ABCC2, is an unprecedented finding in the literature, and further studies are needed to understand the differences found. According to our findings and previous studies in ovaries, it is believed that these proteins are involved in the transport of steroid hormones, ions and other substances necessary for ovarian follicular development. In addition, they may also be linked to follicular detoxification, which according to Brayboy et al. (2013) the ABC transporters promote an increase in follicular resistance when exposed to toxic substances such as metabolites, chemicals and chemotherapeutics.

We found that in the primordial follicles, Independently of the protein evaluated, the ABCs transporters were predominantly expressed in oocytes. In the more advanced stages, the ABCs transporters were found in both oocytes and granulosa cells. These findings may be related to the fact that more developed follicles have a greater number of granulosa cells and consequently more organelles such as the endoplasmic reticulum, which is responsible for protein synthesis (Lucci et al 2001). Trezise et al. (1992) identified for the first time the expression of the ABCB1 protein in granulosa cells of rat antral follicles. Increased expression in granulosa cells in the developing stages may be related to increased steroid production by granulosa and theca cells during follicular development. It is natural that with the advancement of follicular development these proteins are found in granulosa cells because evidences suggest that these proteins are involved with the process of luteinization. In addition, deregulation in ABCB1 is able to prevent ovarian folliculogenesis and steroidogenesis (Bloise et al., 2016). ABCB1 itself has been reported as a carrier of steroids and their metabolites on the cell

membrane in human colon carcinoma cell lines SW-620 (Barnes et al., 1996; Edelmann et al., 1999).

In conclusion, goat ovarian tissue expresses mRNA for the ABCB1, ABCC2 and ABCG2 transporters and the expression of these proteins in the preantral follicles is dependent upon stage of follicular development. Although the ABC transporters are distributed in different follicular compartments, they are found predominantly in oocytes of primordial follicles. The results of the present study support the hypothesis that these transporters may play a crucial role in the development of ovarian follicles, although its biological activity and regulation still remain largely unexplored. A better understanding of the role of ABC transporters in the ovary may help in elucidating the regulation of key reproductive events such as preantral folliculogenesis and steroidogenesis. Full understanding of these proteins in the ovarian follicles may further improve and minimize the adverse effects of toxic substances such as chemotherapeutics used in the treatment of neoplasms and other disorders.

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8 CAPÍTULO 3:

Estudo *in vitro* da toxicidade do Vitanolido D em folículos pré-antrais de cabra e seus efeitos no ciclo celular

"In vitro study of Withanolide D toxicity on goat preantral follicles and its effects on the cell cycle*"*

Resumo

O objetivo deste estudo foi avaliar a toxicidade de um novo candidato a fármaco antineoplásico, o Vitanolido D (WD), na integridade morfológica, desenvolvimento (ativação e proliferação de células da granulosa) e expressão gênica da proteína ABCB1 de folículos pré-antrais caprinos. Os fragmentos ovarianos foram cultivados *in vitro* por 2 ou 6 dias em α-MEM ou α-MEM adicionado com paclitaxel (PTX - 0,1 µg / mL; controle negativo) e diferentes concentrações de WD (WD1.5, WD3.0 ou WD6.0). WD6.0 mostrou um efeito tóxico semelhante ao PTX e maior (P <0,05) do que outros tratamentos após 2 e 6 dias. Além disso, o WD6.0 reduziu a proliferação de células em comparação com o PTX ou o WD3.0. A expressão de ABCB1 permaneceu inalterada na presença dos agentes quimioterápicos (PTX e WD) durante todo o período de cultura. Em conclusão, o WD exerceu um efeito tóxico sobre os folículos caprinos pré-antrais semelhantes ao PTX, cujo efeito negativo sobre a foliculogênese já é amplamente conhecido.

Palavras-chave: folículos pré-antrais, falha ovariana, quimioterapia, câncer, vitanolido D, cabras, ABCB1

In vitro study of Withanolide D toxicity on goat preantral follicles and its effects on the cell cycle

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Abstract

The aim of this study was to evaluate the toxicity of a new candidate to anticancer drug, Withanolide D (WD) on morphologic integrity, development (activation and granulosa cell proliferation) and gene expression of ABCB1 protein of caprine preantral follicles. Ovarian fragments were cultured in vitro for 2 or 6 days in α-MEM or α-MEM added with paclitaxel (PTX - 0.1 µg/mL; negative control) and different concentrations of WD (WD1.5, WD3.0 or WD6.0). WD6.0 showed a toxic effect similar to PTX and higher ($P < 0.05$) then other treatments after 2 and 6 days. In addition, WD6.0 reduced the cell proliferating compared to PTX or WD3.0. The expression of ABCB1 remained unchanged in the presence of the chemotherapeutic agents (PTX and WD) throughout the culture period. In conclusion, WD exerted a toxic effect on preantral caprine follicles similar to PTX, whose negative effect on folliculogenesis is already widely known.

Keywords: Preantral follicles, ovarian failure, cancer chemotherapy, withanolide D; goat, ABCB1

1. Introduction

The considerable increase in the number of new cases of cancer, parallel to the increase in the number of survivors has been the focus of several studies worldwide which aim to develop new therapies with minimal side effects, ensuring the quality of life of patients undergoing treatment [1]. Among these side effects, we can highlight female infertility or premature ovarian failure, since most drugs used during treatment can cause damage to the ovarian reserve of preantral follicles [2]. In this way, many efforts have been made to find new drugs that have fewer side effects, especially on the reproductive function.

Currently, many plants have been investigated as a source of new compounds, as they are excellent raw material sources of substances with cytotoxic potential. Paclitaxel/taxol (PTX), for example, is a chemotherapeutic agent from the *Taxus brevifolia* plant [3] and has a potent cytotoxic effect on several tumor cell lines [4,5]. Although it is widely used for the treatment of several kinds of cancer, such as breast cancer [6], PTX is extremely gonadotoxic [7], being directly associated with cell division blockade [6], and has been associated with ovary failure as a consequence of the reduction of the population of the primordial follicles [[6], [8], [9]]. In addition, this chemotherapeutic agent is related to increased expression of the drug efflux pump (ABCB1 / P-glycoprotein / MDR1-P-gp) which is an ATP-dependent membrane protein [10]. This protein in the form of a channel allows expel of PTX out of the cell, reducing the intracellular concentrations of this drug [11,12]. As a consequence, the phenomenon of multidrug resistance has been developed. However, in order to minimize or negate these drawbacks, new chemical entities have been extensively investigated.

Withanolide D (WD) is a steroidal lactone isolated from leaves of *Acnistus arborescens* (Solanaceae family), a medicinal plant indicated to the treatment of cancer and diseases related to liver and spleen, widespread in the Central and South America [13,14]). Batista et al. [15] demonstrated the chemotherapeutic potential of WD against the human tumor cell lines: leukemia (HL-60), colon adenocarcinoma (HCT-116), glioblastoma (SF-268) and pancreas carcinoma (PANC-1). Despite the relevant results related to the antiproliferative properties of WD on different human cancer cell lines, there no report on the effect of this compound on ovarian function or preantral folliculogenesis. Therefore, the goal of this study was to evaluate the toxic effects on the goat ovary exposure to WD during in vitro culture on: 1) follicular morphology, 2) proliferative capacity of the granulosa cells of preantral follicles, 3) gene expression of positive factors involved in cell proliferation and 4) expression of the ABCB1 protein.

2. Material and methods

2.1 Source of ovaries

Ovaries were collected from six adult cross-bred goats (*Capra hircus*) at a local abattoir. Immediately postmortem, ovaries were washed once in 70% (v/v) ethanol and then washed twice in HEPES-buffered minimum essential medium (MEM) supplemented with 100 µg/mL penicillin and 100 µg/mL streptomycin. The ovaries were then transported to the laboratory in MEM at 4 ºC within 1 h.

2.2 Culture medium

Unless mentioned otherwise, the culture media and other chemicals used in the present study were purchased from Sigma Chemical Co. (St Louis, USA). The basic *in situ* culture medium (control culture) consisted of α-MEM (pH 7.2 - 7.4) supplemented with 1.25 mg/mL bovine serum albumin (BSA), insulin 10 µg/mL, transferrin 5.5 µg/mL, and selenium 5 ng/mL, 2 mM glutamine and 2 mM hypoxanthine, which was called α-MEM$^+$.

2.3 Experimental protocol

The ovarian cortex of each pair (n = 6) was removed with a sterile scalpel and divided into 13 fragments (3 x 3 x 1 mm) one of which was randomly taken and immediately fixed as described below and designed as Control. The remaining fragments were individually cultured in a 24-well plate containing 1 mL α-MEM$^+$, α-MEM$^+$ supplemented with PTX 0.1 µg/mL (positive control) or WD at different concentrations (1.5, 3.0, and 6.0 µM), resulting in the follow treatments: MEM, PTX, WD1.5, WD3.0 and WD6.0. The *in vitro* culture was carried out for two or six days at 39 °C and 5% CO_2. Every two days, whole culture medium was replaced and each treatment was replicated six times.

2.4 Analyze of the toxicity of withanolide D

2.4.1. Follicular morphology, activation and in vitro growth

The control and *in vitro* cultured ovarian fragments were fixed in buffered 4 % paraformaldehyde (PAF) in phosphate-buffered saline (PBS) for 4 h at room temperature (RT), dehydrated in a graded series of ethanol, clarified with xylene, embedded in paraffin wax, and serially sectioned into 7 µm thickness. The sections were stained with periodic acid Schiff

(PAS) - hematoxylin. For morphological evaluation, coded anonymized slides were examined on a microscopy (Nikon, Japan) under 400X magnification.

The follicles were classified according to the integrity and developmental stage, defined previously [16] as *primordial* (one layer of flattened granulosa cells around the oocyte) or *growing follicles* (*intermediate*: one layer of flattened to cuboidal granulosa cells; *primary*: one layer of cuboidal granulosa cells; and *secondary*: two or more layers of cuboidal granulosa cells around the oocyte). These follicles were still classified individually as *morphologically normal* or *atretic follicles*. The first one, those follicles with an intact oocyte surrounded by granulosa cells well organized in one or more layers and that had no pyknotic nucleus. *Atretic follicles* were defined as those with a retracted oocyte, pyknotic nucleus, and/or disorganized granulosa cells detached from the basement membrane. All the follicles present on the slide were evaluated. Each follicle was examined in every section in which it appeared and matched with the same follicle on adjacent sections to avoid double counting, thus ensuring that each follicle was only counted once, regardless of its size.

To evaluate follicular activation and growth, only intact follicles with a visible oocyte nucleus were recorded and the proportion of primordial and growing follicles were calculated on day 0 (*Control*) and after two or six days of culture in all tested treatments.

2.4.2 Immunoassays

For analyses of cellular proliferation (Ki67), apoptosis (TUNEL assay) and multidrug resistance protein 1 (P-glycoprotein), were performed three additional repetitions. After, the cultured tissue samples from the control and cultured groups were fixed with 4% PAF in PBS (pH 7.2), subsequently dehydrated and embedded in paraffin wax. Tissue sections (5 mm) mounted on Superfrost Plus slides (Knittel Glass, Bielefeld, NW, Germany) were deparaffinized with Citrisolve (Fisher Scientific, Ottawa, Ontario, Canada) and rehydrated in a graded ethanol series.

