UNIVERSIDADE ESTADUAL DO CEARÁ
PRÓ-REITORIA DE PÓS-GRADUAÇÃO E PESQUISA
FACULDADE DE VETERINÁRIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS

GILDAS MBEMYA TETAPING

EXTRATO AQUOSO DE *JUSTICIA INSULARIS:* UM COMPONENTE ALTERNATIVO NO MEIO DE CULTIVO *IN VITRO* DE FOLÍCULOS PRÉ-ANTRAIS OVINOS

FORTALEZA – CEARÁ
2019

GILDAS MBEMYA TETAPING

EXTRATO AQUOSO DE *JUSTICIA INSULARIS:* UM COMPONENTE ALTERNATIVO NO MEIO DE CULTIVO *IN VITRO* DE FOLÍCULOS PRÉ-ANTRAIS OVINOS

Tese apresentada ao Curso de Doutorado em Ciências Veterinárias do Programa de Pós-Graduação em Ciências Veterinárias da Faculdade de Veterinária da Universidade Estadual do Ceará, como requisito parcial para a obtenção do título de Doutor em Ciências Veterinárias. Área de Concentração: Reprodução e Sanidade Animal.

Orientador: Profa. Dra. Ana Paula Ribeiro Rodrigues

FORTALEZA – CEARÁ
2019

Dados Internacionais de Catalogação na Publicação

Universidade Estadual do Ceará

Sistema de Bibliotecas

Mbemya Tetaping, Gildas .
Extrato aquoso de Justicia insularis: um componente alternativo no meio de cultivo in vitro de folículos pré-antrais ovinos [recurso eletrônico] / Gildas Mbemya Tetaping. - 2019.
1 CD-ROM: il.; 4 ¾ pol.

CD-ROM contendo o arquivo no formato PDF do trabalho acadêmico com 174 folhas, acondicionado em caixa de DVD Slim (19 x 14 cm x 7 mm).

Tese (doutorado) - Universidade Estadual do Ceará, Faculdade de Veterinária, Programa de Pós-Graduação em Ciências Veterinárias, Fortaleza, 2019.
Área de concentração: Reprodução e Sanidade Animal..
Orientação: Prof.ª Ph.D. Ana Paula Ribeiro Rodrigues.
Coorientação: Prof. Dr. José Ricardo de Figuereido.

1. Justicia insularis. 2. Antioxidante. 3. PSH. 4. Cultivo in vitro de dois passos. 5. Folículos pré-antrais. I. Título.

GILDAS MBEMYA TETAPING

EXTRATO AQUOSO DE *JUSTICIA INSULARIS:* UM COMPONENTE ALTERNATIVO NO MEIO DE CULTIVO *IN VITRO* DE FOLÍCULOS PRÉ-ANTRAIS OVINOS

Tese apresentada ao Curso de Doutorado em Ciências Veterinárias do Programa de Pós-Graduação em Ciências Veterinárias da Faculdade de Veterinária da Universidade Estadual do Ceará, como requisito parcial para a obtenção do título de Doutor em Ciências Veterinárias. Área de Concentração: Reprodução e Sanidade Animal.

Aprovada em: 23/01/2019.

Profa. Dra. Ana Paula Ribeiro Rodrigues - Orientadora
(Universidade Estadual do Ceará)

Prof. Dr. José Ricardo de Figueiredo - Co-orientador
(Universidade Estadual do Ceará)

Dra. Francisca Geovânia Canafístula de Sousa - Co-orientador
(Universidade Estadual do Ceará).

Profa. Dra. Maria Helena Tavares de Matos
(Universidade Federal do Vale do São Francisco)

Profa. Dra. Alexsandra Fernandes Pereira
(Universidade Federal Rural do Semi-Árido)

Profa. Dra. Cristiana Libardi Miranda Furtado
(Universidade Federal do Ceará)

Prof. Dr. José Roberto Viana Silva
(Universidade Federal do Ceará)

Prof. Dr. Luis Alberto Vieira
(Universidade de Murcia)

AGRADECIMENTOS

A Deus, por ter me dado o bem mais precioso que é a vida, além de força de vontade, iluminação, saúde, paciência e determinação para enfrentar todas as dificuldades da vida.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pelo incentivo financeiro, através da bolsa de doutorado concedida, no Programa de Estudantes-Convênio de Pós-Graduação (PEC-PG).

À Universidade Estadual do Ceará (UECE) e ao Programa de Pós-Graduação em Ciências Veterinárias (PPGCV), por terem me proporcionado a oportunidade de cursar o doutorado durantes esses 4 anos, com uma ótima infraestrutura e acima de tudo, pelos professores que me fizeram evoluir, intelectualmente.

Ao Laboratório de Manipulação de Oócitos e Folículos Ovarianos Pré-Antrais (LAMOFOPA) da UECE por todo suporte para a realização dessa tese.

À minha orientadora, Profa. Dra. Ana Paula Ribeiro Rodrigues, pela excelente orientação, confiança e oportunidades oferecidas. Muito obrigado por todos os conhecimentos compartilhados, pelo exemplo de profissionalismo e dedicação à ciência.

Ao Professor Dr. José Ricardo de Figueiredo pela oportunidade e acolhimento no LAMOFOPA.

À Profa. Dra. Otília Deusdênia Loiola Pessoa e sua equipe no Laboratório de Análise Fitoquímica de Plantas Medicinais II da Universidade Federal do Ceará, por toda colaboração.

À minha esposa Denise Damasceno Guerreiro, pelo amor, apoio e incentivo, para que eu pudesse ter um caminho mais fácil e prazeroso durante esses anos. E ao fruto do nosso amor (Johan Guerreiro Mbemya) que me faz sempre ir em busca do meu melhor.

À minha mãe Lucienne Madjio (Em memória) e ao meu pai Thomas Tetaping, por serem minha fonte de inspiração. Agradeço enormemente, pelos ensinamentos e por terem apoiado todos os meus sonhos.

Aos meus queridos irmãos e irmãs, Rodric Tetaping Tatsinkou, Raoul Tetaping Sonhafouo, Ghislain Tetaping Djou, Laure Tetaping Tatiana, Pharel Tetaping, Cris Tetaping cujos exemplos sempre busco seguir e, por acreditarem em meu potencial; sempre me incentivando a lutar pelos meus sonhos.

À família da minha esposa, Ana Vládia, Gilberto Guerreiro, Liz Guerreiro e Sra. Maria Damasceno, por todo amparo e apoio imprescindível. Deus foi muito generoso, quando me abençoou com pessoas tão especiais.

Ao Prof. Dr. Benner Geraldo Alves, pelas mil e uma análises estatísticas. Obrigado pela paciência.

À banca examinadora, por ter aceito prontamente o convite, sobretudo pela colaboração na correção deste trabalho.

À toda equipe do LAMOFOPA, por todo auxílio e por terem feito dos meus dias mais felizes. Agradeço enormemente aos pós-doutores: Ana Beatriz Graça Duarte; Daniele Calado; Francisco Léo Aguiar; Giovanna Quintino Rodrigues; Heline Hellen Moreira; Laritza Lima; Luis Alberto Vieira; Sylvain Nguedia Njina e, especialmente à Geovania Canafístula de Sousa, por toda receptividade, suporte e ajuda durante todos os experimentos.

À Dra. Rebeca Rocha pela amizade e colaboração nessa tese.

À Dra. Nathalie Donfack Njiatsa pela amizade de muitos anos e pela disponibilidade de me ajudar sempre.

Aos doutorandos e companheiros de jornada: Anna Clara Ferreira; Juliana Zani; Naiza Arcângela Ribeiro de Sá; Hudson Vieira Correia; Renato Félix da Silva; Victor Macêdo Paes; Thalles Gothardo e Yago Pinto.

Aos mestrandos e amigos: Ana Flávia Bezerra da Silva; Ana Normélia Morais; Everton Pimentel Lopes; Gabriel Las Heras de Alcântara e Lucy Vanessa Sulca.

Aos alunos de iniciação científica: Amanda Mendes Gomes; Andreza de Sá Nunes; Daniel Amed; José Guilherme; Layla Cely; Mariana Silva Ramyres Diego e Natália Soltys.

Aos funcionários, Sr. João Batista e Sra. Alzenira Ferreira, pelo carinho e convivência durante todo o período de realização do doutorado.

Por fim, agradeço a todos aqueles que direta ou indiretamente, contribuíram para a realização desse trabalho, bem como para o meu aperfeiçoamento profissional.

RESUMO

O objetivo do presente trabalho foi avaliar o efeito do extrato aquoso de Justicia insularis (JI) como fitormônio e como antioxidante sobre o cultivo in vitro de folículos pré-antrais (primordiais, primários e secundários) ovinos (in situ e/ou isolados). Para isso, o estudo foi dividido em três fases, a saber: Fase I – Cultivo dos folículos presentes em fragmentos ovarianos (FO) ou cultivo in situ; Fase II - Cultivo de folículos secundários (FS) isolados e Fase III – Cultivo de folículos pré-antrais em dois passos, isto é, in situ (passo 1) e isolados (passo 2). Na Fase I, em um primeiro experimento, FO foram cultivados por 7 dias na presença de FSH (50 ng/mL) ou de JI em diferentes concentrações (0,3; 1,25 ou 5 mg/mL). No segundo experimento, os FO foram cultivados na presença de FSH (50 ng/mL) + anetol (300 µg/mL) ou de JI (0,3 mg/mL) + anetol (300 µg/mL). Em ambos os experimentos, FO frescos foram utilizados como controle e foram avaliados os seguintes parâmetros: sobrevivência, ativação e crescimento folicular; níveis de espécies reativas de oxigênio (EROs - experimento 1) e a densidade de células do estroma (experimento 2). Na Fase II, FS foram cultivados por 18 dias na presença de FSH (100 ng/mL) ou de JI em diferentes concentrações (0,3; 1,25 ou 2.5 mg/mL). Ao final do cultivo foram avaliados, a morfologia, sobrevivência e crescimento folicular; antro e níveis de EROs. Complexos cumulus oócito foram maturados para avaliação da cromatina e as paredes foliculares foram analisadas quanto à expressão de RNAm para glutationa peroxidase, Kit ligand, cyclina B1 e a hialuronano sintase 2. Na Fase III, FO foram cultivados por 7 dias (passo 1), em seguida, FS foram isolados desses FO e cultivados por 6 dias (passo 2). Em ambos os passos, o meio de cultivo foi suplementado com JI (0,3 mg/mL). Além dos parâmetros já mencionados, foi avaliada também a capacidade antioxidante total (passo 1) e a distribuição e atividade mitocondrial (passo 2). Na Fase I, a JI 0,3 mg/mL manteve a porcentagem de folículos morfologicamente normais semelhante ao controle. O uso de JI e anetol aumentou os níveis de EROs. Na Fase II, a taxa de antro com a JI 0,3 mg/mL foi maior ($P < 0,05$) do que nos demais tratamentos. Além disso, a taxa de oócitos viáveis nesse tratamento foi superior ($P < 0,05$) à observada com FSH. Os níveis de EROs e a expressão gênica não foram influenciados pelos tratamentos, em relação ao controle. Na Fase III, a JI 0,3 mg/mL aumentou o potential antioxidante, promoveu a ativação e crescimento folicular nos FO, bem como promoveu o desenvolvimento dos FS isolados. De acordo com os resultados, conclui-se que, o extrato aquoso de JI pode ser utilizado como um suplemento alternativo ao FSH, no meio de cultivo in

vitro de folículos pré-antrais ovinos, seja em um sistema simples ou em um sistema de dois passos.