2.4.2.1 Granulosa cells proliferation test (KI67)

To determine the effect of Withanolide D on granulosa cells proliferation, we used the antigen Ki67 which is a nuclear protein expressed in all the active phases of the cell cycle (G1, S, G2 and mitosis), however, this protein is not expressed in resting cell phase (G0). The antigen

retrieval was performed by incubating the tissue sections in 0.01 M sodium citrate buffer (pH 6.0) for 5 min, in a pressure cooker. To block endogenous peroxidase, the slides were incubated with 3% H_2O_2 in methanol and then blocked with avidin and biotin. After, non-specific blocking was performed using PBS containing 5 % bovine serum albumin (BSA) and 3 % Triton X for 1 h at RT. Then, the slides were incubated overnight at 4 °C with rabbit polyclonal anti-Ki67 (1:4000 – ab15580, Abcam Inc., Cambridge, MA, USA) followed by incubation with the biotinylated anti-rabbit immunoglobulin G (IgG) secondary antibody (1:500 – ab97049, Abcam Inc., Cambridge, MA, USA). Next, the slides were washed, allowed to react with 3,3'-diaminobenzidine in chromogen solution (DAB) in Imidazole-HCl buffer, pH 7.5, containing H_2O_2 (Dako, Inc., Carpinteria, CA, USA), and finally, the sections were counterstained with haematoxylin. Negative control was performed by omitting the primary antibody. Cell proliferation quantification was evaluated as a percentage of Ki67 granulosa cells relative to all the granulosa cells in each follicle [17].

2.4.2.2 DNA fragmentation assay for the detection of apoptotic cells (TUNEL staining)

The DNA fragmentation was analyzed by TUNEL (terminal deoxynucleotidyl transferase-mediated biotinylated deoxyuridine triphosphates nick end-labeling) assay, using the In Situ Cell Death Detection Kit, POD (Roche Applied Science, Mannheim, BW, Germany), according to the manufacturer's instructions. Antigen retrieval was performed by incubating the tissue sections in 0.01 M sodium citrate buffer (pH 6.0) for 5 min, in a pressure cooker. To block endogenous peroxidase the slides were incubated with 3 % H_2O_2 in methanol and then blocked for 1 h at room temperature (RT) using PBS containing 5 % BSA and 3 % Triton X. After washing, the slides were incubated with the TUNEL reaction mixture (50 µL) for 1 h at 37 ºC. Converter POD was added and the location of the protein expression was demonstrated by incubation with 3 DAB. Finally, the sections were counterstained with hematoxylin. The follicles were considered with fragmented DNA when the oocytes were detected having dark brown stained nuclei (Yucebilgin et al., 2004). As an internal positive control, the sections were treated with 10 U/mL DNase I (InvitrogenTM, Carlsbad, CA) for 15 min at RT, before incubation with the TUNEL reaction mixture to induce the nonspecific breaks in the DNA. The negative control sections omitted the terminal deoxynucleotidyl transferase enzyme.

2.4.2.3 Immunofluorescence detection of multidrug resistance protein 1 (ABCB1)

For this analysis, we used a mouse monoclonal primary antibody for the detection of P-glycoprotein (1:300 ab3083, Inc. Abcam, Cambridge, MA, EUA), an important protein of the cell membrane that pumps many foreign substances out of cells. The secondary antibody was Alexa 488 Fluor® anti-mouse IgG (ab150113, Abcam Inc., Cambridge, MA, USA) diluted 1:500. The negative control was obtained by omitting the primary antibody

Antigen retrieval was performed by incubating tissue sections in 0.01M sodium citrate buffer (pH 6) at 95–100 °C for 5 min in a pressure cooker. After cooling until, sections were washed in PBS and blocked for 1 h at RT using PBS containing 1% (w/v) BSA. Follow antigen retrieval, slides were incubated overnight at 4 °C with primary antibody (P-glycoprotein). Then, slides were incubated with the secondary antibody Alexa Fluor® 488 for 1 h at RT and stained with Evans blue (1: 2000). The slides were mounted with Fluoroshield Mounting Medium with DAPI (ab104139, Abcam Inc., Cambridge, MA, USA). Immunostaining was evaluated using a confocal laser scanning microscope (LSM 710, Zeiss, Oberkochen, Germany). All analyzes were performed using the same configurations.

2.5 RNA extraction and real-time PCR (qPCR) of cyclins (A, B1, D1 and E) and cyclin-dependent kinases (1, 2, 4 and 6)

In order to verify the cell proliferating potential, the mRNA of genes involved in the positive control of the cell cycle were analyzed. For this, two ovarian fragments (3 x 3 x 1 mm) of three animals were stored in microcentrifuge tubes (1.5 mL) containing 100 μL of trizol in ice and stored at −80 °C until RNA extraction. Total RNA was isolated with Trizol® Plus RNA Purification Kit (Invitrogen, São Paulo, SP, Brazil). The isolated RNA preparations were treated with DNase I and Pure Link™ RNA Mini Kit (Invitrogen, São Paulo, SP, Brazil). Complementary DNA (cDNA) was synthesized from the isolated RNA using SuperscriptTM II RNase H-Reverse Transcriptase (Invitrogen, São Paulo, Brazil). The quantitative PCR (qPCR) reactions had a final volume of 20 μL and contained the following components: 1 μL cDNA as a template in 7.5 μL of SYBR Green Master Mix (PE Applied Biosystems, Foster City, CA, USA), 5.5 μL of ultra-pure water and 0.5 μL of each primer. The primers were designed to perform the amplification of cyclins A (CCNA), B1 (CCNB1), D1 (CCND1), E1 (CCNE1) and cyclin-dependent kinase 1 (CDK1), 2 (CDK2), 4 (CDK4), 6 (CDK6) mRNA levels (Table 1). Two candidate reference genes, glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and peptidylprolyl Isomerase A (PPIA), were selected as endogenous controls to study the expression, stability and for normalization of gene expression in all samples. Primer

specificity and amplification efficiency were verified for each gene. The expression stability of these genes was analyzed using BestKeeper software. BestKeeper highlighted PPIA as the reference gene with the least overall variation. The thermal cycling profile for the first round of RT-PCR was as follows: initial denaturation and activation of the polymerase for 15 min at 94 °C, followed by 40 cycles of 15 s at 94 °C, 30 s at 60 °C and 45 s at 72 °C. The final extension was for 10 min at 72 °C. All reactions were performed in a real-time PCR Mastercycler (Eppendorf, Germany, Hamburg, Germany, USA). The delta-delta-CT method (Livak and Schmittgen, 2001) was used to transform threshold cycle values (Ct) into normalized relative expression levels of mRNA [18].

Table 1. Primer pairs used for real-time reverse-transcriptase PCR analysis.

Target gene	Primer sequence (5'→3')	Sense Anti-sense[a]	Genbank accession nos.
GAPDH	ATGCCTCCTGCACCACCA AGTCCCTCCACGATGCCAA	S AS	GI: 327679 (Bos taurus)
CCNA	TGGACCTTCACCAGACCTACCT GTGGGTTGAGGAGAGAAACAC	S AS	GI: 281667 (Bos taurus)
CCNB1	AGCGGATCCAAACCTTTGTAGTG CAATGAGGATGGCTCTCATGTTT C	S AS	GI: 327679 (Bos taurus)
CCND1	GGTCCTGGTGAACAAACTC TTGCGGATGATCTGCTT	S AS	GI: 100144763 (Ovis aries)
CCNE1	GGGACAAGCACCTTATGCAAC GTGTTGCCATATACCGATCAAAG A	S AS	GI: 533526 (Bos taurus)
CDK1	CCAATAATGAAGTGTGGCCAGA AG AGAAATTCGTTTGGCAGGATCAT AG	S AS	GI: 281061 (Bos taurus)
CDK2	CTGCACCGAGACCTTAAACCTCA GCTCGGTACCACAGAGTCACCA	S AS	GI: 519217 (Bos taurus)
CDK4	TGAGCATCCCAATGTTGT CCTTGTCCAGATACGTCCT	S AS	GI: 100144756 (Ovis aries)

| CDK6 | AGAGTGATTGCAGCTTTATGTCCA TGCCCAGGTTGCTCACTTC | S AS | GI: 102180182 |

[a]S, sense; AS, anti-sense.

2.6 Statistical analyses

Data are presented as percentage and mean ($\pm$ SEM), and the results were considered different when $P < 0.05$. Chi-square and Fisher's exact tests were used to compare percentage variables among the treatments. All statistical analyses were performed with Sigma Plot software version 11.0 (Systat Software Inc., USA).

3. Results

3.1 Toxicity of Withanolide D on caprine preantral follicles enclosed in ovarian tissue

3.1.2 Morphology, activation and in vitro follicular growth

A total of 5.607 follicles were analyzed by classical histology. The data in figure 2 show that after 2 days of culture there was a significant reduction in the percentage of morphologically normal follicles in the presence of PTX, WD1.5 or WD6.0 as compared to control or tissue cultured in MEM. Similar results ($P < 0.05$) were observed after 6 days of *in vitro* culture in all tested treatments. It was noted that after 2 days of culture, WD6.0 showed a toxic effect similar to PTX, whereas after 6 days, the toxicity of WD6.0 was higher ($P < 0.05$) not only to PTX, but also to other concentrations tested (1.5 and 3.0) of this drug. It was also observed that, in the presence of PTX and WD, regardless of the concentration tested, the toxic effect on follicular morphology increased with the *in vitro* culture period.
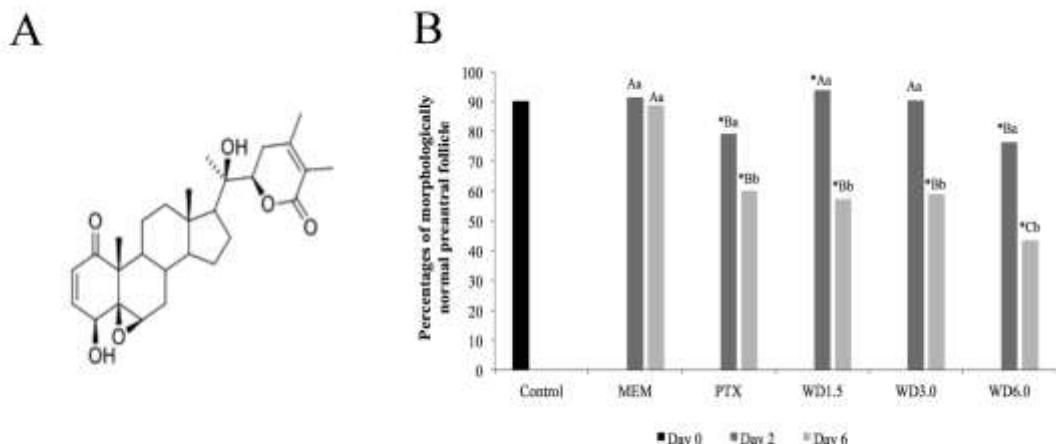
Figure 1. Chemical structure of withanolide D (A) and percentage of morphologically normal preantral follicles on ovarian fragments non-cultured (control) or *in vitro* cultured for 2 or 6 days (B). *Differ from control (P < 0.05). A,B,C indicate differences between treatments (P < 0.05). a,bindicate differences between days of culture (Day 2 and Day 6) (P < 0.05).

The percentage of activation of the preantral follicles in control or after *in vitro* culture for 2 and 6 days is shown in Figure 1. After 2 days of *in vitro* culture, when preantral follicles were analyzed within the category of quiescent (primordial) or developing follicles, it was observed that follicular activation or growth occurred normally, except in the presence of MEM or PTX. After 2 or 6 days of culture, the percentage of follicles that activated in the presence of MEM was similar (P < 0.05) to that observed in the presence of PTX or WD6.0, whereas in the latter treatment, follicular activation was superior (P < 0.05) than that observed in PTX. Overall, in these three treatments, the lowest rates of follicular activation were observed after 2 or 6 days of in vitro culture (P < 0.05).
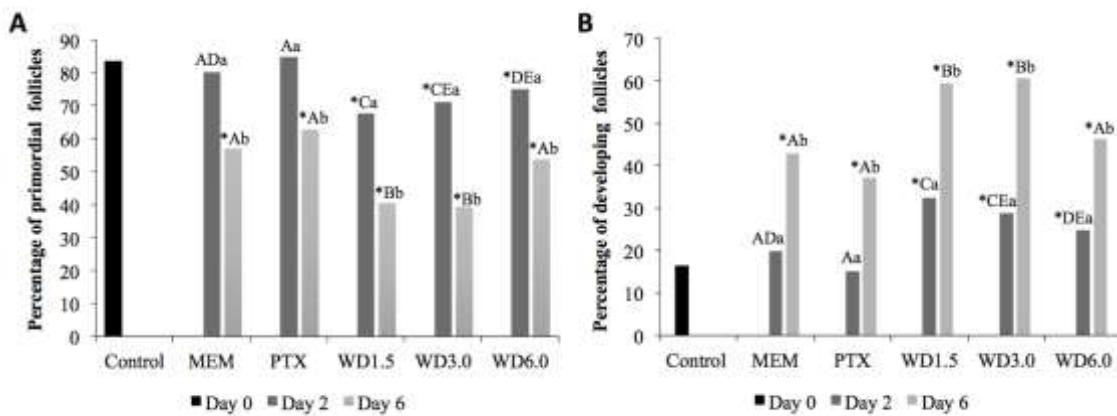
Figure 2. Percentage of primordial **(A)** and growing **(B)** caprine preantral follicles before (control) and after in vitro culture for 2 or 6 days. [A,B,C,D,E]Within a column (P < 0.05). [a,b]Indicate difference between days within the same follicular class (P < 0.05).

3.1.2 Granulosa cells proliferation

The proliferation of granulosa cells was quantified by the immunolocalization of nuclear protein from Ki67 cell proliferation cells (**Fig. 3**). The data showed positive Ki67 labeling in granulosa cells of primordial and developing follicles (**Fig. 3A-G**). The percentage of proliferation of granulosa cells was significantly higher in the presence of PTX (23.6%), WD3.0 (25.5%) or WD6.0 (16.5%) when compared to control follicles (8.5%) or cultured in the absence of drugs (MEM: 4.1%) (**Fig. 3H**). In addition, the presence of WD in the highest concentration showed a lower percentage of proliferating cells compared to PTX or WD3.0.
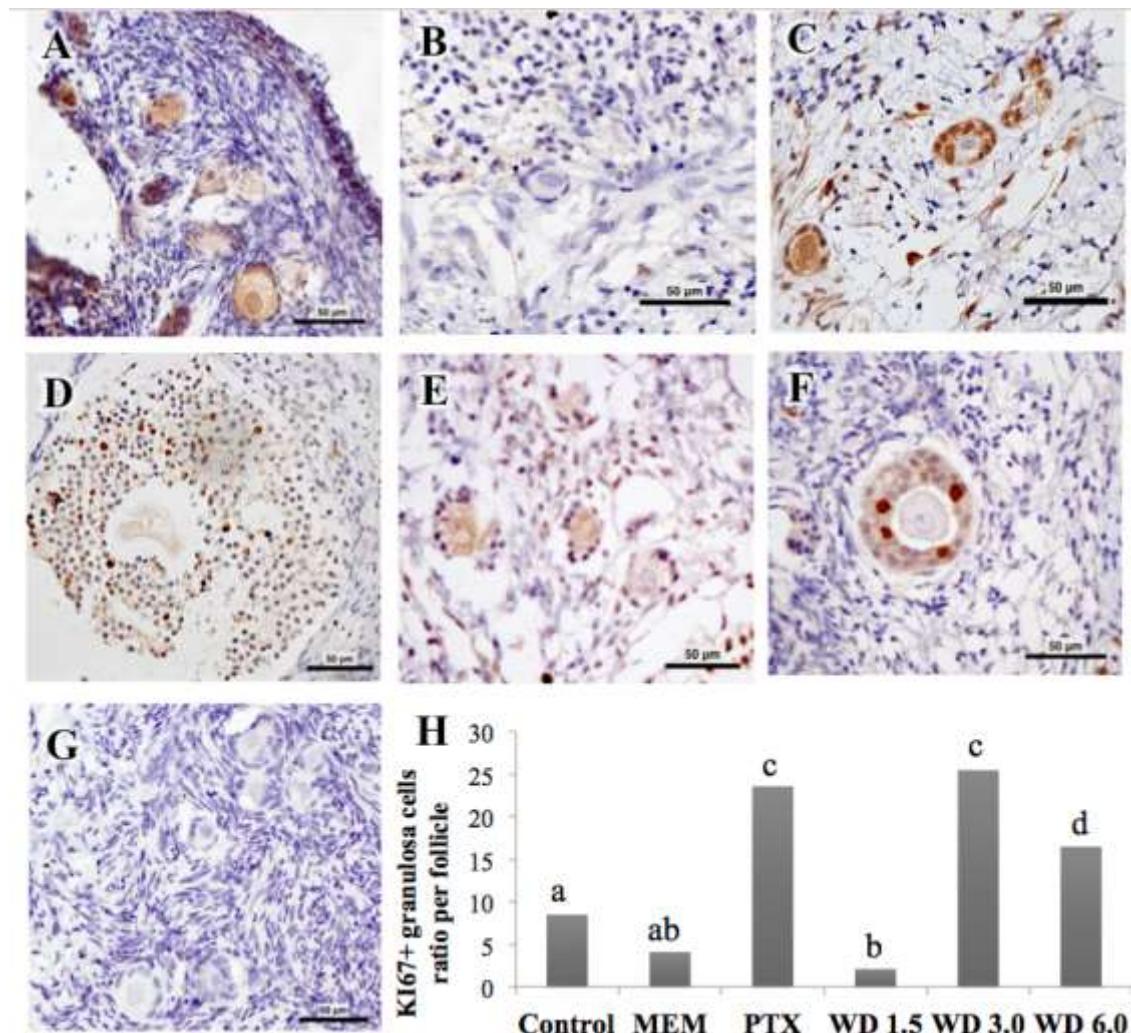
Figure 3. Immunostaining for Ki67 in ovarian tissue. Representative transverse sections of Control (A), MEM (B), PTX (C), WD1.5 (D), WD3.0 (E) and WD6.0 (F) and negative control (G). The black arrows in the granulosa cell indicated the Ki67$^+$. Bar chart showing the percentage of Ki67$^+$ in granulosa cells in the follicles (H). ^{a,b,c,d,e}Within a column (P <0.05). Bars 50 μm.

3.1.3 Detection of apoptotic cells

To evaluate the toxicity of WD on preantral follicles, the TUNEL technique was used, and the follicles were classified as positive when the oocyte nucleus or more than 50% of the granulosa cells were marked in brown. In the primordial and developing follicles of the control, no positive marking was observed for TUNEL (**Fig. 4B**). The data showed a higher percentage of apoptotic follicles (P <0.05) in the presence of PTX (68.7%) or WD1.5 (71.4%), compared to

follicles cultivated in the absence of drugs (MEM: 20.9%) or in the presence of WD3 .0 (31.0%).
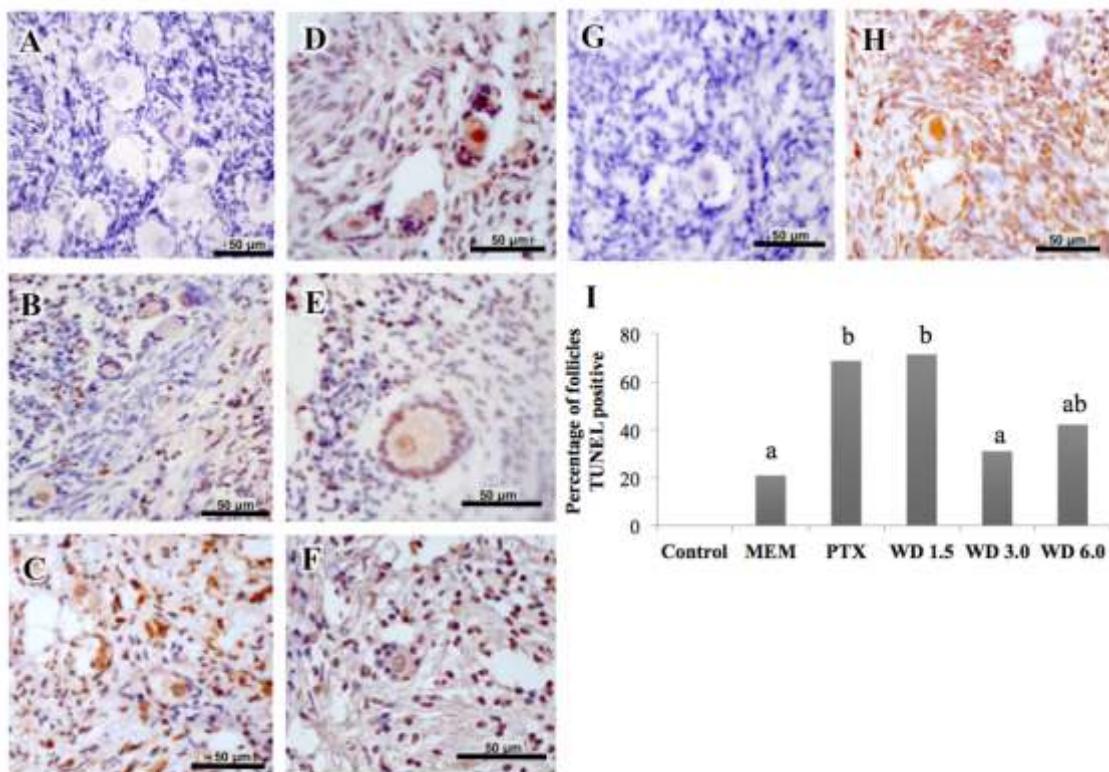
Figure 4. TUNEL assay. Representative transverse sections of control (A), MEM (B), PTX (C), WD1.5 (D), WD3.0 (E) and WD6.0 (F) and negative (G) and positive control (H). The arrow in C indicates TUNEL positive reaction). The black arrows in the oocyte and arrow heads in granulosa cells indicate TUNEL-positive. Bar chart showing the percentage of TUNEL-positive preantral follicles among treatments (I). [a,b,c and d]indicate statistical differences between treatments (P<0.05). Bars 50µm.

3.1.4 Immunodetection of ABCB1 protein

In figure **5A** we can observe positive marking in all treatments for ABCB1 protein in ovarian goat tissue. However, the data showed that in the presence of MEM (21.7%), there was a significant reduction in ABCB1 expression, 6 days after in vitro culture, compared to control or other treatments. On the other hand, expression of this protein remained unaltered in the presence of PTX (60.0%), WD1.5 (54.1%), WD3.0 (57.8%) or WD6.0 (61.5%) throughout the culture *in vitro* (Fig. 5C).
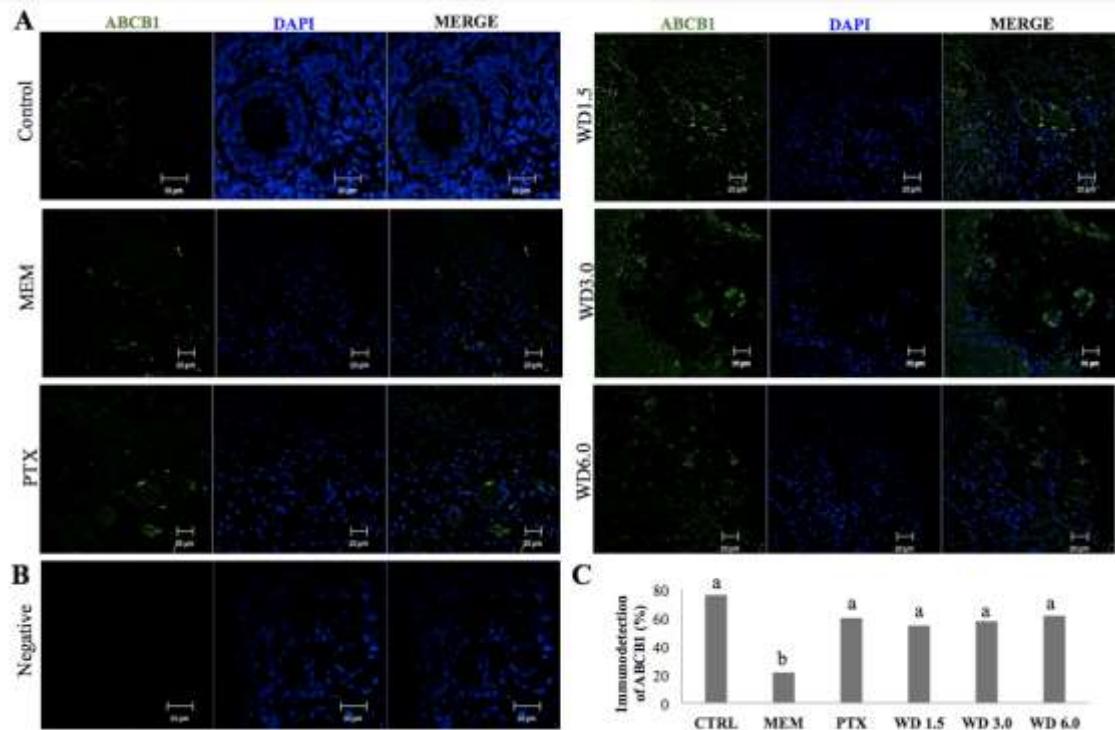
Figure 5. Immunodetection of ABCB1 protein (green bright fluorescent spots) in goat ovarian tissue from the control and after in vitro culture (**A).** Negative control (**B).** Nuclei are labeled with DAPI (blue). Bars 20 μm. Percentage preantral follicles labeled to ABCB1 protein (C). [a,b]indicate statistical differences among treatments (P< 0.05).