Palavras-chave: *Justicia Insularis*. Antioxidante. FSH. Cultivo in vitro de dois passos. Folículos pré-antrais.

ABSTRACT

The objective of the present work was to evaluate the effect of the aqueous extract of J. insularis (JI) as phytohormone and as an antioxidant on the in vitro culture of preantral (primordial, primary and secondary) ovine follicles (in situ and/or isolated). For this, the study was divided into three phases, namely: Phase I - Culture of follicles present in ovarian fragments (OF) or in situ culture; Phase II - Culture of isolated secondary follicles (SF) and Phase III - Culture of preantral follicles in two steps, that is, in situ (step 1) and isolated (step 2). In Phase I, in a first experiment, OF were cultured for 7 days in the presence of FSH (50 ng/mL) or JI at different concentrations (0.3, 1.25 or 5 mg/mL). In the second experiment, OF were cultured in the presence of FSH (50 ng / mL) + anethole (300 μg/mL) or JI (0.3 mg/mL) + anethole (300 μg/mL). In both experiments, fresh OF were used as the control and following parameters were evaluated: survival, activation and follicular growth; levels of reactive oxygen species (ROS - experiment 1) and stromal cell density (experiment 2). In Phase II, SF were cultured for 18 days in the presence of FSH (100 ng/mL) or JI at different concentrations (0.3, 1.25 or 2.5 mg/mL). At the end of the culture, morphology, survival and follicular growth, antrum formation and ROS levels were evaluated. Oocyte cumulus complexes were matured for chromatin evaluation, and follicular walls were analyzed for mRNA expression for glutathione peroxidase, kit ligand, cyclin B1 and hyaluronan synthase 2). In Phase III, OF were cultured for 7 days (step 1), then SF were isolated from these OF and cultured for 6 days (step 2). In both steps, the culture medium was supplemented with JI (0.3 mg/mL). In addition to the previously mentioned parameters, total antioxidant capacity (step 1) and mitochondrial distribution and activity (step 2) were also evaluated. In Phase I, 0.3 mg/mL JI maintained the percentage of morphologically normal follicles similar to the control. The use of JI and anethole increased levels of ROS. In Phase II, the rate of antrum formation with 0.3 mg/mL JI was higher ($P < 0.05$) than in the other treatments. In addition, the rate of viable oocytes in this treatment was higher ($P < 0.05$) than that observed with FSH.. In Phase III, 0.3 mg/mL JI increased the antioxidant potential, promoted follicular activation and growth in OF, as well as promoted the development of isolated SF. According to the results, it is concluded that the aqueous extract of JI can be used as an alternative supplement to FSH in the in vitro culture medium of ovine preantral follicles, either in a single or in two steps system.

Key words: Justicia insularis. Antioxidant. FSH. Two-step in vitro culture. Preantral follicles.

LISTA DE FIGURAS

Figura 1 -	**Imagem ilustrativa da organização e estrutura dos folículos presentes no ovário. Notar a presença de folículos ovarianos em diferentes estágios de desenvolvimento.....**	1
Figura 2 -	**Imagem ilustrativa do processo de oôgenese. Notar a sequência evolutiva desde de células germinativas primordiais até a ovulação.....**	2
Figura 3 -	**Oócitos marcados pelo Hoechst 33342 indicando as diferentes fases do processo de maturação nuclear oocitária. (A) GV: vesícula germinativa, (B) GVBD: quebra da vesícula germinativa, (C) MI: metáfase I, (D)MII: metáfase II.....**	3
Figura 4 -	**Imagem ilustrativa da foliculogênese ovariana. Notar as etapas desde a formação dos folículos primordiais até o desenvolvimento do folículo de De Graaf ou pré-ovulatório.....**	4
Figura 5 -	**Fotografia de Justicia insularis. Uma planta herbácea com folhas verdes, simples e a flor com coloração rosa.....**	5
Figura 6 -	**Diferentes sistemas de cultivo de folículos ovarianos.....**	6
CAPÍTULO 2		
Figure 1	**Representative images of the morphology of ovine preantral follicles before and after in vitro culture……..…**	1
Figure 2	**Stromal cell density (cells/100 µm2). (A) Representative images (400 x), bar 50 µm and (B) mean (± SEM) of ovarian stroma cell density in non-cultured control and after 7 days of in vitro culture in MEM++ANE+FSH and MEM++ANE+JUS treatments……....**	2
Figure 3	**Relationship of stromal cell density with percentage of normal preantral follicles……....**	3
Figure 4	**ROS levels after 1 or 6 days in the absence or presence of FSH or different concentrations of Justicia insularis……..….**	4
Figure 5	**ROS levels after 1 or 6 days in the presence of FSH + anethole and Justicia insularis + anethole……...**	5
Figure 6	**Correlation between the follicular morphology and the reactive oxygen species……...…….....**	6

CAPÍTULO 3

Figure 1	**Percentage of morphological intact follicles at different time point (D0, D6, D12, D18) in MEM+ (Control), FSH or in J. insularis 0.3, 1.25 or 2.5 mg/mL (JI0.3, JI1.25 or JI2.5 respectively)……....**	1
Figure 2	**Percentage of degenerated follicles at different time point (D0, D6, D12, D18) in MEM+ (Control), FSH or in J. insularis 0.3, 1.25 or 2.5 mg/mL (JI0.3, JI1.25 or JI2.5 respectively)…….....**	2
Figure 3	**Follicles diameter at different time point (D0, D6, D12, D18) in MEM+ (Control), FSH or in J. insularis 0.3, 1.25 or 2.5 mg/mL (JI0.3, JI1.25 or JI2.5 respectively).……..**	3
Figure 4	**Percentage of antrum formation (A) at different time point (D0, D6, D12, D18) in MEM^{+} (Control), FSH or in *J. insularis* 0.3, 1.25 or 2.5 mg/mL (JI0.3, JI1.25 or JI2.5 respectively). (B): Normal sheep secondary follicle at D0 (i) or at D18 day in *J. insularis* 0.3 mg/mL (JI0.3) (ii). Note formation of antrum (a) on D18. Representative images of fluorescent metaphase II oocyte grown in vitro (iii) or in vivo (iv)……....**	5
Figure 5	**Percentage of extrusion at different time point (D0, D6, D12, D18) in MEM+ (Control), FSH or in J. insularis 0.3, 1.25 or 2.5 mg/mL (JI0.3, JI1.25 or JI2.5 respectively)……..….**	5
Figure 6	**Relative mean (standard error of the mean) expression of mRNA of GPX (A), KL (B), CCNB1 (C), and HAS2 (D) before (D0) and during 18 days of in vitro culture (D6, D12, D18) in MEM+ (Control), FSH or in JI0.3, JI1.25 or JI2.5……..**	6
Supplementary information 1 Fig.	**Identification of secondary metabolites by thin layer chromatography (TLC)……..……...**	7
Supplementary information 2 Fig.	**Metabolites of J. insularis identified by proton nuclear magnetic resonance (1H NMR) and liquid chromatography-mass spectrometry (LC-MS) ……...…….**	8

CAPÍTULO 4

Figure 1	**Experimental design of two-steps in vitro culture of preantral follicles……..……**	1
Figure 2	**Representative photographs of in vitro culture ovarian. Note that in figures A and B, F indicates an included follicle in tissue. Histological section of ovarian tissue stained with periodic acid Schiff (PAS) - hematoxylin in CTR1 (C) or after 7 days of culture (D). Photomicrographs of normal sheep secondary follicle after trehalose bath (E) or at day 6 in J. insularis 0.3 mg/mL (F). (G): Percentage of normal and developing follicles before and after in vitro culture. F: follicle……...…**	2
Figure 3	**Follicular diameter (mean ± SEM) and antrum formation rate (A), and percentage of intact, extruded, and degenerated follicles (B) at different time points of in vitro culture……**	3
Figure 4	**Total antioxidant capacity (Mean ± SEM) from both in situ and isolated follicles culture on days 2 and 4 (A) and representative image of oocytes (B)……**	4

LISTA DE TABELAS

CAPÍTULO 1

Table 1 **Medicinal plants and their in vivo therapeutic utilization on female reproductive function……..** 1

Table 2 **Basic structures of some pharmacologically plant derived flavonoids, alkaloids and terpenoids…….…...** 2

Table 3 **In vitro implication of some medicinal plants on folliculogenesis and steroidogenesis in different species……** 3

CAPÍTULO 2

Table 1 **Percentage of morphologically normal and growing preantral follicles before (non-cultured control) and after in vitro culture for 1 or 7 days in different treatments, in experiment 1……** 1

Table 2 **Percentage of morphologically normal and growing preantral follicles before (Non-cultured control) and after in vitro culture for 1 or 7 days in MEM++ anethole supplemented with FSH or J. insularis (experiment 2)…….** 2

CAPÍTULO 3

Table 1 **Oligonucleotide primers used for polymerase chain reaction analysis……** 1

Table 2 **Mean (± SEM) daily growth of morphologically intact secondary follicles at different time point (D0, D6, D12, D18) in MEM+ (Control), FSH or in J. insularis 0.3, 1.25 or 2.5 mg/mL……..** 2

Table 3 **Percentage of secondary follicles within a growth category at different time point (D0, D6, D12, D18) in MEM+ (Control), FSH or in J. insularis 0.3, 1.25 or 2.5 mg/mL……** 3

Table 4 **Association analyses between follicular growth categories and percentage of extrusion in different intervals of in vitro follicle culture…….** 4

Table 5 **Level of ROS measured in the culture medium from secondary follicles before (D0) and during 18 days of in vitro culture (D6, D12, D18) in MEM+ (Control), FSH or in JI0.3, JI1.25 or JI2.5…….** 5

Table 6 **Percentage of fully grown oocytes, oocyte diameter, viability rate, percentage of meiotic resumption, and meiotic stages of oocytes matured in TCM 199 (Control), FSH or in J. insularis 0.3, 1.25 or 2.5 mg/Ml……** 6

Table 7 **Percentage of oocyte viability, meiotic resumption, and meiotic stages of oocytes recovery from antral follicles matured in TCM 199 (Control), FSH or in J. insularis 0.3, 1.25 or 2.5 mg/mL……...** 7

CAPÍTULO 4

Table 1 **Mean (± SEM) daily and follicular growth of morphologically normal secondary follicles at different time point (D0, D2, D4, D6)...** 1

LISTA DE ABREVIATURAS E SIGLAS

%	Porcentagem
°C	Graus Celsius
BSA	Bovine Serum Albumin (Albumina Sérica Bovina)
CAPES	Coordoneção de Aperfeicoamento de Pessoal de Nível Superior
CCNB1	Cyclin B1
cDNA	Complementary Deoxyribonucleic Acid (Ácido Desoxirribonucleico Complementar)
CNPq	Conselho Nacional de Desenvolvimento Científíco e Tecnológico
CO_2	Dióxido de Carbono
COCs/CCOs	Cumulus oocyte complex (complexos cumulus-oócitos)
DNA	Deoxyribonucleic Acid (Ácido Desoxirribonucléico)
EROs/ROS	Espécies Reativas de Oxigênio (Reactive Oxygen Species)
FSH	Follicle Stimulating Hormone (Hormônio Folículo Estimulante)
GPx	Glutathione Peroxidase (Glutationa Peroxidase)
HAS2	Hyaluronan Synthase *2 (*hialuronano sintase 2*)*
HEPES	2-[4-(2-hydroxyethyl)-1-piperazine]ethanesulfonic Acid (ácido 4-(2- hidroxietil)-1-piperazina-etanosulfónico)
IVF	*In vitro* Fertilization (Fertilização *in vitro*)
Kg	Kilogramas
KL	Kit Ligand
L	Litro
LAMOFOPA	Laboratório de Manipulação de Oócitos e Folículos Pré-antrais
LIF	Fator Inibitório de Leucemia (Fator Inibitório de Leucemia)
M	Molar
MEM	Minimum Essential Medium (Meio Essencial Mínimo)
mg	Miligramas
min	Minuto
mL	Mililitro
mM	Millimolar

mm	Milímetro
MOIFOPA	Manipulação de Oócitos Inclusos em Folículos Ovarianos Pré-antrais
ng	Nanogram (Nanograma)
nmol	Nanomol
$P < 0.05$	Probabilidade de erro menor que 5%
$P > 0.05$	Probabilidade de erro maior que 5%
PAF	Paraformaldehyde (Paraformaldeído)
PAS	Periodic Acid Schiff (Ácido Periódico Schiff)
PBS	Phosphate Buffered Saline (Tampão Fosfato Salina)
PCR	Polimerase Chain Reaction (Reação em Cadeia da Polimerase)
pH	Potencial Hidrogeniônico
PPGCV	Programa de Pós-Graduação em Ciências Veterinárias
qPCR	Quantitative Polimerase Chain Reaction (Reação em Cadeia Polimerase Quantitativa)
RNA	Ribonucleic Acid (Ácido Ribonucléico)
RNAm	Messenger Ribonucleic Acid (Ácido Ribonucléico mensageiro)
RT	Room temperature (temperature ambiente)
RT-PCR	Reverse Transcription Polymerase Chain Reaction (Transcrição Reversa - Reação em Cadeia da Polimerase)
s	Segundo
TRA	Técnica de Reprodução Assistida
TCM-199^{+}	Tissue culture medium 199
UECE	Universidade Estadual do Ceará
α-MEM	Minimum Essential Medium Alpha (Meio Essencial Mínimo alfa)
µg	Microgramas

SUMÁRIO

1	**INTRODUÇÃO.....**	25
2	**REVISÃO DE LITERATURA.....**	26
2.1	O OVÁRIO MAMÍFERO E OS PROCESSOS DE OOGÊNESE E FOLICULOGÊNESE.....	27
2.2	CARACTERIZAÇÃO E CLASSIFICAÇÃO DOS FOLÍCULOS OVARIANOS.....	28
2.3	CULTIVO IN VITRO DE FOLÍCULOS PRÉ-ANTRAIS.....	29
2.3.1	**Importância para a pesquisa básica e para a preservação da função reprodutiva das fêmeas.....**	30
2.3.2	**Papel do Hormônio Folículo Estimulante (FSH) no cultivo *in vitro* de folículos pré-antrais.....**	31
2.3.3	**Substâncias fitoterápicas alternativas como estimulantes do desenvolvimento de folículos pré-antrais.....**	32
2.4	SISTEMAS DE CULTIVO IN VITRO DE FOLÍCULOS PRÉ-ANTRAIS...	33
3	**JUSTIFICATIVA.....**	34
4	**HIPÓTESES CIENTÍFICAS.....**	35
5	**OBJETIVOS.....**	36
5.1	OBJETIVO GERAL.....	37
5.2	OBJETIVOS ESPECÍFICOS.....	38
6	**CAPÍTULO 1: Reports on *in vivo* and *in vitro* contribution of medicinal plants to improve the female reproductive function……...**	39
7	**CAPÍTULO 2: *Justicia insularis* improves the *in vitro* survival and development of ovine preantral follicles enclosed in ovarian tissue……**	40
8	**CAPÍTULO 3: Supplementation of *in vitro* culture medium with FSH to grow follicles and mature oocytes can be replaced by extracts of *Justicia insularis*.....**	41
9	**CAPÍTULO 4: Early preantral follicles have a potential to grow until antral stage in two-step culture system in the presence of aqueous extract *Justicia insularis*. .....**	42
10	**CONCLUSÕES.....**	43
11	**PERSPECTIVAS.....**	44
	REFERÊNCIAS.....	45

1 INTRODUÇÃO

O cultivo in vitro de folículos pré-antrais, também conhecido como ovário artificial, é uma parte importante na biotécnica de Manipulação de Oócitos Inclusos em Folículos Ovarianos Pré-antrais (primordiais, intermediários, primários e secundários), também conhecida como MOIFOPA. Esta biotecnologia tem se tornado uma importante ferramenta para a elucidação dos mecanismos básicos, envolvidos na foliculogênese ovariana inicial ou pré-antral (ARUNAKUMARI et al., 2010). Uma vez que os eventos, fatores e mecanismos envolvidos na transformação de um folículo primordial até o seu estágio final de desenvolvimento (folículo pré-ovulatório), sejam conhecidos e, possam ser controlados in vitro, o cultivo de folículos pré-antrais, especialmente, os secundários, pode se tornar uma alternativa para a preservação da fertilidade feminina. Dessa forma, essa biotécnica poderá ser indicada para mulheres com dificuldades na função reprodutiva natural, como aquelas submetidas a tratamentos contra o câncer ou outras enfermidades e, que por alguma razão, não podem receber um transplante ovariano (SHEA et al., 2014). Sob o aspecto da reprodução animal, o cultivo in vitro de folículos secundários também poderá ser uma excelente alternativa para a reprodução assistida de espécies em risco de extinção ou economicamente valiosas, como a espécie ovina.

O completo crescimento in vitro de folículos pré-antrais ainda seja um grande desafio para a reprodução assistida, alguns casos de sucesso já foram relatados na literatura. Por exemplo embriões já foram obtidos após fecundação in vitro (FIV) de oócitos oriundos de folículos secundários cultivados in vitro em espécies como murinos (camundongos: CORTVRINDT et al., 1996 e ratos: KIM et al., 2009), suínos (WU et al., 2001), bubalinos (AGARWAL et al., 2014) e caprinos (SARAIVA et al., 2010; MAGALHÃES et al., 2011). Em ovinos, folículos secundários cultivados por 18 dias na presença do fator inibitório de leucemia (LIF) e do hormônio folículo estimulante (FSH) deram origem a oócitos, que após ativação partenogenética, resultaram na produção de embriões partenotos de 8 células (LUZ et al., 2012). Todos esses resultados tenham sido animadores, muitos fatores durante o cultivo in vitro são preocupantes e necessitam ser controlados para a obtenção de melhores resultados. Dentre esses fatores, destaca-se a produção de espécies reativas de oxigênio (Reactive Oxigem Species - ROS), as quais podem afetar o crescimento e a sobrevivência folicular. O ácido ascórbico, geralmente, tem sido o antioxidante de eleição nos protocolos de cultivo in vitro de folículos pré-antrais (MURRAY et al., 2001; TALEBI et al., 2012; LUZ et al., 2012), porém substâncias

naturais com intensa ação antioxidante, como o extrato aquoso de Justicia insularis, podem ser uma excelente alternativa às substâncias sintéticas, obtidas comercialmente.

A Justicia insularis T. Anders (família Acanthaceae) é uma planta herbácea e perene, amplamente distribuída na área tropical da África (GOKA et al., 2016), com o gênero Justicia sendo também encontrado em outros continentes, incluindo a América do Sul (OLIVEIRA et al., 2000; CORRÊA; ALCÂNTARA, 2012). Além de atuar como antioxidante, já foi relatado que a J. insularis exerce um efeito estimulador sobre a foliculogênese e a esteroidogênese ovariana in vivo (TELEFO et al., 2012), atuando como fitohormônio (KURZER; XU, 1997). Estudos realizados por TELEFO et al. (1998, 2012) e GOKA et al. (2016, 2018) revelaram que a J. insularis promoveu o desenvolvimento folicular in vivo. Portanto, com base nos efeitos positivos da J. insularis observados in vivo, acreditamos que o extrato aquoso dessa planta, se adicionado ao meio de cultivo in vitro de folículos pré-antrais ovinos, poderá também estimular o desenvolvimento dessa categoria folicular, seja como fitohormônio, seja como agente antioxidante.

Além do meio de cultivo, o sistema de cultivo de folículos ovarianos deverá ser o mais apropriado possível, ou mais próximo da condição fisiológica do indivíduo. Um estudo realizado no início dos anos 2000 relatou o nascimento de camundongos a partir de folículos pré-antrais desenvolvidos in vitro, utilizando um sistema de cultivo de dois passos. Nesse sistema, incialmente, o tecido ovariano foi cultivado por 8 dias, em seguida, folículos secundários foram isolados do ovário e cultivados por um período de 14 dias até a obtenção dos oócitos aptos à maturação, seguido de FIV (O'BRIEN et al., 2003). Outras tentativas foram relatadas nas espécies bovina (MCLAUGHLIN et al., 2010) e humana (MCLAUGHLIN et al., 2018) e, embora, esses estudos não tenham obtido nascimentos, os dados mostraram que os folículos primordias destas espécies podem se desenvolver in vitro e resultar na produção de oócitos com diamêtro superior a 100 μm. Portanto, nós acreditamos que o cultivo de dois passos também pode ser o caminho a ser seguido para o cultivo de folículos pré-antrais ovinos, o qual também foi realizado na presente tese.

Com a finalidade de mostrar a importância da realização deste trabalho, inicialmente, será apresentada uma revisão de literatura abordando alguns aspectos sobre a gônada feminina e os processos de oogênese e foliculogênese; o papel do FSH na foliculogênese; relatos do efeito da Justicia insularis sobre a função reprodutiva da fêmea; as ROS; o cultivo in vitro de folículos pré-antrais e os diferentes sistemas de cultivo in vitro. Além

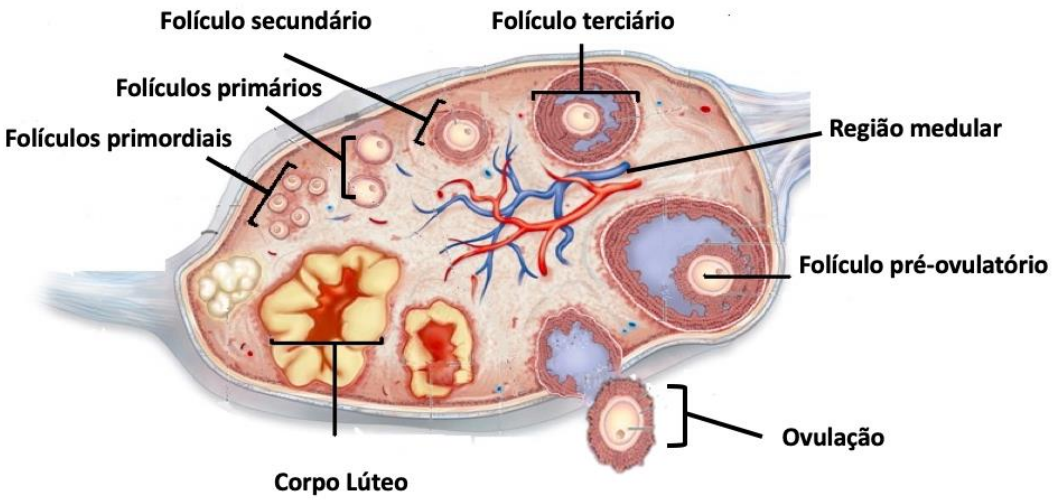
disso, será mostrada também a contribuição científica desta tese para o uso de Justicia insularis como suplementação alternativa para o meio de cultivo in vitro de folículos pré-antrais ovinos.