3.1.5 mRNA levels for genes involved in the positive control of the cell cycle

Cyclin mRNA expression levels (CCNA, CCNB1, CCND1, CCNE1) and cyclin-dependent kinases (CDK1, CDK2, CDK4, CDK6) are shown in figure 6 (**A-G**). After 6 days of *in vitro* culture, there was a significant reduction in mRNA levels for CCNA and CCND1 (**Fig. 6A-C**) in all treatments when compared in the presence of MEM. At the highest concentrations of withanolide D (WD3.0 and WD6.0), a reduction in CCNB1 expression was observed when compared to the other treatments (**Fig.6B**). Regarding mRNA levels for CCNE1, a significant reduction in the presence of WD1.5 or WD3.0 was observed when compared to MEM (**Fig. 6D**).

With respect to cyclin-dependent kinases, the results showed a significant reduction in mRNA levels for CDK1 in the presence of PTX, WD3.0 and WD6.0, compared to MEM. Similar results were observed for CDK2 and CDK6 at all concentrations of WD when compared to MEM (**Fig. 6F-H**).
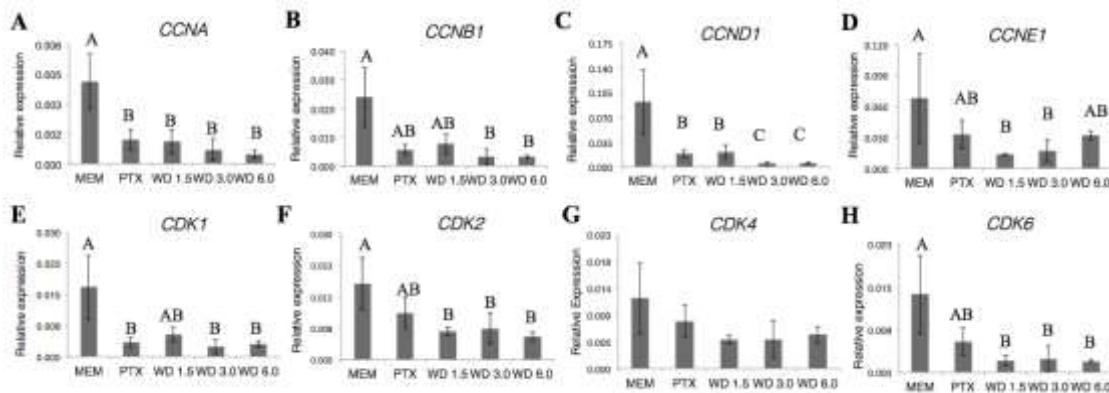
Figure 6. Relative mean (standard error of the mean) expression of mRNA of CCNA (**A**), CCNB1 (**B**), CCND1 (**C**), CCNE1 (**D**), CDK1 (**E**), CDK2 (**F**), CDK4 (**G**), CDK6 (**H**) in caprine preantral follicles. Different letters denote significant differences ($P < 0.05$).

4. Discussion

Conventional chemotherapies used in cancer treatment have many side effects, such as damage to the follicles and consequently premature ovarian failure, interfering negatively in reproductive function, often leading to infertility [2]. Thus, several researchers have employed many efforts in the attempt to identify new antitumor compounds such as WD and offer less side effects. The WD is an ergostane-type steroidal lactone extracted from the *Acnistus arborescens* (L.) Schlecht (Solanaceae), a medicinal plant common to South and Central Americas. In addition to their pharmacological properties, such as cytotoxicity, anti-inflammatory and immunosuppressive activities, this secondary metabolite have attracted particular attention due its anticancer property [15]. However, there is still no report on the effect of WD on ovarian function or preantral folliculogenesis. Therefore, in this study, we evaluated the toxic effects of goat ovary exposure to WD at different concentrations during *in vitro* culture for up to 6 days and compared with the PTX (negative control) whose negative effects on ovarian function are already widely known to cause follicular death *in vivo* [19] and *in vitro* [7].

Data from the present study showed a significant reduction in the percentage of morphologically normal follicles in all ovarian fragments treated with PTX or WD, compared to control or MEM after 6 days of culture. Analyzing the data of both substances, it was observed that already on 2 days of culture, the WD6.0 presented toxic effect similar to the PTX. After 6 days, the toxicity of WD6.0 was higher ($P < 0.05$) not only to PTX but also to other

treatments (MEM, WD1.5, and WD3.0), indicating that this WD concentration was extremely toxic to the tissue ovarian. The increase in the number of degenerated follicles was also a characteristic observed when exposed to other chemotherapics, such as Anthracyclines, Cisplatin, Cyclophosphamide, Gemcitabine, Mitomycin C, Taxanes. These drugs generally cause cell death by triggering the activation of cellular apoptosis [20], which also leads us to believe that WD stimulates this pathway of degeneration.

Despite the negative effect of WD observed on follicular morphology, activation of primordial follicles was not inhibited, ranging from 46.3% (WD6.0) to 60.7% (WD3.0), of follicles activated after 6 days of culture *in vitro*. Our results are similar to those reported in the literature after *in vitro* culture of in the absence of chemotherapeutic agents, which vary from 60% [21] to about de 80% [[7],[15],[22],[23]] of activated follicles. Therefore, according to the result of this study, we suggest two hypotheses for follicular activation: 1) or factors and mechanisms responsible for this *in vitro* process were not negatively influenced by WD or 2) activation of primordial follicles may have been stimulated by the death of developing follicles already present at the beginning of *in vitro* culture. It is known that developing follicles produce substances that inhibit the mass activation of the primordial follicles, such as the Anti-Müllerian Hormone [24], with the objective of avoiding the ovarian reserve depletion [25, 26]. Therefore, the initial death of the developing follicles would result in a lower production of these inhibitory factors, leading to follicular mass activation [27] . Once activated, these follicles would die due to WD toxicity, thus reducing the percentage of morphologically normal follicles [[28], [29], [30]] also observed that ovarian exposure of mouse chemicals such as Bisphenol A, 7,12-Dimethylbenz [a] anthracene, 4-vinylcyclohexene, methoxychlor and menadione diepoxide during *in vitro* culture may stimulate activation of primordial follicles, associated with an extensive atresia of developing follicles.

In relation to the cell proliferation rate, by the immunolocalization of the Ki67 protein, our results showed an increase in the number of positive granulosa cells, in the treatments in which degeneration was most evident (PTX, WD3.0 and WD6.0) when compared to other treatments (control, MEM and WD1.5). Ki-67 is a proliferation marker that is often used to estimate the cell proliferation. This antigen is expressed during all phases (G1-S-G2-M) of the cell cycle, but not in quiescent G0 cells or cells stimulated to enter the G phase [31]. Thus, we believe that the increase of Ki67 positive cells observed in our study is related to the entry of granulosa cells into the process of cell division. However, with the toxicity of PTX and WD in high

concentration, these cells would be blocked during some phase of this process and then enter into apoptosis, causing follicular degeneration.

The results of the present study showed a significant increase in the percentage of apoptotic follicles evaluated by TUNEL assay in the presence of PTX and WD1.5 when compared to MEM or WD3.0 Modal et al. [32] also observed that death of several cancer cell lines (chronic myelogenous leukemia, colorectal carcinoma, brain carcinoma and lung carcinoma) caused by WD at different concentrations (2, 3 and 4 μM) occurs by apoptosis. Curiously, no positive labeling was observed for TUNEL in primordial follicles treated with WD, but we found a predominant marking in the nucleus of oocytes from developing follicles, suggesting that oocytes from these follicles are more susceptible to WD. This may be due to the quiescent state of the primordial follicles, which have low metabolism, lower number of cells, less transcription and protein synthesis, consequently less susceptible to damage. Other studies have also shown that mouse primordial follicles are less sensitive to the toxic effects caused by chemicals such as Bisphenol A; 7,12-Dimethylbenz-[a]anthracene; 4- vinylcyclohexene; methoxychlor and menadione diepoxide [[28], [29], [30]].

In order to have a greater accuracy of the negative effect of WD on preantral follicles, mRNA expression for cell cycle regulatory proteins (cyclins and cyclin-dependent kinases - CDKs) was also evaluated. The cell cycle is positively controlled by mechanisms ensuring the cell proliferation, i.e. regulation of CDKs (CDK1, CDK2, CDK4 and CDK6) by cyclins (A, B, D and E). The first are constantly expressed in cells, but need to bind cyclins to become active [33]. On the other hand, cyclins are synthesized and degraded according to the need of each cell cycle (G1, S, G2 and M), so that the cyclin/Cdk complex becomes specific for each of these phases [34,35] In summary, the CDK4/6 kinases and cyclin D are required for progression through G1. CDK2 associated with cyclin E regulates the progression from G1 into S phase, while cyclin A binds with CDK2 and this complex is required during all S phase. In late G2 and early M phase, cyclin A complexes with CDK1 to promote entry into M. Mitosis is further regulated by cyclin B in complex with CDK [36]. According to these protein complexes, in general, our results showed that, in the same way as the negative control (PTX), WD altered the cell cycle characterized by the reduction of expression of both cyclins and CDKs. WD, as well as PTX, significantly reduced gene expression for CDK1 and cyclin A, indicating a probable blockage in progression to phase M of the cycle. However, only the WD caused a reduction of the CDK2 and CDK6 genes in relation to the MEM, suggesting a block in the progression of S phase and G1, respectively. Reduced expression of genes involved in cell cycle

progression may be a major factor in preventing development and stimulating follicular apoptosis during WD exposure.

In the present work, we also evaluated the immunoblotting for ABCB1, a member of the transmembrane family of proteins responsible for the multidrug resistance mechanism, such as chemotherapeutics. According to Giacomini and Sugiyama [37], there are 49 known genes for ABC proteins that can be grouped into 7 subclasses or families (ABCA to ABCG). Although differences are observed in their functions, substrate specificity, molecular mechanism, *in vivo* localization, the families of ABC proteins share a high degree of structural homology [38]. The ABCB1 evaluated in this work is among the main ABC transporter proteins linked to the mechanism of cell resistance to chemotherapeutics. Thus, increased expression of this protein in tumor cells reduces drug efficacy during cancer treatment [39]. In our study, the *in vitro* culture of the follicles in the medium without the presence of drugs (MEM) significantly reduced the expression of ABCB1, whereas in the presence of PTX or WD, this protein remained unchanged throughout the culture period in relation to control. This result suggests that WD does not induce cell resistance to multidrug at concentrations tested in normal cells. Although studies on WD are still scarce, Issa et al. 40 found that exposure of tumor cells (chemotherapeutic resistant) at different concentrations (0, 25, 100, 200, 400 and 600 nM) of WD, isolated from *Withania somnifera* L. for 24 h, resulted in death, however, did not alter expression of ABCB1.