2 REVISÃO DE LITERATURA

2.1 O OVÁRIO MAMÍFERO E OS PROCESSOS DE OOGÊNESE E FOLICULOGÊNESE

O ovário (gônada feminina) é uma palavra derivada do termo “ovum” que em latim significa ovo (EDSON et al., 2009). Trata-se de um órgão complexo que assegura várias funções essenciais para os processos reprodutivos (VANORNY; MAYO, 2017). Esse orgão é constituído por duas regiões diferenciadas: a medula, que na maioria das espécies ocupa a região interna do ovário, formada principalmente por tecido conjuntivo, nervos e vasos sanguíneos; e o córtex, é a região externa do ovário, em que se situam os folículos ovarianos e corpos lúteos em diferentes estágios de desenvolvimento (GEORGES et al., 2014), como mostra a figura 1. 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 (CORTVRINDT; SMITZ, 2001).

Figura 1 - Imagem ilustrativa da organização e estrutura dos folículos presentes no ovário. Notar a presença de folículos ovarianos em diferentes estágios de desenvolvimento.



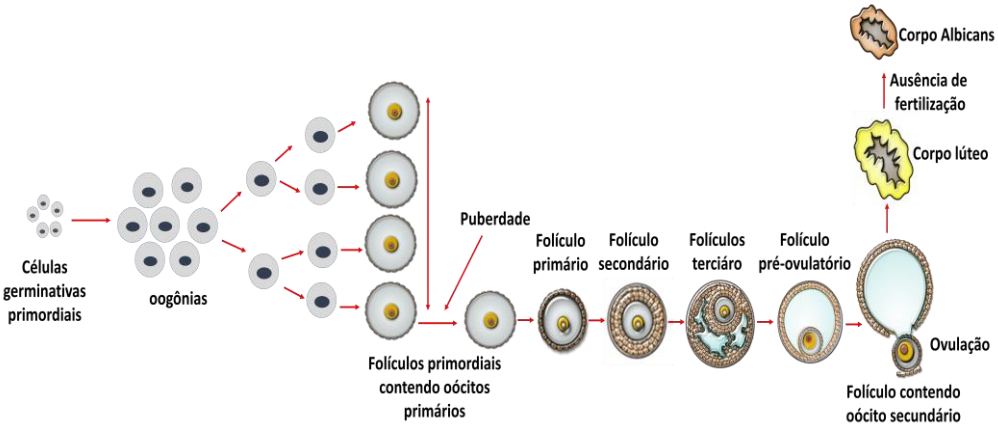
Fonte: Adaptado http://faculty.southwest.tn.edu/rburkett/A&P2 reproductive system lab.htm

O ovário é responsável pela importante função de produção e liberação de gametas funcionais, isto é, os oócitos. Além disso, a gônada feminina também desenvolve uma função

endócrina crítica, incluindo a produção de estrógenos, os quais são essenciais para o desenvolvimento da genitália feminina, e progesterona, que é responsável pelo estabelecimento da gestação (GEORGES et al., 2014). Essas funções são fortemente coordenadas por dois processos que ocorrem simultaneamente no ovário, ou seja, os processos de oogênese e foliculogênese.

De uma maneira bem resumida, a oogênese consiste na formação e diferenciação das células germinativas primordiais (CGP), culminando com a formação do oócito haplóide fecundado (VAN DEN HURK; ZHAO, 2005). No embrião, as CGP presentes na parede do saco vitelínico, deslocam-se para as gônadas em desenvolvimento, sofrem extensiva proliferação mitogênica e redistribuição das organelas citoplasmáticas, transformando-se em *oogônias* (SADEU et al., 2006). As oogônias, por sua vez, proliferam-se por mitose, entrando em seguida em meiose e, então, diferenciam-se em oócitos primários, os quais passam pelos estádios da prófase I (leptóteno, zigóteno, paquíteno e diplóteno) da primeira divisão meiótica (HIRSHFIELD, 1991). No estádio de diplóteno ou vesícula germinativa, ocorre a primeira interrupção da divisão meiótica e os oócitos permanecem neste estádio até a puberdade (Figura 2).

Figura 2 - Imagem ilustrativa do processo de oôgenese. Notar a sequência evolutiva desde células germinativas primordiais até a ovulação.

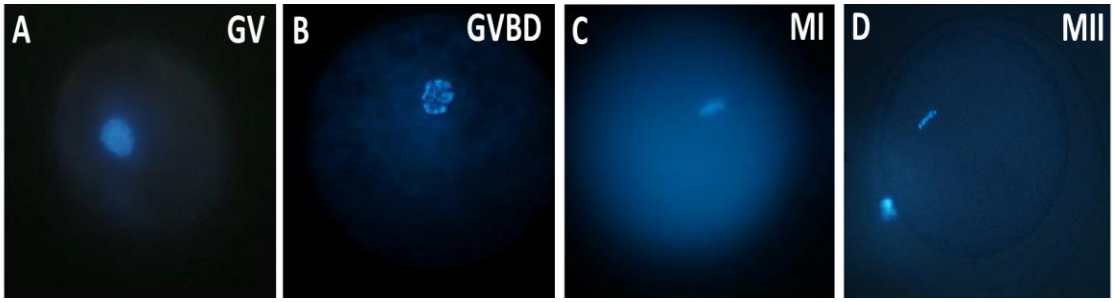


Fonte: Adaptado de GEORGES et al., 2014.

A partir deste período, com o pico do FSH e do hormônio luteinizante (LH) durante a puberdade, os oócitos crescidos retomam a meiose e o núcleo passa do estádio de vesícula germinativa para diacinese. Em seguida, ocorre a ruptura da vesícula germinativa, progressão para metáfase I, anáfase I, telófase I, expulsão do primeiro corpúsculo polar e formação do oócito secundário, iniciando a segunda divisão meiótica. Nesse momento, o núcleo do oócito

evolui até o estádio de metáfase II (Figura 3), quando ocorre a segunda interrupção da meiose (SÁNCHEZ; SMITZ, 2012). O oócito permanece neste estádio até ser fecundado pelo espermatozóide, quando então completa a meiose e expulsa o segundo corpúsculo polar, formando o oócito haplóide fecundado. Vale ressaltar que na maioria das espécies, oogênese acontece de forma simultânea à foliculogênese (VAN DEN HURK; ZHAO, 2005).

Figura 3 - Oócitos marcados pelo Hoechst 33342, indicando as diferentes fases do processo de maturação nuclear oocitária. (A) GV: vesícula germinativa, (B) GVBD: quebra da vesícula germinativa, (C) MI: metáfase I, (D) MII: metáfase II.

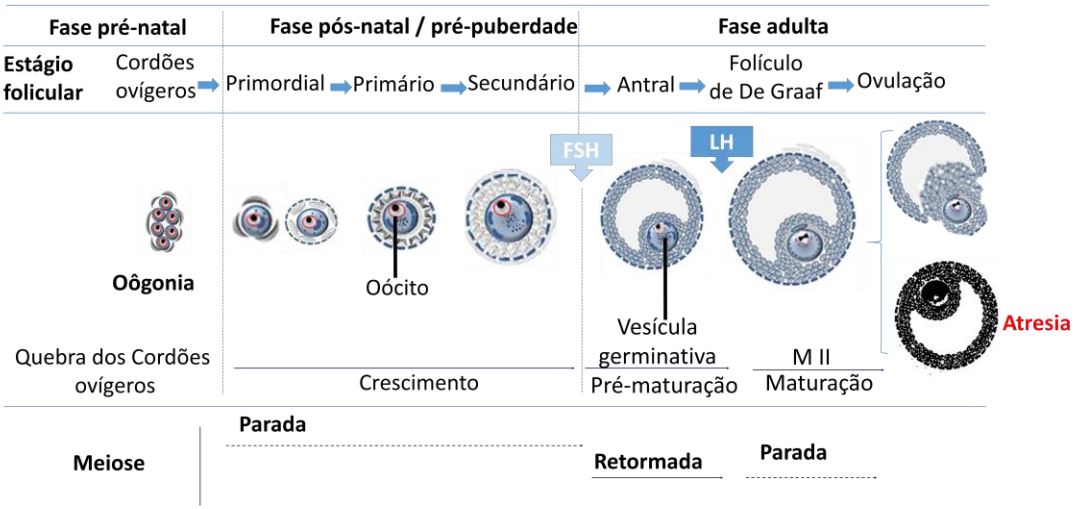


Fonte: Elaborado pelo próprio autor.

A foliculogênese, por sua vez, 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, como mostrado na figura 4 (VAN DEN HURK; ZHAO, 2005). Os foliculos ovarianos podem ser classificados de acordo com a ausência ou a presença do antro, 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* e estão detalhadamente descritos nos tópicos a seguir.

Em relação aos folículos antrais, à medida que o folículo cresce e se desenvolve, uma cavidade repleta de fluido folicular denominada antro começa a se formar e, a partir deste estádio, os folículos passam a ser classificados como antrais. Os folículos antrais, por sua vez, podem ainda ser classificados em terciário e pré-ovulatório. O fluido antral é um composto rico em substâncias reguladoras derivadas do sangue ou secreções das células foliculares, como por exemplo, gonadotrofinas, esteróides e fatores de crescimento. A produção desse fluido é intensificada pelo aumento da vascularização folicular e permeabilidade dos vasos sangüíneos (VAN DEN HURK; ZHAO, 2005), que ocorre com o desenvolvimento do folículo. Neste estádio, as células da granulosa são diferenciadas em células do cumulus (mais próximas ao oócito) e células murais.

Figura 4 - Imagem ilustrativa da foliculogênese ovariana. Notar as etapas desde a formação dos folículos primordiais até o desenvolvimento do folículo de De Graaf ou pré-ovulatório.



Fonte: Adaptado de KNOBIL; NEILL, 2015.