In conclusion, this study showed that, in general, WD exerted a toxic effect on preantral caprine follicles cultured in vitro in the ovarian tissue, similar to that exerted by PTX, whose negative effect on folliculogenesis is already widely known. In addition, WD can also dramatically alter the cell cycle, as indicated by the reduction of cyclins and Cdks, responsible by its positive control. As the expression of ABCB1 was not altered by the presence of WD throughout the culture period, this may be an indication that the phenomenon of multidrug resistance can be controlled according to the condition of use of a certain chemotherapeutic. According to these results, we believe that WD is a promising chemotherapeutic agent of natural origin. However, as apoptosis has not been strongly evidenced as shown by TUNEL assay, we suggest that further studies need be performed in an attempt to identify not only the mechanism of action of WD, but also the effect on preantral follicles in more advanced stages of development, aiming at a deeper investigation on the reproductive function of the female.

Acknowledgments

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9 CAPÍTULO 4:

Resposta dos folículos pré-antrais à quinoxalina: um novo composto com

potencial anticancerígeno

"Response of preantral follicles exposed to Quinoxaline: a new compound with

anticancer potential"

Periódico: Submetido em dezembro à Chemico-Biological Interactions

(Qualis A2)

Resumo

Atualmente, existem constantes investigações para a identificação de novos medicamentos contra o câncer com menos efeitos colaterais. A cultura de folículos pré-antrais como modelo in vitro para avaliar a toxicidade do novo fármaco antineoplásico foi estabelecida, o que indiretamente determina o potencial antiproliferativo de novos compostos. Portanto, o objetivo deste estudo foi avaliar o efeito de um novo candidato ao fármaco antineoplásico, o derivado da quinoxalina 2 (XYZC 6 H 3 -CH = N-NH) -quinoxalina, 1 (Quinonoxalina (QX) em caprinos). Avaliamos a morfologia e ativação folicular, proliferação e apoptose de células da granulosa e finalmente a expressão de proteínas (ABCB1) e genes (ciclina / Cdks) envolvidos na resistência a múltiplos fármacos e progressão do ciclo celular. Folículos em desenvolvimento foram expostos (cultivo *in vitro*) a diferentes concentrações de QX (QX1.5, QX3.0 ou QX6.0 μM / mL) durante 2 e 6 dias. Para avaliar o efeito da QX, o tecido ovariano foi exposto ao Paclitaxel 0,1 μg / mL (PTX - controle negativo) ou em meio de cultura sem QX (MEM). Ao final do tempo de exposição, o QX (todas as concentrações) alterou ($P < 0,05$) a morfologia normal dos folículos pré - antrais controle (tecido ovariano não tratado) ou MEM. No entanto, QX6.0 mostrou um efeito mais forte ($P < 0,05$) sobre a ativação folicular (bournout) e apoptose QX1.5 e QX3.0. Expressão de ABCB1 foi semelhante entre QX1.5 e QX6.0 e ambos foram inferiores ao controle, MEM e PTX. Curiosamente, a taxa de apoptose em QX3.0 foi semelhante ao controle e MEM e inferior a QX1.5; QX6.0 e PTX. Concluímos que a quinoxalina pode ser um agente quimioterápico promissor, no entanto, outras concentrações dentro de uma faixa definida (2-5,5 μM) podem ser amplamente investigadas.

Palavras-chave: Agentes quimioterápicos, folículos primordiais, citotoxicidade folicular, preservação da fertilidade, ABCB1

Response of preantral follicles exposed to Quinoxaline: a new compound with anticancer potential

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Abstract

Currently, there are constant investigations for the identification of new drugs against cancer with fewer side effects. Culture of preantral follicles as an in vitro model to evaluate the toxicity of new anticancer drug has been established, which indirectly determines the antiproliferative potential of new compounds. Therefore, the aim of this study was to evaluate the effect of a new candidate to anticancer drug, the quinoxaline derivative the 2 2- (XYZC 6 H 3 –CH=N-NH) -quinoxaline, 1 (Quinonoxaline (QX) on caprine preantral follicles. We evaluate the follicular morphology and activation, proliferation and apoptosis of granulosa cells and finally

the protein (ABCB1) and genes expression (cyclin/Cdks), respectively involved in involved in multidrug resistance and cell cycle progression. Ovarian fragments containing primordial and developing follicles were exposed (in vitro culture) to different concentrations of QX (QX1.5, QX3.0 or QX6.0 μM/mL) during 2 or 6 days. To evaluate the effect of QX, the ovarian tissue was exposed to Paclitaxel 0.1 μg/mL (PTX – negative control) or in culture media without QX (MEM). At the end of exposure time, we realized that the QX (all concentrations) changed (P< 0.05) the normal morphology of preantral follicles compared to control (not treated ovarian tissue) or MEM. However, QX6.0 showed a stronger (P< 0.05) effect on follicular activation (*bournout*) and apoptosis then QX1.5 and QX3.0. Expression of ABCB1 was similar between QX1.5 and QX6.0 and both were lower than control, MEM and PTX. Interestingly, the apoptosis rate in QX3.0 was similar to control and MEM and lower then QX1.5; QX6.0 and PTX. We conclude that quinoxaline may be a promising chemotherapeutic agent, however, other concentrations within a defined range (2-5.5 μM) could be widely investigated.

Key words: Chemotherapeutic agents, primordial follicles, follicular cytotoxicity, fertility preservation, ABCB1

1. INTRODUCTION

Several reports have shown that the cancer rate survivors are increasing each year. According to the American Cancer Society [1], the expectation is that in 2026, the number of cancer survivors is 20 million. This phenomenon is due to early diagnosis, associated with treatments, as new, more effective chemotherapeutics [2]. Based on this, a study reported that the 2 2-($XYZC_6H_3$–CH=N-NH)-quinoxaline, 1 (QX) is a promise and potent new class of anticancer agent, since its cytotoxicity activity was evaluated against four human cancer cell lines: OVCAR-8 (human ovary), SF-295 (glioblastoma), HCT-116 (colon) and HL-60 (leukemia) (Figure 1). The authors showed that PJOV 56 - (*E*)-2-[2-(2-pyridin-2-ylmethylene)hydrazinyl]quinoxaline (Figure 1) had a potent cytotoxicity activity compared to Doxorubicin [3], a widely used chemotherapeutic for the treatment of ovarian, breast, liver, lung and lymphoma [4, 5].
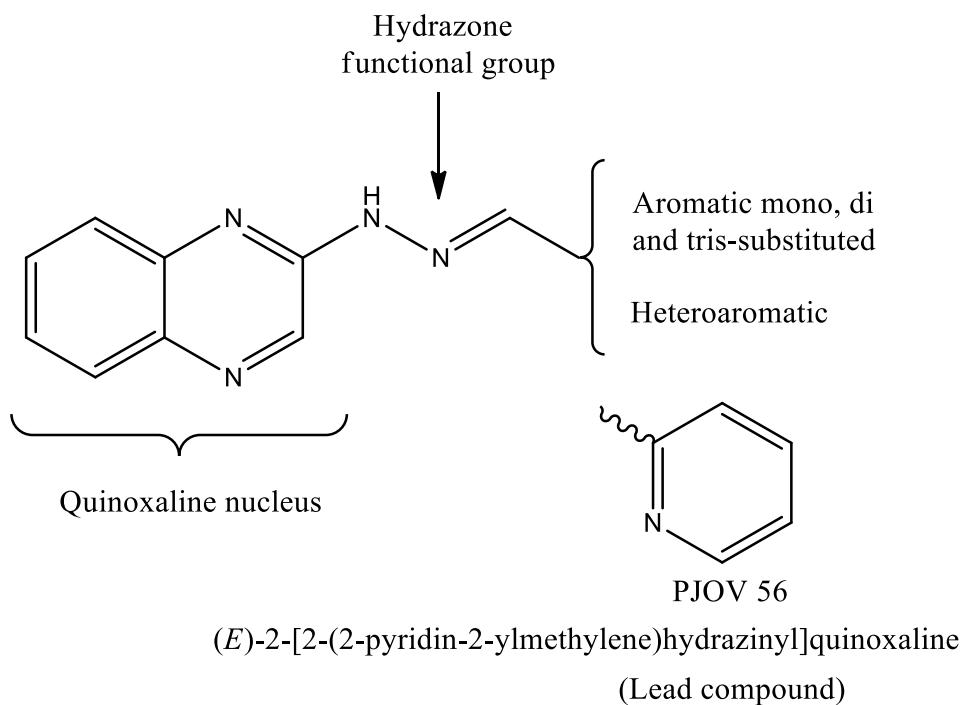
Figure 1. The lead compound PJOV 56 (*E*)-2-[2-(2-pyridin-2-ylmethylene)hydrazinyl]quinoxaline.

Despite the positive effects against cancer cells, chemotherapeutic agents such as Doxorubicin (DXR), Paclitaxel (PTX), Cisplatin (CP) and 5-Fluorouracil (5-FU), among others, also have antiproliferative or cytotoxicity effects on healthy body cells. Thus, these agents are extremely gonadotoxic and lead to ovarian failure, which is one of the most detrimental consequences of cancer treatment procedures in women who could overcome their

cancer disease [6]. Previous studies have shown that DXR has increased the breakdown of double-stranded DNA from granulosa cells and caused dose-dependent cytotoxicity in rodent ovaries [7,8], caprine [9] and non-human primates [10]. In another study, Sanchez et al. [11] showed that DXR, PTX, and CP led to apoptosis of human ovarian luteinized granulosa cells, regulating negatively the estrogen receptor β and the follicle stimulating hormone receptor in a dose-dependent manner. Recently, it has also been reported that 5-FU was severely toxic to developing ovarian follicles, inducing DNA damage and apoptosis of granulosa cells [12].

In addition to the cytotoxicity observed in normal cells, the multidrug resistance (MDR) in cancer leads to synchronous resistance of cancer cells to structurally unrelated anticancer drugs, and as a result, chemotherapy fails [13]. One important mechanism of MDR is mediated by the efflux pump protein, known as the ATP-binding cassette (ABC) transporters, which are located on the membrane of cancer cells [14]. The ABC transporters comprise 49 members, one of the largest known protein families and are divided into seven subfamilies from ABCA to ABCG [15]. Among the members of these families, we highlight ABCB1, also known as P-glycoprotein or multidrug resistance protein 1, MDR1. ABCB1 is known to transport a variety of hydrophobic drugs outside the cancer cells, including Paclitaxel [13] and steroids [16]. Studies performed with cancer cells have shown that ABCB1 expression can be inhibited or increased, resulting in cell cycle arrest [17] and lower responsiveness of cells to apoptotic stimuli [18] respectively. Recently, we immunolocalized ABCB1 in the ovary of healthy goats [19], which may be an indication that the presence of this protein in this organ can act as a defense mechanism against the toxic effect of chemotherapeutic agents.

Considering the impact that chemotherapeutic agents with anticancer potential exert on female fertility, the objective of this study was to evaluate the effect of 2 2-($XYZC_6H_3$–CH=N-NH)-quinoxaline, 1, on preantral follicles (morphology, activation, antiproliferative and apoptosis on granulosa cells) present in the ovarian cortex cultured in vitro for 2 or 6 days. In addition, the expression of ABCB1 was also verified under the action of this compound.

MATERIAL AND METHODS

Ovaries and culture medium

Ovaries were collected from six adults cross-bred goats (*Capra hircus*) at a local abattoir. Immediately postmortem, ovaries were washed once in 70% (v/v) ethanol for 10 seconds and then washed twice in HEPES-buffered minimum essential medium (MEM)

supplemented with 100 µg/mL penicillin and 100 µg/mL streptomycin. The ovaries were then transported to the laboratory in MEM at 4 ℃ within 1 h.