2.2 CARACTERIZAÇÃO E CLASSIFICAÇÃO DOS FOLÍCULOS OVARIANOS

Os folículos primordiais são constituídos por um oócito primário circundado por uma camada de células da pré-granulosa planas e são os folículos de menor tamanho e mais numerosos no ovário mamífero (90% da reserva folicular), os quais constituem o *pool* de folículos quiescentes (BECKERS et al., 1996). Durante toda a vida da fêmea, um pequeno grupo de folículos primordiais é gradualmente estimulado a crescer, constituindo a etapa de ativação folicular. Após a ativação, ocorre uma série de eventos que promovem a mudança da morfologia e o aumento do número de células da granulosa, formação da zona pelúcida e aumento do diâmetro oocitário. Esses eventos levam à formação das demais categorias de folículos pré-antrais: os folículos de transição (oócito circundado por células da granulosa pavimentosas e cúbicas), primários (oócito circundado por uma camada completa de células da granulosa de morfologia cúbica) e secundários (várias camadas de células da granulosa cúbicas ao redor do oócito com a zona pelúcida claramente identificada ao redor do oócito) (SILVA et al., 2004). Uma vez ativados, os folículos são recrutados para o desenvolvimento e maturação, ou alternativamente, são perdidos pelo processo fisiológico denominado atresia (Figura 4) (MCGEE; HSUEH, 2000). Com o crescimento dos folículos secundários e organização das células da granulosa em várias camadas, ocorre a formação do antro. A partir deste estágio, os folículos passam a ser denominados de folículos terciários ou antrais, cujo diâmetro folicular

aumenta acentuadamente devido ao crescimento do oócito, multiplicação das células da granulosa, da teca e aumento do fluido na cavidade antral (DRIANCOURT, 1991). Pequenos folículos antrais podem ser similares aos folículos secundários quanto ao diâmetro (~200 μm), mas eles aumentam rapidamente em tamanho com o contínuo acúmulo de fluido folicular. Os grandes folículos antrais geralmente possuem diâmetro acima de três milímetros e contêm células da granulosa diferenciadas em células do cumulus e células murais e muitas camadas de células tecais (BRISTOL-GOULD; WOODRUFF, 2006). Contudo, durante toda a vida reprodutiva da fêmea, apenas um pequeno grupo de folículos, aproximadamente 0,1%, chega à ovulação (NUTTINCK et al., 1993), diminuindo assim o seu potencial reprodutivo. É importante destacar que do total da população de folículos presentes em um ovário, 90% é composta por folículos pré-antrais.

Considerando a grande população de folículos pré-antrais presentes no ovário mamífero, dezenas de estudos desde a década de 90 (HIRSHFIELD, 1991; NUTTINCK et al., 1993; BECKERS et al., 1996; LIU et al., 2001; CLARK et al., 2004) até os dias de hoje (TELFER; ZELINSKI, 2013; WINKLER-CREPAZ et al., 2014; SILVA et al., 2015; BERTOLDO et al., 2018) têm tentado elucidar como ocorre *in vivo*, o processo de desenvolvimento de folículos pré-antrais, através de sistemas de cultivo *in vitro*. As descobertas realizadas por esses estudos têm indicado que, uma vez que o ovário esteja em plena condição de higidez, os folículos pré-antrais podem ser recuperados e cultivados *in vitro*. Essa técnica pode ser uma alternativa promissora para a perpetuação de gerações futuras de uma fêmea, mesmo após a ovariectomia ou após a morte.

2.3 CULTIVO IN VITRO DE FOLÍCULOS PRÉ-ANTRAIS

Nos últimos anos, muitos avanços foram alcançados através da aplicação do cultivo *in vitro* de folículos ovarianos pré-antrais, sobretudo no que concerne à identificação de substâncias necessárias ao meio de cultivo visando a ativação de folículos primordiais (cultivo de tecido ovariano), bem como, o crescimento e o desenvolvimento de folículos, especialmente os folículos secundários, fora do ambiente ovariano. Até o momento, os resultados mais animadores foram obtidos em camundongos. Inicialmente, em 1996, foi relatado o nascimento de duas crias vivas (EPPIG; O'BRIEN, 1996), porém, em 2003 o protocolo foi aprimorado e foram obtidas 59 crias (O'BRIEN et al., 2003). No entanto, em mamíferos de grande porte, os principais resultados podem ser resumidos somente à produção de um pequeno e variado

número de oócitos em metáfase II (MII) e embriões (suíno: WU; EMERY; CARRELL, 2001; bubalino: GUPTA et al., 2008; ovino: ARUNAKUMARI; SHANMUGASUNDARAM; RAO, 2010; caprino: MAGALHÃES et al., 2011; SILVA et al., 2015). No entanto, os esforços e investigações envolvendo a MOIFOPA, continuam e têm fornecido informações importantes, seja na tentativa de usá-la como técnica de reprodução assistida viável, seja para a elucidação dos mecanismos envolvidos na foliculogênese e sua aplicação no futuro.

2.3.1 Importância para a pesquisa básica e para a preservação da função reprodutiva das fêmeas

Do ponto de vista da preservação da função reprodutiva, a MOIFOPA pode ser uma excelente alternativa de modo a fornecer milhares de oócitos viáveis, inclusos em folículos pré-antrais em vários estágios de desenvolvimento, para outras técnicas reprodutivas, contribuindo assim para a produção *in vitro* de embriões de espécies domésticas ou ameaçadas de extinção. Além da aplicação na reprodução animal, a MOIFOPA também pode ser aplicada em tratamentos de infertilidade, na área de reprodução assistida em humanos (FIGUEIREDO et al., 2008). Uma vez que seja estabelecido um sistema de cultivo que garanta o crescimento e desenvolvimento de folículos pré-antrais *in vitro*, capazes de fornecer oócitos fertilizáveis, a MOIFOPA também poderá ser de grande interesse para manter e preservar a função reprodutiva de mulheres que sobrevivem ao câncer, porém não podem se submeter a um transplante de tecido ovariano.

Com relação à pesquisa básica, os estudos já demonstraram alguns fatores de crescimento e hormônios que controlam o desenvolvimento de folículos pré-antrais. Dentre os fatores, destacam-se o fator de crescimento e diferenciação 9 (GDF-9); a proteína morfogenética óssea 15 (BMP-15); o fator de crescimento semelhante à insulina-1 (IGF-1); o fator de crescimento epidermal (EGF); o kit ligand (KL), dentre outros. Estes fatores de crescimento também são envolvidos na proliferação de células da granulosa e sobrevivência folicular, sobretudo na relação molecular estabelecida entre oócito e células da granulosa durante o progresso do crescimento folicular, aumentando dessa forma o diâmetro folicular e oocitário, bem como a taxa de formação antro (ITOH et al., 2002; PANGAS, 2012). Estudos mostraram que o GDF-9 e a BMP-15 induzem a ativação de folículos primordiais em roedores (NILSSON; SKINNER, 2002), humanos (HREINSSON et al. 2002) e caprinos (MARTINS et al., 2007; CELESTINO et al., 2011; ALVES et al., 2013). Estes fatores também desempenham

um papel durante a maturação *in vitro* dos oócitos e subsequente viabilidade fetal (YEO et al., 2008; HUSSEIN et al., 2005). Adicionalmente, a BMP-15 tem mostrado uma potente estimulação do RNAm para KL nas células da granulosa, que é necessário para o crescimento de oócitos de folículos pré-antrais (OTSUKA; SHIMASAKI, 2002). O KL, por sua vez, tem sido implicado na migração, proliferação e sobrevivência de CGP (ZAMA; HUDSON; BEDELL, 2005), na proliferação das células da granulosa (OTSUKA; SHIMASAKI, 2002), no crescimento e sobrevivência do oócito (JIN et al., 2005) e na ativação de folículos primordiais (PARROTT; SKINNER, 1999), dentre outras funções.

Nos ovários, o GDF-9 é expresso nos oócitos de folículos primordiais de ovelhas e vacas (BODENSTEINER et al., 1999). Entretanto, em mulheres (AALTONEN et al., 1999), camundongos (ELVIN et al., 1999) e ratas (JAATINEN et al., 1999), a expressão desta proteina começa no folículo primário. Já em caprinos, SILVA et al. (2004) revelou a presença de mRNA de GDF-9 em folículos primordiais, primários, secundários; oócitos e células da granulosa de folículos antrais e também no corpo lúteo. Nesta mesma espécie, a expressão da proteina BMP-15 foi encontrada nos oócitos de todos os tipos de folículos, mas não em folículos primordiais (SILVA et al., 2004). Ambos os receptores de IGF-1 e EGF estão presentes em células da granulosa de folículos pré-antrais e antrais em caprinos (SILVA et al., 2004; 2006) e em ovinos (ARMSTRONG et al., 2000). O KL, por sua vez é expresso nas células da granulosa de várias espécies (rato: ISMAIL; DUBE; VANDERHYDEN, 1996; ovino: TISDALL et al., 1999; camundongo: DONEDA et al., 2002; humano: HOYER; BYSKOV; MOLLGARD, 2005 e caprino: SILVA et al., 2006).

Além dos fatores de crescimento, o papel do hormônio folículo estimulante (FSH) tem sido amplamente investigado em diferentes estudos, em função do seu importante efeito sobre o desenvolvimento dessa categoria folicular, razão pela qual será discutido no tópico abaixo.

2.3.2 Papel do Hormônio Folículo Estimulante (FSH) no cultivo in vitro de folículos pré-antrais

O FSH é um hormônio glicoproteico heterodimérico secretado por células gonadotrópicas na hipófise anterior que coordena, não somente a foliculogênese, como também a ovulação (FILATOV et al., 2017). Este hormônio consiste em uma família de isoformas e é liberado na velocidade em que é produzido, embora uma pequena parcela possa ser armazenada

para ser liberada em resposta ao hormônio liberador de gonadotrofinas, ou seja, o GnRH (FARNWORTH, 1995; FILATOV et al., 2017). O FSH atua através da ligação com os seus receptores, que estão acoplados à proteína G (HAUACHE, 2001; FILATOV et al., 2017). De acordo com alguns estudos, os receptores de FSH (R-FSH) são expressos nas células da granulosa de folículos primordiais, primários, secundários (XU et al., 1995; BARROS et al., 2013) e também em oócitos de folículos primordias e primários (ROY, 1993; BARROS et al., 2013). A interação do FSH com seu receptor inicia uma cadeia de reações intracelulares que incluem a ativação de mais de 100 genes que codificam diferentes respostas (HUNZICKER-DUNN; MAIZELS, 2006), tais como a estimulação da proliferação celular, a síntese de esteroides e a expressão de receptores para o EGF, o IGF-1 e o hormônio luteinizante ou LH (VAN DEN HURK; ZHAO, 2005). O FSH é conhecido por ser o principal regulador do desenvolvimento folicular *in vivo* e i*n vitro*. *In vivo*, os R-FSH podem regular as várias fases do desenvolvimento do folículo em resposta à liberação periódica do FSH pituitário (WIGGLESWORTH et al., 2015). Segundo FOREST e BERTRAND (1986), em ratas pré-púberes, em sinergia com o estrógeno, o FSH estimula a proliferação de células da granulosa durante o desenvolvimento folicular e a consequência desse efeito é o aumento do peso e síntese de proteínas nos ovários.

Os melhores resultados com cultivo *in vitro* de folículos ovarianos pré-antrais na literatura têm em comum a presença de FSH adicionado ao seu meio de cultivo. Tanto o nascimento de crias vivas em camundongos a partir de folículos primordiais (EPPIG; O'BRIEN, 1996, O'BRIEN, 2003), como a produção de embriões oriundos de folículos pré-antrais em ovinos (ARUNAKUMARI et al., 2010), caprinos (SARAIVA et al., 2010; SILVA et al., 2015) e búfalas (GUPTA et al., 2008) usaram FSH como suplemento do meio de cultivo *in vitro*. Além disso, em bovinos o FSH promoveu crescimento, sobrevivência, formação de antro e secreção de esteroides em folículos primários e secundários isolados (GUTIERREZ et al., 2000; ITOH et al., 2002). Em equinos, o FSH promoveu a ativação e desenvolvimento de folículos primordiais, aumentou a sobrevivência dos folículos pré-antrais e manteve a produção de estradiol e ROS no tecido ovariano após 7 dias de cultivo *in vitro* (AGUIAR et al., 2016).