Unless mentioned otherwise, the culture media and other chemicals used in the present study were purchased from Sigma Chemical Co. (St Louis, USA). The basic in situ culture medium (culture control) consisted of α-MEM (pH 7.2 - 7.4) supplemented with 1.25 mg/mL bovine serum albumin BSA, insulin 10 ng/mL, transferrin 5.5 µg/mL, and selenium 5 ng/mL, 2 mM glutamine and 2 mM hypoxanthine, which was called α-MEM$^+$.

Ovarian cortex exposure to quinoxaline and histological processing

In the laboratory, three pairs of ovaries (n = 6) were cut into 13 pieces (3 x 3 x 1 mm) of which one was randomly taken and immediately fixed as described below and designed as *Control*. The remaining were individually cultured in a 24-well plate containing 1 ml α-MEM$^+$ culture medium without (MEM); Paclitaxel (PTX 0.1 µg/mL – negative control) or in the presence of Quinoxaline (QX) in three different concentrations (1.5, 3.0, and 6.0 µM), resulting in the following treatments: MEM, PTX, QX1.5, QX3.0 and QX6.0). The in vitro culture was carried out for two or six days at 39 °C and 5% CO_2 in air and the medium was replaced with fresh culture medium every two days. Each treatment was replicated six times.

Control and in vitro cultured pieces of ovarian cortex were fixed in buffered 4 % paraformaldehyde (PAF) in phosphate-buffered saline (PBS) for 4 h at room temperature (RT), dehydrated in a graded series of ethanol, clarified with xylene, embedded in paraffin wax, and serially sectioned into 7 µm thickness. The sections were stained with periodic acid Schiff (PAS) - hematoxylin. For morphological evaluation, coded anonymized slides were examined on a microscope (Nikon, Japan) under 400X magnification.

The follicles were classified according to integrity and developmental stage, defined previously (Hulshof et al., 1994) as *primordial* (one layer of flattened granulosa cells around the oocyte) or *growing follicles* (*intermediate*: one layer of flattened to cuboidal granulosa cells; *primary*: one layer of cuboidal granulosa cells; and *secondary*: two or more layers of cuboidal granulosa cells around the oocyte). These follicles were still classified individually as *morphologically normal* or *atretic follicles*. The first one, those follicles with an intact oocyte surrounded by granulosa cells well organized in one or more layers and that had no pyknotic nucleus. *Atretic follicles* were defined as those with a retracted oocyte, pyknotic nucleus, and/or disorganized granulosa cells detached from the basement membrane. All the follicles present on the slide were evaluated. Each follicle was examined in every section in which it appeared

and matched with the same follicle on adjacent sections to avoid double counting, thus ensuring that each follicle was only counted once, regardless of its size.

To evaluate follicular activation and growth, only intact follicles with a visible oocyte nucleus were recorded and the proportion of primordial and growing follicles were calculated on day 0 (Control) and after two or six days of culture in all tested treatments.

Detection of granulosa cells proliferation and DNA fragmentation (apoptosis)

For analyses of cellular proliferation (Ki67), apoptosis (TUNEL assay) and multidrug resistance protein 1 (P-glycoprotein), were performed in 3 additional repetitions. After the cultured tissue samples from the control and cultured groups were fixed with 4% PAF in PBS (pH 7.2) and subsequently dehydrated and embedded in paraffin wax. Tissue sections (5 mm) mounted on Superfrost Plus slides (Knittel Glass, Bielefeld, NW, Germany) were deparaffinized with Citrisolve (Fisher Scientific, Ottawa, Ontario, Canada) and rehydrated in a graded ethanol series.

To determine the effect of Quinoxaline on granulosa cells proliferation we used the antigen Ki67 which is a nuclear protein expressed in all the active phases of the cell cycle (G1, S, G2, and mitosis), however, this protein is not expressed in resting cell phase (G0). The antigen retrieval was performed by incubating the tissue sections in 0.01 M sodium citrate buffer (pH 6.0) for 5 min, in a pressure cooker. To block endogenous peroxidase, the slides were incubated with 3% H_2O_2 in methanol and then blocked with avidin and biotin. After non-specific blocking was performed using PBS containing 5 % bovine serum albumin (BSA) and 3 % Triton X for 1 h at RT. Then, the slides were incubated overnight at 4 °C with rabbit polyclonal anti-Ki67 (1:4000 – ab15580, Abcam Inc., Cambridge, MA, USA) followed by incubation with the biotinylated anti-rabbit immunoglobulin G (IgG) secondary antibody (1:500 – ab97049, Abcam Inc., Cambridge, MA, USA). Next, the slides were washed, allowed to react with 3,3'-diaminobenzidine in chromogen solution (DAB) in Imidazole-HCl buffer, pH 7.5, containing H_2O_2 (Dako, Inc., Carpinteria, CA, USA), and finally, the sections were counterstained with haematoxylin. The Negative control was performed by omitting the primary antibody. Cell proliferation qnantification was evaluated as a percentage of Ki67 granulosa cells relative to all the granulosa cells in each follicle [20].

The DNA fragmentation was analyzed by TUNEL (terminal deoxynucleotidyl transferase-mediated biotinylated deoxyuridine triphosphates nick end-labeling) assay, using the In Situ Cell Death Detection Kit, POD (Roche Applied Science, Mannheim, BW, Germany), according to the manufacturer's instructions. Antigen retrieval was performed by

incubating the tissue sections in 0.01 M sodium citrate buffer (pH 6.0) for 5 min, in a pressure cooker. To block endogenous peroxidase the slides were incubated with 3 % H_2O_2 in methanol and then blocked for 1 h at room temperature (RT) using PBS containing 5 % BSA and 3 % Triton X. After washing, the slides were incubated with the TUNEL reaction mixture (50 μL) for 1 h at 37 °C. Converter POD was added and the location of the protein expression was demonstrated by incubation with 3 DAB. Finally, the sections were counterstained with hematoxylin. The follicles were considered with fragmented DNA when the oocytes were detected having dark brown stained nuclei (Yucebilgin et al., 2004). As an internal positive control, the sections were treated with 10 U/mL DNase I (InvitrogenTM, Carlsbad, CA) for 15 min at RT, before incubation with the TUNEL reaction mixture to induce the nonspecific breaks in the DNA. The negative control sections omitted the terminal deoxynucleotidyl transferase enzyme.

Immunofluorescence detection of multidrug resistance protein 1 (ABCB1)

For this analysis, we used a mouse monoclonal primary antibody for the detection of P-glycoprotein (1:300 ab3083, Inc. Abcam, Cambridge, MA, EUA), an important protein of the cell membrane that pumps many foreign substances out of cells. The secondary antibody was Alexa 488 Fluor® anti-mouse IgG (ab150113, Abcam Inc., Cambridge, MA, USA) diluted 1:500. The negative control was obtained by omitting the primary antibody

Antigen retrieval was performed by incubating tissue sections in 0.01M sodium citrate buffer (pH 6) at 95–100 °C for 5 min in a pressure cooker. After cooling, sections were washed in PBS and blocked for 1 h at RT using PBS containing 1% (w/v) BSA. Follow antigen retrieval, slides were incubated overnight at 4 °C with primary antibody (P-glycoprotein). Then, slides were incubated with the secondary antibody Alexa Fluor® 488 for 1 h at RT and stained with Evans blue (1: 2000). The slides were mounted with Fluoroshield Mounting Medium with DAPI (ab104139, Abcam Inc., Cambridge, MA, USA). Immunostaining was evaluated using a confocal laser scanning microscope (LSM 710, Zeiss, Oberkochen, Germany). All analyzes were performed using the same configurations.

Real-time RT-PCR analysis

We utilized real-time RT-PCR to determine tissue mRNA levels for cyclins (A, B1, D1, and E) and cyclin-dependent kinases (1, 2, 4 and 6), genes involved in the positive control of the cell cycle. For this, two ovarian fragments (3 x 3 x 1 mm) of three animals were stored in

microcentrifuge tubes (1.5 mL) containing 100 μL of trizol in ice and stored at −80 °C until RNA extraction. Total RNA was isolated with Trizol® Plus RNA Purification Kit (Invitrogen, São Paulo, SP, Brazil). The isolated RNA preparations were treated with DNase I and Pure Link™ RNA Mini Kit (Invitrogen, São Paulo, SP, Brazil). Complementary DNA (cDNA) was synthesized from the isolated RNA using SuperscriptTM II RNase H-Reverse Transcriptase (Invitrogen, São Paulo, Brazil). The quantitative PCR (qPCR) reactions had a final volume of 20 μL and contained the following components: 1 μL cDNA as a template in 7.5 μL of SYBR Green Master Mix (PE Applied Biosystems, Foster City, CA, USA), 5.5 μL of ultra-pure water and 0.5 μL of each primer. The primers were designed to perform the amplification of cyclins A (CCNA), B1 (CCNB1), D1 (CCND1), E1 (CCNE1) and cyclin-dependent kinase 1 (CDK1), 2 (CDK2), 4 (CDK4), 6 (CDK6) mRNA levels (Table 1). Two candidate reference genes, glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and peptidylprolyl Isomerase A (PPIA), were selected as endogenous controls to study the expression, stability and for normalization of gene expression in all samples. Primer specificity and amplification efficiency were verified for each gene. The expression stability of these genes was analyzed using BestKeeper software. BestKeeper highlighted PPIA as the reference gene with the least overall variation. The thermal cycling profile for the first round of RT-PCR was as follows: initial denaturation and activation of the polymerase for 15 min at 94 °C, followed by 40 cycles of 15 s at 94 °C, 30 s at 60 °C and 45 s at 72 °C. The final extension was for 10 min at 72 °C. All reactions were performed in a real-time PCR Mastercycler (Eppendorf, Germany, Hamburg, Germany, USA). The delta-delta-CT method (Livak and Schmittgen, 2001) was used to transform threshold cycle values (Ct) into normalized relative expression levels of mRNA [21].

Table 1 Real-time RT-PCR oligonucleotide primer sequences and cycling conditions.

Target gene	Primer sequence (5′ →3′)	Sense Anti-sense[a]	Genbank accession nos.
GAPDH	ATGCCTCCTGCACCACCA AGTCCCTCCACGATGCCAA	S AS	GI: 327679 (Bos taurus)
CCNA	TGGACCTTCACCAGACCTACCT GTGGGTTGAGGAGAGAAACAC	S AS	GI: 281667 (Bos taurus)
CCNB1	AGCGGATCCAAACCTTTGTAGTG CAATGAGGATGGCTCTCATGTTT C	S AS	GI: 327679 (Bos taurus)
CCND1	GGTCCTGGTGAACAAACTC TTGCGGATGATCTGCTT	S AS	GI: 100144763 (Ovis aries)
CCNE1	GGGACAAGCACCTTATGCAAC GTGTTGCCATATACCGATCAAAG A	S AS	GI: 533526 (Bos taurus)
CDK1	CCAATAATGAAGTGTGGCCAGA AG AGAAATTCGTTTGGCAGGATCAT AG	S AS	GI: 281061 (Bos taurus)
CDK2	CTGCACCGAGACCTTAAACCTCA GCTCGGTACCACAGAGTCACCA	S AS	GI: 519217 (Bos taurus)
CDK4	TGAGCATCCCAATGTTGT CCTTGTCCAGATACGTCCT	S AS	GI: 100144756 (Ovis aries)
CDK6	AGAGTGATTGCAGCTTTATGTCC A TGCCCAGGTTGCTCACTTC	S AS	GI: 102180182

[a]S, sense; AS, anti-sense.

Statistical analyses

Data are presented as percentage and mean (± SEM), and the results were considered different when $P < 0.05$. Chi-square and Fisher's exact tests were used to compare percentage variables among the treatments. All statistical analyses were performed with Sigma Plot software version 11.0 (Systat Software Inc., USA).

RESULTS

Effects of Quinoxaline on preantral follicles

We analyzed a total of 5.903 follicles and found that after 2 days of in vitro culture, only PTX significantly reduced the percentage of morphologically normal follicles compared to control or other treatments. However, at the end of 6 days of ovarian tissue exposure to both PTX and QX at all concentrations, there was an expressive reduction ($P < 0.05$) of morphologically normal follicles compared to control or in the absence of drugs (MEM). In addition, PTX was more aggressive ($P < 0.05$) than QX. The lower concentrations of QX had similar effects, but QX1.5 was more aggressive ($P < 0.05$) than QX6.0. In addition, the extended exposure period to both drugs resulted in a larger ($P < 0.05$) number of follicles with altered morphology (Fig. 2A).

After 2 days of in vitro culture in the presence of QX1.5 and QX6.0 a greater number ($P < 0.05$) of activated primordial follicles was observed, compared to the control. Similar results were found in all treatments after 6 days of drug exposure. However, the higher concentration (6.0) of QX stimulated a greater percentage ($P < 0.05$) of follicular activation in comparison to the other treatments. In addition, this effect in the presence of QX (all concentrations) was more intense than in PTX (Fig. 2B).
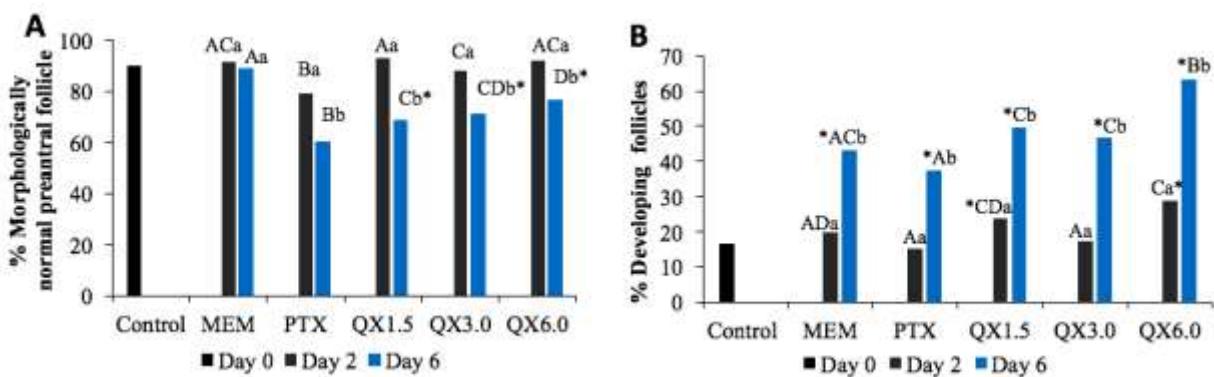
Figure 2. Percentage of morphologically normal (A) and growing (B) preantral caprine preantral follicles on ovarian fragments non-cultured (control) or in vitro cultured for 2 or 6 days. [*] Differ from Control ($P < 0.05$). [A,B,C] indicate differences between treatments ($P < 0.05$). [a,b] indicate differences between days (Day 2 and Day 6) of culture ($P < 0.05$).

Immunodetection to granulosa cell proliferation, apoptosis and ABCB1 protein

Immunohistochemistry showed positive labeling for Ki67 in granulosa cells from both primordial and developing follicles. The rate of cell proliferation was more expressive in the presence of PTX and QX6.0 than in control or other treatments (Fig 3A).

Follicles were considered apoptotic when the oocyte nucleus and/or more than 50% of the granulosa cells were labeled in brown. The percentage of apoptotic follicles was higher (P <0.05) in the presence of PTX, QX1.5 and QX6.0, compared to that observed in the control or in the presence of QX3.0 (Fig. 3B).

After 6 days of *in vitro* culture, the ABCB1 protein was immunolabelled in the control follicles, as well as all treatments, except in QX3.0. However, the percentage of follicles marked in the MEM, PTX, and QX, was lower than the control. On the other hand, although the expression of this protein in the PTX was similar to that observed in the MEM, it was higher to that found in the follicles exposed to QX (all concentrations).
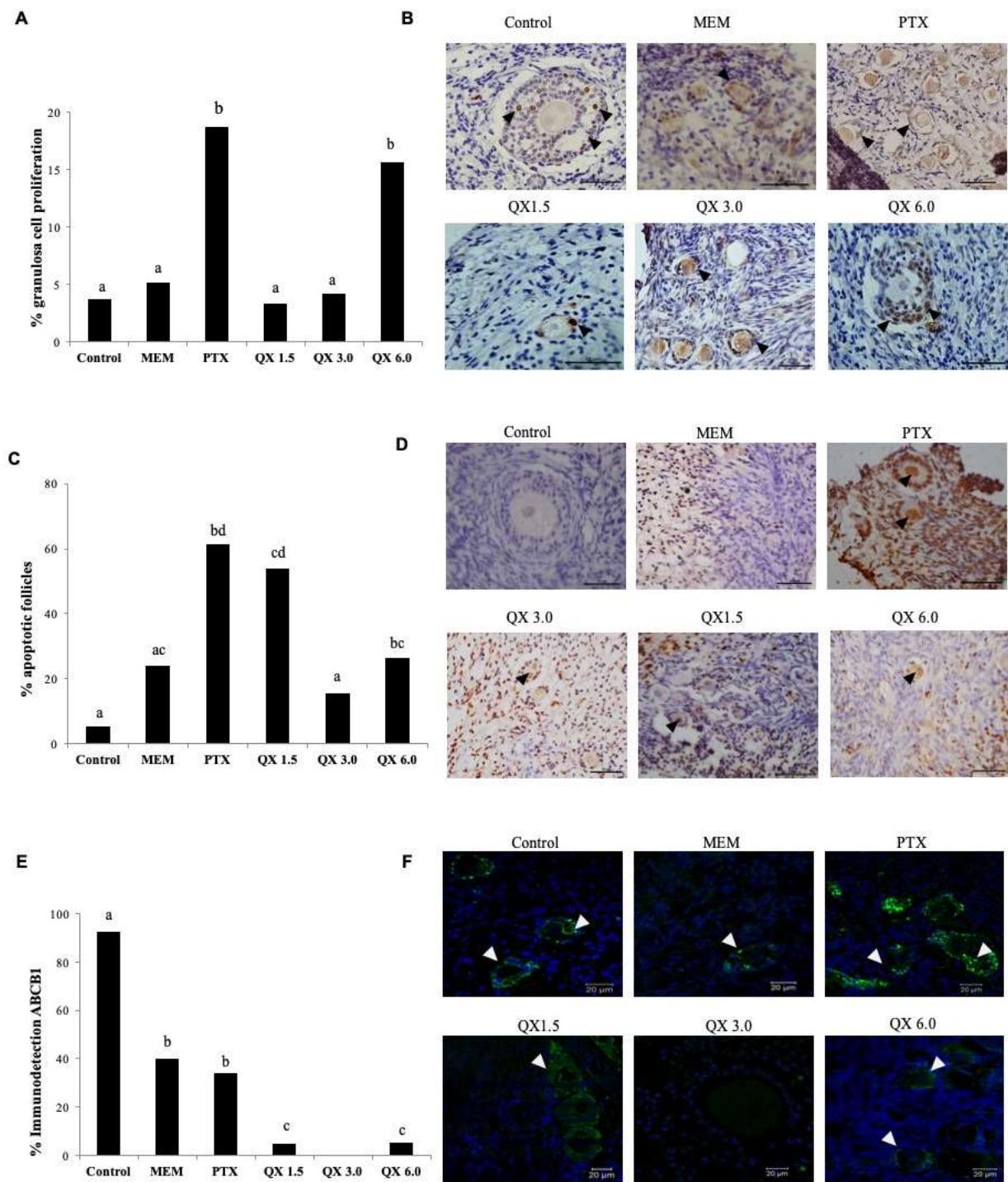
Figure 3. Immunostaining for Ki-67 (A and B), TUNEL assay (C and D) and ABCB1 (E and F) in ovarian tissue. [a,b,c,d,e] indicate differences within a column (P < 0.05). arrowhead

d) mRNA levels for genes involved in the positive control of the cell cycle

The expression levels of mRNA for cyclins (CCNA, CCNB1, CCND1, CCNE1) and cyclin-dependent kinases (CDK1, CDK2, CDK4, CDK6) are shown in Figure 4 (A-G). Regarding cyclins, the expression of CCNA in the presence of QX1.5 was higher (P < 0.05)

than in the MEM. The expression of CCNB1 was also higher ($P < 0.05$) in QX1.5, compared not only to MEM but also to PTX. However, the CCND1 and CCNE cyclins had their expression increased ($P < 0.05$) in the lowest concentrations of QX (1.5 and 3.0), compared to QX6.0, MEM and PTX. Concerning cyclin-dependent kinases, we observed that expression of CDK1 had no effect during in vitro culture in all experimental conditions tested. On the other hand, expression of CDK2 and CDK4 was significantly higher in the presence of QX1.5, compared to MEM or PTX. CDK6 expression was higher ($P < 0.05$) at all concentrations of QX than in PTX. In addition, CDK6 also had its expression, significantly increased in the presence of QX1.5, compared to MEM.
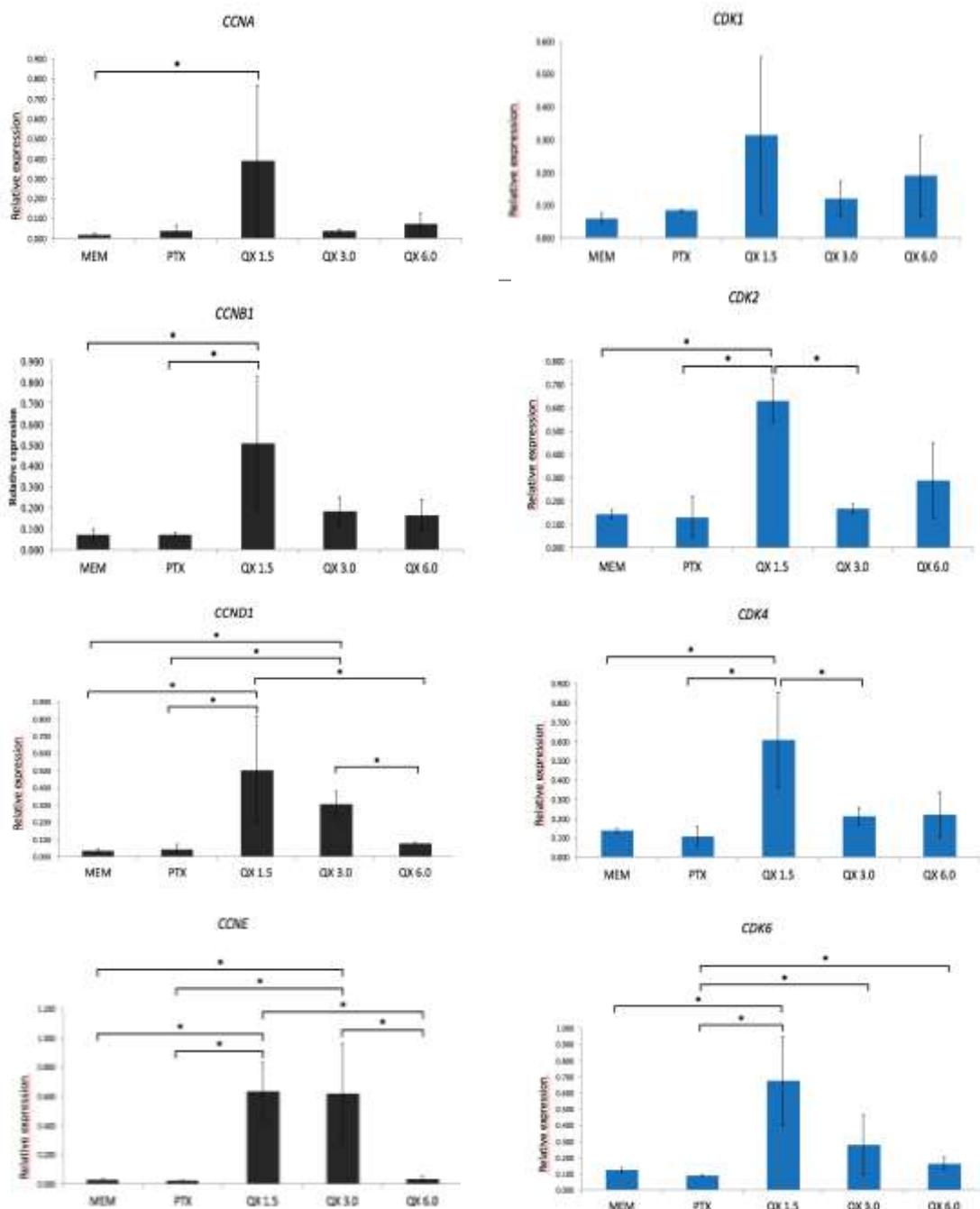
Figure 4. Relative mean (standard error of the mean) expression of mRNA of CCNA (**A**), CCNB1 (**B**), CCND1 (**C**), CCNE1 (**D**), CDK1 (**E**), CDK2 (**F**), CDK4 (**G**), CDK6 (**H**) in caprine preantral follicles. *(P < 0.05).