Devido à indisponibilidade de FSH para cada espécie estudada, o FSH recombinante tem sido amplamente utilizado, como alternativa. Nesse sentido, fitohormônios, sobretudo com ação similar à do FSH também têm sido investigados com a finalidade de serem também utilizados, alternativamente a este hormônio.

2.3.3 Substâncias fitoterápicas alternativas como estimulantes do desenvolvimento de folículos pré-antrais

A fitoterapia pode ser definida como o uso de plantas medicinais na prevenção, alívio ou cura de doenças. Uma planta pode ser considerada medicinal quando pelo menos uma de suas partes tem uma ou mais propriedades médicas (AKOKA; AKOKA, 1972). Existe um interesse crescente no uso de produtos naturais, principalmente os derivados de plantas. De acordo com GURIB-FAKIM (2006), os produtos naturais e seus derivados representam mais de 50% de todas as fármacos utilizadas no mundo, e as plantas medicinais contribuem com 25% deste total.

Plantas medicinais são usadas por curandeiros tradicionais para resolver problemas de infertilidade e isso é devido ao seu poderoso efeito regulador na função reprodutiva, atuando direta ou indiretamente no eixo hipotálamo-hipófise-ovário por indução ou inibição da ovulação e esteroidogênese, interrompendo assim o funcionamento hormonal do hipotálamo e hipófise (TELEFO, 1998). Seu uso pode trazer respostas diretas para alguns problemas de saúde, como distúrbios reprodutivos. O uso de plantas medicinais em resposta a problemas reprodutivos pode ser visto como uma alternativa aos medicamentos industrializados, especialmente nos países em desenvolvimento, onde estes fármacos são caros e/ou inacessíveis (RATES, 2001). Diversos estudos mostraram a implicação do uso de compostos naturais na reprodução da mulher, mostrando a sua atuação direta nos órgãos reprodutivos ou indireta no processo fisiológico relacionado. Mais informações sobre a contribuição *in vivo* e *in vitro* de plantas medicinais para melhorar a função reprodutiva de fêmeas encontram-se compiladas no artigo de revisão (Capítulo 1), que compõe a presente tese.

Embora a função ovariana seja um processo controlado por hormônios secretados essencialmente por células animais, como é o caso do FSH e outros hormônios, algumas substâncias produzidas por células vegetais podem exercer algum efeito sobre a função reprodutiva da fêmea, como é o caso de alcalóides, flavonoides, terpenóides e glicosídios, presentes em plantas como a *Justicia insularis.* Esses alcaloides, flavonoides, terpenoides e glicosídios atuam como antioxidantes.

Os antioxidantes naturais são moléculas encontradas livremente na natureza em plantas, frutas e sementes (BAUER et al., 2001). Eles possuem várias estruturas e são compostos de metabólitos secundários que podem atuar como antioxidantes, generalmente através de sua capacidade de eliminar as ROS (HARBORNE, 1999). A classificação de metabolitos secundários é derivada de vias biossintéticas. Na farmacognosia, os metabólitos

secundários podem ser classificando em compostos: fenólicos, alcalóides e compostos terpenoides (HARBORNE, 1999), os quais são encontrados no extrato aquoso de *Justicia insularis* (TELEFO et al., 2004; GOKA et al., 2016).

Muitos compostos naturais com capacidade antioxidante têm sido extraídos e utilizados no cultivo *in vitro* de folículos ovarianos (HUTT; ALBERTINI, 2007; RAJABI-TOUSTANI et al., 2013; ABEDI et al., 2014). Dentre eles, o anetol ou trans-anetol, um composto natural derivado da planta *Croton zehntneri*, caracterizado por ser uma substância antioxidante aromática natural derivada do fenilpropano, e que está presente numa variedade de extratos de plantas medicinais (POLZIN *et al.*, 2007). A adição de diferentes concentrações de anetol (30, 300, 2000 μg/mL) ao meio de cultivo de folículos secundários caprinos foi capaz de produzir um maior *pool* de oócitos meioticamente competentes, sinalizando, assim, para o importante papel do anetol no desenvolvimento folicular e oocitário *in vitro* (SÁ et al., 2016). Neste estudo, vale resaltar que somente a concentração de 300 μg/mL reduziu o nível de ROS no dia 6 de cultivo.

Outro composto natural usado com meio de base de folículos pré-antrais caprinos e ovinos é a *Amburana cearensis* (Allemão) A.C. Smith (Fabaceae). BARBERINO et al. (2015) mostraram que 0.1 mg/mL do extrato etanólico bruto das folhas desta planta manteve a sobrevivência folicular e promoveu desenvolvimento *in vitro* de folículos secundários isolados de ovinos. Já em caprinos, 0.2 mg/mL do mesmo extrato mostrou resultados semelhantes aos ovinos (GOUVEIA et al., 2016). Além disso, 0,2 mg/mL do extrato manteve a morfologia folicular durante o transporte de folículos pré-antrais caprinos inclusos no tecido ovariano por até 6 h a 4 °C (GOUVEIA et al., 2015). Na espécie caprina, um trabalho realizado por nosso grupo investigou o efeito da *Auxemma oncocalyx* (A. oncocalyx) e seu composto ativo, a *Oncocalyxone A* (onco A), sobre o cultivo *in vitro* de folículos pré-antrais (LEIVA-REVILLA et al., 2016). Os resultados mostraram que a *Auxemma oncocalyx* (1,2; 12; e 34 g/mL) e *Oncocalyxone A* (1, 10, e 30 g/mL) afetaram de uma forma concentração dependente a sobrevivência e desenvolvimento de folículos pré-antrais caprinos inclusos no tecido ovariano cultivados por 1 e 7 dias. Em um outro estudo, *Auxemma oncocalyx* (1,2 g/mL) e *Oncocalyxone A* (1 g/mL) não apresentaram efeito tóxico sobre o desenvolvimento de folículos secundários isolados nem sobre as taxas de maturação *in vitro* de COCs (LEIVA-REVILLA et al., 2017). No entanto, estes fármacos apresentaram uma menor percentagem de oócitos viáveis (*Auxemma oncocalyx*: 55,84 % ; *Oncocalyxone A*: 54,65%) quando comparadas ao controle (90,67%) após a MIV (LEIVA-REVILLA et al., 2017). Recentemente, SOARES et al. (2018) avaliaram o

efeito da frutalina (lectina extraída das sementes da fruta-pão de caroço-*Artocarpus incisa L*.) sobre o desenvolvimento folicular *in vitro*. Neste trabalho, o cultivo do tecido ovariano caprino por 6 dias na presença desta droga em diferentes concentrações (1, 10, 50, 100 e 200 µg/mL) resultou na redução da sobrevivência folicular em todas as concentrações testadas, sendo seus efeitos mais pronunciados em altas concentrações (50, 100 e 200 µg/mL).

Um outro exemplo de planta medicinal é a *Justicia insularis*, que é uma planta herbácea e perene de 30 a 75 cm de altura com ramos ascendentes opostos (Figura 5), amplamente distribuída na região tropical da África (ADJANOHOUN et al., 1989). Suas folhas são simples e a flor com coloração branca, rosa ou púrpura (BERHAUT, 1971). A *J. insularis* é cultivada durante a estação chuvosa e é facilmente encontrada em savanas arborizadas e ravinas (AJIBESIN et al., 2008). No Senegal, a decocção de folhas de *J. insularis* é oferecida às mulheres durante o último mês de gravidez para reduzir as dores do parto. Na Nigéria, uma sopa feita a partir de suas folhas é usada para o tratamento de enxaquecas ou como laxante, e também como alimento substituto após o desmame (AJIBESIN et al., 2008). Na região ocidental dos Camarões, é utilizado em associação com as folhas de outras três plantas medicinais (*Aloe buettneri, Hibiscus macranthus e Dicliptera verticillata*), para tratar a dismenorreia e alguns casos de infertilidade feminina.

Figura 5 - Fotografia de *Justicia insularis* que é uma planta herbácea com folhas verdes, simples e a flor com coloração rosa.

Fonte: Elaborada pelo próprio autor.

Estudos realizados na província ocidental de Camarões mostraram o efeito positivo sobre a função ovariana de um extrato aquoso denominado ADHJ, composto, respectivamente, por 23, 20, 15 e 42% das plantas *Aloe buettneri (A), Dicliptera verticillata (D)* e *Hibiscus macranthus (H)* e *Justicia insularis (J)* (TELEFO et al., 1998; 2002; 2004). TELEFO et al. (2002) relataram que o extrato aquoso ADHJ administrado por via oral às ratas imaturas, nas doses de 13, 49 e 94 mg/kg por dia, durante 15 dias, induziu um aumento significativo do peso dos ovários e do útero, bem como induziu a produção de estradiol sérico e ovariano. Posteriormente, um estudo *in vitro* com a mistura ADHJ mostrou um efeito indutivo sobre a produção *in vitro* de estradiol em ratas imaturas, usando a concentração de 130 µg/mL (TELEFO et al., 2004).

O extrato aquoso de *J. insularis* também foi administrado sozinho à ratas imaturas a uma concentração de 5 mg/mL e induziu a abertura vaginal precoce, bem como aumento no número de pontos hemorrágicos, corpo lúteo, locais de implantação, peso ovariano, proteínas uterinas e ovarianas. Esses resultados apontaram para um efeito indutor de *J. insularis* sobre a foliculogênesis ovariana (TELEFO et al., 2012). Além disso, todos os resultados até agora obtidos foram uma indicação clara da presença de compostos estrogênicos nos extratos vegetais com efeitos semelhantes ao FSH. A análise fitoquímica dessas plantas revelou a presença de alcalóides, flavonoides, terpenóides e glicosídios (TELEFO et al., 2004; GOKA et al., 2016), os quais são moléculas com atividade antioxidante e de eliminação de ROS. Tais compostos são conhecidos por apresentarem uma atividade antioxidante contra os radicais livres (CAI; SUN; CORKE, 2003).

Embora, a presença de fatores de crescimento e hormônios sejam um dos fatores fundamentais para o sucesso do cultivo de folículos pré-antrais, o sistema de cultivo, ou seja, a forma como os folículos são cultivados, é também extremamente fundamental para o sucesso da técnica.

2.4 SISTEMAS DE CULTIVO IN VITRO DE FOLÍCULOS PRÉ-ANTRAIS

Diferentes protocolos de cultivo de folículos pré-antrais têm sido descritos na literatura e, conforme mostrado na figura 5, o cultivo *in vitro* desses folículos pode ser realizado no interior do tecido ovariano, conhecido também como cultivo *in situ*, o qual pode ser realizado com o ovário inteiro (DEMEESTERE et al., 2005; ABIR et al., 2006) ou em pequenos fragmentos (3 x 3 mm com 1 ou 0.5 mm de espessura) de ovário (LIMA et al., 2013;

ESMAIELZADEH et al., 2013; GUEDES et al., 2017; SILVA et al., 2018). Além do cultivo *in situ*, os folículos pré-antrais, predominantemente, os secundários podem ser cultivados isolados ou fora do ambiente ovariano, que também pode variar, ou seja, podendo ser um cultivo bi ou tridimensional, conforme descrito abaixo. Na tentativa de obter melhores resultados, os folículos pré-antrais podem ser incialmente cultivados *in situ* por um período que pode variar de 6 a 8 dias e em seguida, os folículos maiores (111 ± 1.46 µm) podem ser isolados e cultivados por um período adicional que pode variar de 4 a 15 dias. Nesse caso, o sistema é conhecido como cultivo de dois ou multi passos (EPPIG; O´BRIEN, 1996; O´BRIEN et al., 2003; TELFER et al., 2008; MCLAUGHLIN; TELFER, 2010; MCLAUGHLIN; et al., 2018).

O cultivo *in situ* é considerado de extrema importância para o estudo da ativação de folículos primordiais e do posterior crescimento de folículos primários, em várias espécies, como caprinos (CELESTINO et al., 2009), bovinos (JIMENEZ et al., 2016), babuínos (WANDJI et al., 1997), equinos (AGUIAR et al., 2016) e humanos (ZHANG et al., 2004). Além da praticidade, o cultivo *in situ* apresenta também como vantagem a manutenção do contato celular (ABIR et al., 2006) e da integridade tridimensional dos folículos. No entanto, apesar do êxito já relatado, poucos folículos primários cultivados progridem até o estágio de folículo secundário (FORTUNE, 2003).

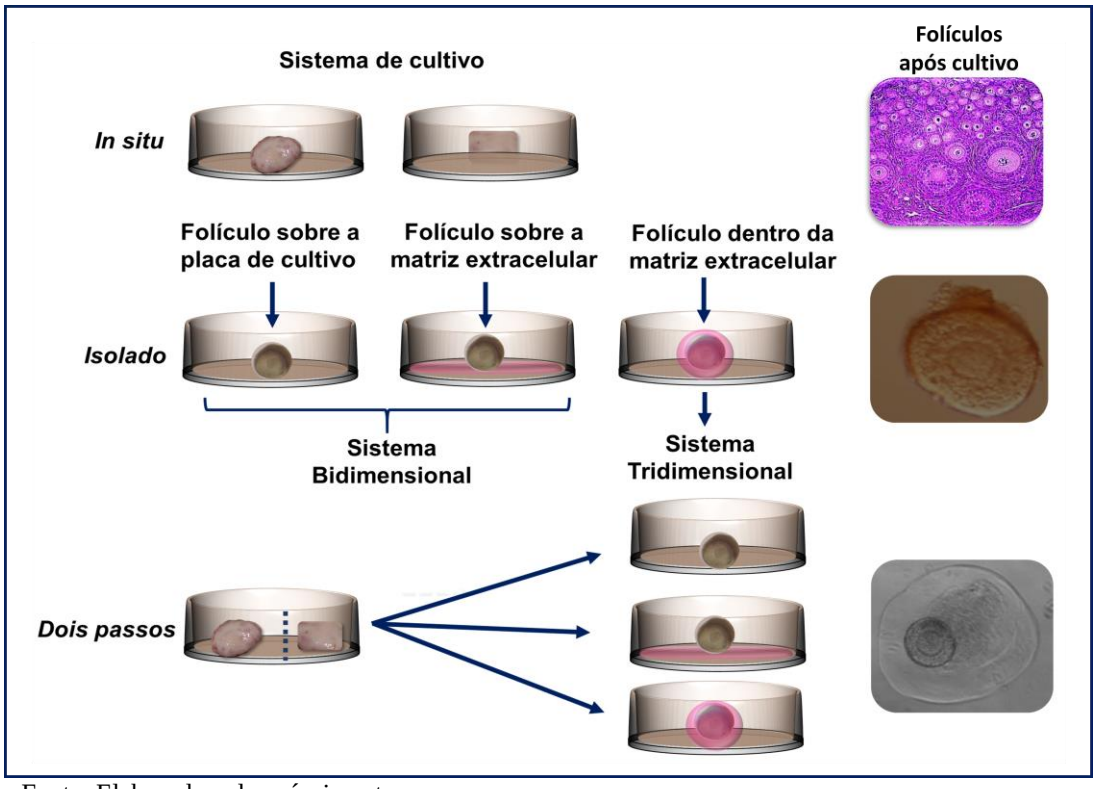
O cultivo de folículos isolados apresenta como vantagens a possibilidade do acompanhamento individual dos folículos durante o cultivo, além de oferecer também uma melhor perfusão do meio para cada folículo na placa de cultivo (ABIR et al., 2006). Outro aspecto favorável é que se caso o folículo sofra atresia durante o cultivo, as substâncias produzidas em decorrência desta morte, não afetarão outros folículos. O cultivo de folículos isolados pode ser *bidimensional* ou *tridimensional*.

No sistema bidimensional, o folículo é colocado diretamente sobre uma placa de cultivo (LUZ et al., 2012; SILVA et al., 2015; APOLLONI et al., 2015), ou sobre uma camada de células somáticas ou de matriz extracelular, como o *alginato* (VANACKER e AMORIM, 2017). Estudos em diferentes espécies revelaram que folículos secundários isolados são capazes de crescer e formar antro nesse tipo de cultivo (caprina: SARAIVA et al., 2010; ovina: ARUNAKUMARI et al., 2010; suína: WU; TIAN, 2007; bubalina: GUPTA et al., 2008).

No sistema tridimensional, os folículos, inicialmente são inseridos em uma matriz extracelular (*colágeno*: CARROLL et al., 1991; HIRAO et al., 1994; *fibrina*: BRITO et al., 2014; *alginato*: WEST et al., 2007; XU et al., 2009) ou até mesmo uma cobertura de alginato já aderida à placa de cultivo, como descrito, recentemente por XU et al. (2018) com a finalidade

de manter a morfologia original do folículo. Esse cuidado é fundamental para garantir a comunicação entre o oócito e as células somáticas que o circundam (SPEARS et al., 1994), via junções *gap*, pelas quais circulam fatores parácrinos essenciais que proporcionam crescimento e maturação do oócito (CARABATSOS et al., 2000). A perda desse contato causa a liberação de oócito atrésico (EPPIG et al., 2005). Alguns estudos mostraram que o co-cultivo com células da granulosa utilizando gel de colágeno em sistema tridimensional foi capaz de suportar o crescimento folicular em humanos (ABIR et al., 1999), suínos (HIRAO et al., 1994), murinos (OKTEM; OKTAY, 2007) e bovinos (ALM et al., 2006), possibilitando a manutenção da estrutura tridimensional do folículo até a fase antral. Embora os resultados com esse tipo de sistema tenham sido animadores, o único relato de nascimento obtido a partir de folículos pré-antrais desenvolvidos *in vitro* foi obtido utilizando o sistema de dois passos (EPPIG, O´BRIEN, 1996; O´BRIEN et al., 2003). A figura 6 representa os diferentes sistemas de cultivo de folículos ovarianos.

Figura 6 - Diferentes sistemas de cultivo de folículos ovarianos



Fonte: Elaborado pelo próprio autor.

Em 1996, os pesquisadores EPPIG e O'BRIEN publicaram a prova de que é possível obter nascimento a partir de folículos primordiais crescidos e desenvolvidos totalmente *in vitro*. Nesse estudo, os autores cultivaram incialmente folículos primordiais de camundongos, no

interior do ovário ou *in situ* (primeiro passo) durante um perído de oito dias. Posteriormente, os folículos secundários avançados foram isolados do tecido ovariano previamente cultivado e foram cultivados por um período adicional de 14 dias (segundo passo). Após o crescimento e desenvolvimento dos oócitos *in vitro*, os complexos oócito-células da granulosa foram maturados e fertilizados *in vitro*. Os embriões que clivaram para o estágio de 2 células foram transferidos para os ovidutos de fêmeas e após 19 dias de gestação, duas fêmeas produziram um filhote cada, porém apenas um sobreviveu. Resultados após um cultivo de dois passos foram relatados em humanos por TELFER et al. (2008), os quais mostraram que, sob certas condições, é possível alcançar o desenvolvimento acelerado de oócitos a partir de folículos primordiais/primários. Nesse trabalho, biópsias ovarianas de mulheres com idades entre 26 e 40 anos foram obtidas e fragmentadas. Os fragmentos foram cultivados *in vitro* por 6 dias (primeiro passo) e no final deste período, os folículos secundários (100 μm ± 3,4) foram isolados e cultivados por 4 dias (segundo passo). Os folículos secundários cresceram (diamêtro médio 143 μm ± 7.4) e formaram o antro. Recentemente, também em humanos (MCLAUGHLIN et al., 2018), biópsias ovarianas de mulheres com idades entre 25 e 39 anos foram obtidas, fragmentadas e cultivadas *in vitro* por 8 dias (primeiro passo). Após esse período, os folículos secundários com diamêtro variando entre 100–150 μm foram isolados e cultivados por 8 dias (segundo passo). Em seguida os complexos cumulus-oócitos foram recuperados e cultivados por mais 4 dias (terceiro passo). Os complexos contendo oócitos com diâmetro superior a 100 μm foram selecionados e maturados *in vitro* por 24 horas (quarto passo). A taxa de maturaçao obtida foi de 28%. Os resultados mostraram que o desenvolvimento de oócitos até a maturação meiótica pode ser alcançado a partir folículos pré-antrais presentes no tecido ovariano humano (MCLAUGHLIN et al., 2018).

3 JUSTIFICATIVA

Desde meados dos anos 1990 até os dias de hoje, muitos esforços têm sido realizados por várias equipes de pesauisadores utilizando a técnica do cultivo *in vitro* de folículos pré-antrais, em diferentes espécies (camundongos: EPPIG; SCHROEDER, 1989; HIRSHFIELD, 1991; caprinos: SILVA et al., 2015; humanos: MCLAUGHLIN et al., 2018). Devido ao grande número de folículos pré-antrais presentes no ovário, essa técnica visa o crescimento e o desenvolvimento desses folículos e, consequentemente, a obteção de um grande número de oócitos aptos à maturação e fecundação *in vitro*, culminando com a produção de embriões produzidos também *in vitro*. Uma vez que essa técnica esteja plenamente dominada, poderá ser aplicada na reprodução assistida contribuindo, portanto, para a preservação da função reprodutiva, mesmo após o abate ou morte de animais valiosos ou em risco de extinção. Sob o ponto de vista da reprodução humana, a obtenção de embriões a partir de folículos pré-antrais desenvolvidos *in vitro*, também representará um grande impacto, sobretudo, no caso de mulheres com problemas reprodutivos ou que realizaram ovariectomia e que por alguma razão não podem receber um transplante ovariano. Apesar dessa grande expectativa, apenas dois resultados impactantes em animais de laboratório (EPPIG; O'BRIEN, 1996; O'BRIEN et al., 2003) foram relatados até o presente momento.