Discussion

According to Bedoschi et al. [22], cancer is a major public health problem around the world. However, recent developments in cancer diagnostics combined with modern chemoradiotherapy strategies have considerably improved long-term survival rates [23]. On the other hand, besides the impact on the physical and psychosocial health of many patients, within the ovarian follicle, both oocyte and granulosa cells are vulnerable to damage caused by chemotherapy [22]. Therefore, several researchers have devoted huge efforts to the development of new therapies that ensure the quality of life and have minimal side effects, especially on reproductive function [2, 24, 25]. There is an extensive list of substances with potential chemotherapeutic potential, among them we can highlight the drugs derived from synthetic <u>heterocyclic</u> compounds, such as quinoxalines. Therefore, in this study, we evaluated the effects of this compound on preantral follicles after 2 or 6 days of in vitro culture into the ovarian cortex.

Our data showed that PTX and QX (1.5, 3.0 and 6.0) after 6 days of culture showed a great impact on the reduction of morphologically normal follicles compared to control or to MEM. These results are consistent with other studies that have demonstrated that chemotherapy may induce abnormal morphology in ovarian follicles [22]. The mechanism by which this can occur are varied and depends on each chemotherapeutic agent [22]. In general, chemotherapeutics cause DNA breakage in the oocyte and granulosa cells [26, 27]. These are the most detrimental injuries, although some follicles may be able to survive due to the ability to repair DNA damage [28]. In the case of quinoxalines, the exact mechanism is still not well elucidated, and changes in the chemical structure, like the addition of new groups, can not only produce new varieties of quinoxaline, but can also alter the mechanism of action of these molecules [29].

Although the chemotherapeutics used (QX and PTX) in the present study reduced the percentage of morphologically normal follicles at the end of the culture period, all treatments showed a higher number of activated follicles or development compared to control. This effect was more significant in the presence of QX6.0. We suggest two hypotheses for follicular activation found here: 1) or factors and mechanisms responsible for this in vitro process, were not negatively influenced by the substances used or 2) the activation of primordial follicles may have been stimulated by the death of the developing follicles, already present from the beginning of the culture, whose effect is known as *burnout*. It has been reported in the literature that some xenobiotic substances, such as 7.12, dimethylbenz (A) anthracene (DMBA) and 4-

Vinylcyclohexene diepoxide (VCD) [30], and some chemotherapeutics, such as DXR, PTX [9] and cyclophosphamide [31] may cause burnout of the primordial follicle pool [32]. *In vivo*, the process of follicular activation is regulated by a balance between regulatory factors and stimulators secreted around the primordial follicle [33]. However, *in vitro*, uncontrolled activation of follicular growth caused by the early differentiation of granulosa cells [34] due to the absence of these factors. In the presence of chemotherapeutics, this process is intensified, as they lead to the degeneration and death of activated follicles, which are in high mitotic activity [35]. In addition, levels of the anti-Müllerian hormone (AMH) and other inhibitors of follicular activation, secreted by the developing follicles, are also reduced, consequently leading to massive follicular activation [30]. In the present study, we suggested that once activated, these follicles would die due to QX effect, which could explain, therefore, the low percentage of morphologically normal follicles observed in all concentrations (1.5, 3.0 and 6.0 μM) tested, of similar when compared to taxol.

Our data also showed that QX6.0 (as well as PTX 0.1 μM) after 6 days increased cell proliferation, in relation to the other treatments, demonstrated by immunolocalization of Ki67. This proliferation marker antigen is expressed during all phases (G1, -S-G2-M) of the cell cycle but not in quiescent G0 cells [36]. However, some studies in the literature have shown that this protein can be expressed in degenerating cells when there is an interruption in the cell cycle during the division process [37-39]. Thus, in the present study, we reinforce the cytotoxic potential of QX6.0 and PTX, these cells would be blocked during some phase of the division process and then enter into apoptosis causing follicular degeneration. This result corroborates our hypothesis about the *burnout* effect because, for the proliferation of granulosa cells to occur, the follicle needs to be activated. However, these structures would die due to cytotoxicity of QX, stimulating the activation of new follicles and, consequently, an increase in the marking index for Ki67. The literature shows that some quinoxalines are able to stop cell growth, presenting an antiproliferative profile [41, 42]. Maranhão et al. [43], verified by flow cytometry that the cancer cells in the presence of QX remain retained in the S and G2 / M phases of the cell cycle and degenerate, leading to a reduction in the number of cells.

The molecular evidence of apoptosis, evaluated by terminal nucleotidyl transferase-mediated nick end labeling (TUNEL) assay, was stronger in the follicles exposed to QX1.5 and QX6.0 (idem to PTX). Ovarian xenograft models have shown that the death of preantral follicles (primordial) caused by chemotherapeutic agents, such as cyclophosphamide, occurs by apoptosis [26, 45]. *In vivo,* granulosa cells of secondary follicle, preantral and DXR-treated

mouse anthrax were TUNEL-positive [46, 47]. Zhang et al. [25] have suggested that apoptosis in the granulosa cells caused by DXR is associated with ROS accumulation and the decline of mitochondrial membrane potential. However, as mentioned above, the mechanisms by which QX acts, leading to apoptosis of the ovarian follicles, are still unknown.

In our study, we also evaluated immunostaining for ABCB1, a member of the transmembrane family of proteins responsible for the cellular detoxification system. This protein is expressed in tumor cells and in normal tissues [48]. In cancer cells, the overexpression of ABCB1 is a major cause of multidrug resistance during chemotherapy [49]. Currently, there is a concern to identify new drugs that do not increase the expression of ABCB1 to be more effective in eradicating the tumor [16]. Our results showed that all treatments reduced ABCB1 expression in relation to the control. In addition, in the presence of QX the expression for this protein was lower than MEM and PTX. These results suggest that at the concentrations tested, QX may not be eliminated from normal cells, present in the ovary, at least through ABCB1. This fact may be indicative of the presence of follicular apoptosis mentioned above.

Expression of mRNA for cell cycle regulatory proteins (cyclins and cyclin- dependent kinases - CDKs) was also evaluated. The role of some cyclins (A, B, D, and E) and CDKs (1, 2, 4, and 6) as cell cycle positive regulators. It is also known that, while the cyclins are synthesized and degraded according to the need of each cycle (G1, S, G2 and M), the CDKs are constantly produced by the cell. However, CDKs are active only when bound to cyclins [50], forming a specific (cyclin / CDK) complex [51] for each phase of the cell cycle (G1: Cyclin D / CDK4 / 6; G1 / S: Cyclin E / CDK2; S: Cyclin A / CDK2; G2 / M: Cyclin B / CDK1). In general, in this study, QX1.5 significantly increased the expression of all cyclins and cdks (except for Cdk1) in relation to MEM and PTX. Already QX3.0 increased the cyclins D and E when compared to the MEM, PTX and QX6.0, whereas the latter did not affect the expression neither of the cyclins nor of the CDKs.

According to the results obtained in the present study, we can conclude that in general, the exposure of goat ovarian tissue to Quinoxaline presented a very significant cytotoxicity to preantral follicles, especially in the highest concentration (QX6.0 µM). Considering practically all parameters analyzed (morphology and follicular activation, proliferation, apoptosis and expression of ABCB1, we suggest that QX6.0 can cause follicular burnout in a more evident and intense way. In some way, we do not know, both Quinoxaline at the lowest (1.5 µM) and at the highest (6.0 µM) concentration, seem to inhibit the expression of ABCB1, which could imply in reducing the expulsion of the drug by normal ovary cells (by this way), further

increasing its cytotoxicity. On the other hand, the data also showed us that the intermediate concentration (3.0 µM) of Quinoxaline may be a starting point for a more detailed study on its use as a chemotherapeutic agent, since its effect on follicular activation, proliferation of granulosa cells and apoptosis were less intense than QX6.0 and closer to that observed in the absence of the drug (in vitro culture in MEM).

Acknowledgments

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10 CONCLUSÕES

- O tecido ovariano de cabra expressa RNAm e proteínas para os transportadores ABCB1, ABCC2 e ABCG2. A localização destas proteínas nos diferentes compartimentos foliculares varia de acordo com estágio de desenvolvimento folicular, mas se encontra predominantemente nos oócitos dos folículos primordiais;

- O Vitanolido D exerceu um efeito tóxico sobre os folículos caprinos pré-antrais cultivados in vitro no tecido ovariano, semelhante ao exercido pela PTX, cujo efeito negativo sobre a foliculogênese já é amplamente conhecido;

- O Vitanolido D reduziu a expressão de ciclinas e Cdks, responsáveis pelo controle positivo do ciclo celular;

- Ao contrário do Vitanolido D, que não apresentou efeito sobre a expressão de ABCB1, a Quinoxalina inibiu a expressão dessa proteína nos folículos pré-antrais, cultivados in vitro, inclcusos no tecido ovariano caprino;

- A Quinoxalina exerceu um efeito tóxico sobre os folículos caprinos pré-antrais cultivados in vitro no tecido ovariano, principalmente na concentração de 6.0 μM e pode levar ao *bournout* folicular.

11 PERSPECTIVAS

Esse trabalho mostrou pela primeira vez que três membros da familia dos ABC Transportadores foram identificados no ovário caprino, os quais estão envolvidos no fenômeno da resistencia a multi drogas. Os resultados desse estudo também nos mostraram que os diferentes compostos investigados (Vitanolido D e Quinoxalina) exerceram diferentes efeitos, pelo menos sobre a expressão do ABCB1. Esse achado sugere que a partir da investigação dessas proteínas, é possível predizer sobre a capacidade de um determinado tipo celular apresentar ou não resistência a um determinado agente quimioterápico. No entanto, mais estudos nesse direcionamento serão necessários.

No que concerne especificamente aos compostos investigados, nós sugerimos que outros estudos sejam realizados não apenas para identificar o seu mecanismo de ação, mas também para identificar o seu efeito sobre folículos ovarianos em estágios mais avançados de desenvolvimento. Esses futuros estudos poderão ser de grande interesse para as equipes que acreditam no Vitanolido D como um promissor agente quimioterapéutico natural, visando um menor efeito sobre a função reprodutiva, sobretudo de mulheres jovens, submetidas a tratamentos contra o câncer.

Já com relação à Quinoxalina, nós podemos agora hipotetizar que a concentração de 3.0 μM desse composto pode ser um ponto de partida para um próximo estudo mais detalhado sobre o seu uso como agente quimioterapêutico sintético com menos efeito adverso. Essa inferência é baseada no fato de que os efeitos dessa concentração sobre a ativação folicular, bem como sobre a proliferação e apoptose das células da granulosa foram menos agressivos do que o observado com a concentração mais alta (6.0 μM) e, mais próximos do que os encontrados na ausência de drogas.

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