Dentre os grandes desafios da técnica, destacam-se o *meio*, ou seja, as substâncias necessárias para a nutrição, bem como o *sistema de cultivo*, capazes de manter a sobrevivência, promover o crescimento e o desenvolvimento desses folículos e, consequentemente dos seus oócitos, *in vitro*. No que se refere ao meio de cultivo, dentre as principais substâncias adicionadas ao meio de base, merece destaque o FSH, pois estimula o desenvolvimento folicular inicial em diferentes espécies (murino: MCGEE et al., 1997; humano: WRIGHT et al., 1999; bovino: GUTIERREZ et al., 2000; suíno: MAO et al., 2002; ovino: CECCONI et al., 1999, ESMAIELZADEH et al., 2013). Geralmente, tem sido utilizado o FSH recombinante, cujo valor é elevado, em função da tecnologia empregada para produzí-lo, além disso, não existe FSH recombinante para cada espécie. Desta forma, substâncias oriundas de plantas que possam atuar de forma semelhante ao FSH poderiam ser investigadas, visando sua utilização no futuro, como uma alternativa a esse hormônio. Dentre as plantas medicinais estudadas, podemos destacar a *Justicia insularis*. O extrato aquoso dessa planta mostrou ter efeito positivo sobre a foliculogênese e a esteroidogênese ovariana, atuando como fitohormônio (TELEFO ET

al., 2004; 2012). Esses achados, sugerem que a *J. insularis* também possa estimular o desenvolvimento *in vitro* de de folículos pré-antrais.

Embora, em animais domésticos, a obtenção de embriões já tenha sido relatada a partir de folículos secundários desenvolvidos *in vitro* (caprinos: SARAIVA et al., 2010; MAGALHÃES et al., 2011; ovinos: ARUNAKUMARI et al., 2010; LUZ et al., 2012), os únicos relatos de nascimento foram em camundongos, utilizando um sistema de cultivo em dois passos (EPPIG; O'BRIEN, 1996; O'BRIEN et al., 2003). No entanto, até o presente momento, na espécie ovina ainda não foi relatada a tentativa de desenvolvimento *in vitro* de folículos pré-antrais em um sistema de dois passos.

A espécie ovina foi utilizada nesse estudo, considerando dois fatores de extrema relevância. Primeiro, por ser um modelo experimental para a espécie humana, pois o ovário de ambas as espécies apresentam similaridades em vários aspectos, como, tamanho, textura, bem como a duração do processo de foliculogênese *in vivo* (aproximadamente 180 dias). Desse modo, os dados obtidos nesse estudo poderão contribuir de maneira significativa para o avanço da MOIFOPA como uma técnica de reprodução assistida (TRA) para mulheres que necessitam de tratamentos para a infertilidade. Segundo, os resultados obtidos também poderão ser de extrema relevância para a própria espécie ovina, visando a preservação do material genético de animais de grande valor zootécnico ou risco de extinção. Devido ao valor econômico da espécie ovina para o agronegócio brasileiro, espera-se ainda contribuir com o aumento no número efetivo de animais do rebanho ovino, especialmente na região Nordeste, onde a cultura do consumo da carne ovina é amplamente difundida. O desenvolvimento dessa TRA pode ser também, no futuro, uma ferramenta para evitar a redução de animais de raças ovinas originadas na região, como é o caso de ovinos da raça Morada Nova.

4 HIPÓTESES CIENTÍFICAS

O extrato aquoso de *Justicia insularis* adicionado ao meio de cultivo *in vitro* de folículos pré-antrais inclusos no tecido ovariano ou isolados (folículos secundários), pode conferir bons resultados e apresentar efeitos semelhantes ao FSH;

Um sistema de cultivo *in vitro* em dois passos, na presença de extrato aquoso de *Justicia insularis* pode promover o desenvolvimento de folículos pré-antrais iniciais até o estágio antral.

5 OBJETIVOS

5.1 OBJETIVO GERAL

Utilizar o extrato aquoso de *Justicia insularis* para obter o desenvolvimento de folículos pré-antrais cultivados *in vitro*, inclusos e/ou isolados do ovário ovino.

5.2 OBJETIVOS ESPECÍFICOS

Considerando a complexidade do estudo, a presente tese foi dividida em três fases, portanto, os objetivos específicos serão apresentados de acordo com cada fase.

Fase 1: Avaliar o efeito da adição de *Justicia insularis*, em diferentes concentrações, sobre os folículos pré-antrais ovinos inclusos em tecido ovariano após cultivo *in vitro* de 7 dias

- Investigar o efeito de diferentes concentrações (0,3; 1,25 e 5 mg/mL) do extrato aquoso de *Justicia insularis* no meio de cultivo *in vitro* sobre a morfologia, ativação e crescimento de folículos pré-antrais inclusos no córtex ovariano ovino;
- Identificar a melhor concentração do extrato aquoso de *Justicia insularis* e compará-la com o anetol (300 μg/mL) sobre a morfologia, ativação e crescimento folicular e densidade das células do estroma, após cultivo *in vitro* do tecido ovariano ovino;
- Dosar os níveis de ROS no meio de cultivo *in vitro* de folículos pré-antrais presentes no tecido ovariano.

Fase 2: Estudar o efeito da adição de *Justicia insularis* sobre o cultivo *in vitro* de folículos pré-antrais isolados cultivados por 18 dias

- Avaliar as taxas de sobrevivência, crescimento e formação de antro de folículos secundários isolados, cultivados *in vitro* por 18 dias, na presença de diferentes concentrações (0,3; 1,25 ou 2.5 mg/mL) de extrato aquoso de *Justicia insularis;*
- Dosar os níveis de ROS no meio de cultivo *in vitro* de folículos secundários;
- Determinar a expressão de genes relacionados com o estresse oxidativo (glutationa peroxidase – GPX), crescimento folicular (kit ligand -KL, ciclina B1 - CCNB1) e progressão da maturação oocitária (ácido hialurônico sintase-2 - HAS2) na parede folicular, após o cultivo *in vitro* de folículos secundários;

- Investigar o efeito de diferentes concentrações (0,3; 1,25 ou 2.5 mg/mL) do extrato aquoso de *Justicia insularis* sobre a viabilidade e taxas de retomada da meiose após maturação *in vitro* de oócitos crescidos *in vitro e in vivo*.

Fase 3: Investigar o sistema de cultivo em dois passos de folículos pré-antrais ovinos na presença de *Justicia insularis*

- Desenvolver um sistema de cultivo em dois passos na presença de 0,3 mg/mL de extrato aquoso de *Justicia insularis*, para o crescimento *in vitro* de folículos pré-antrais ovinos;
- Verificar se é possível isolar e cultivar folículos secundários intactos após o cultivo *in vitro* do tecido ovariano (folículos *in situ* - primeiro passo) por 7 dias;
- Avaliar se folículos secundários isolados do tecido ovariano cultivado por 7 dias (primeiro passo) são capazes de crescer e formar antro após 6 dias de cultivo *in vitro* (segundo passo);
- Determinar a capacidade antioxidante total do extrato aquoso de *Justicia insularis* no meio de cultivo *in vitro* de folículos pré-antrais *in situ* (primeiro passo) e isolados (segundo passo);
- Analisar a distribuição e atividade mitocondrial oocitária após 13 dias de cultivo *in vitro* (sistema de dois passos).

6 CAPÍTULO 1:

Relatos sobre a contribuição *in vivo* e *in vitro* de plantas medicinais na melhora da função reprodutiva feminina

"Reports on *in vivo* and *in vitro* contribution of medicinal plants to improve the female reproductive function"

Periódico: Publicado na Reprodução & Climatério, 2017. Pages 109-119.

Qualis: B4

Reports on *in vivo* and *in vitro* contribution of medicinal plants to improve the female reproductive function

Gildas Mbemya Tetaping[1], Luis Alberto Vieira[1], Francisca Geovania Canafistula[1], *Otília* Deusdênia Loiola Pessoa[2], Ana Paula Ribeiro Rodrigues[1,*]

1. Laboratory of Manipulation of Oocyte and Ovarian Preantral Follicles (LAMOFOPA), Faculty of Veterinary (FAVET), State University of Ceará, Fortaleza, Brazil.

2 Laboratory of Phytochemical Analysis of Medicinal Plants (LAFIPLAN), Federal University of Ceará, Fortaleza, Brazil

[*]**Corresponding author.**

E-mail: aprrodriguespapers@gmail.com (A.P.R. Rodrigues)

ABSTRACT

Medicinal plants are known as a prolific source of secondary metabolites which have important function both *in vivo* and *in vitro* during the ovarian folliculogenesis and steroidogenesis in many animal species. Some secondary metabolites can act as antioxidants generally through their ability to scavenge reactive oxygen species (ROS) or can regulate ovarian hormonal production. In general, these properties are responsible for the medicinal functions to treat woman infertility disorder. Some plants are constituted of biological actives substances which have been used to treat reproductive dysfunction. However, until recently, little was known about the implication of plants and/or their secondary metabolites on *in vitro* folliculogenesis and steroidogenesis. With the development of the technology, there is an increase implication of those substances in assisted reproductive technology (ART). The present review highlights some medicinal plants used in the treatment of woman disorders related to infertility. In addition, it provides an *in vivo* and *in vitro* overview of herbs and their active compounds with claims for improvement of ovarian activity thus showing their implication in female reproductive health care.

Key words: Phytotherapy, Antioxidants, Women infertility, Ovarian follicles.

RESUMO

Sabe-se que as plantas medicinais são uma fonte abundante de metabólitos secundários que têm funções importante tanto *in vivo* quanto *in vitro* durante a foliculogênese e aesteroidogênese ovarianas em muitas espécies animais. Alguns metabólitos secundários podem atuar como antioxidantes, geralmente através de sua capacidade de eliminar espécies reativas de oxigênio (ROS) ou podem regular a produção hormonal ovariana. Em geral, essas propriedades são responsáveis pelas func¸ões medicinais usadas para tratar distúrbios da infertilidade feminina. Algumas plantas contêm substâncias biológicas ativas que têm sido utilizadas para tratar a disfunção reprodutiva. No entanto, até recentemente, pouco se sabia sobre o efeito das plantas e/ou seus metabólitos secundários na foliculogênese e na esteroidogênese *in vitro*. Com o desenvolvimento da tecnologia, há uma implicação crescente dessas substâncias na tecnologia de reprodução assistida (TRA). A presente revisão destaca algumas plantas medicinais utilizadas no tratamento de distúrbios femininos relacionados à infertilidade. Além disso, fornece uma visão *in vivo* e *in vitro* de ervas e seus compostos ativos com alegações de melhora da atividade ovariana, mostrando assim seu envolvimento nos cuidados de saúde reprodutiva feminina.

Palavras-chave: Fitoterapia, Antioxidantes, Mulheres, infertilidade, Folículos ovarianos

Introduction

Infertility is a disease of the reproductive system which affects both men and women with almost equal frequency. It is a global phenomenon affecting an average of 10 % of human reproductive age population.[1] Many conditions can be associated to this problem, including intrinsic (anatomic, genetic, hormonal and immunological desorders) and extrinsic factors such as sexually transmitted infections (STIs), infections after parturition or surgery, tuberculosis of the pelvis, and obesity.[2,3]

There are a range of medical treatment options for infertility, such as the use of commercial treatments to stimulate "superovulation" which correspond to the development and release of more than one egg per ovulatory cycle. In addition, ART is commonly applied to solve infertility problems, including procedures to bring about conception without sexual intercourse. Among the available tecniques, *in vitro* maturation (IVM), i*n vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI) and intrauterine insemination (IUI)[4] are frequently applied. As an alternative, medicinal plants can also be used to solve part of the reproductive problems. Due to their chemical composition, many plants have showed beneficial properties in the folliculogenesis and steroidogenesis through their antioxidant properties and regulation of some enzyme of the steroidogenesis.[5-8]

For a better understanding of the medicinal properties of crude plant extract or secondary metabolites on the regulation of reproductive function (folliculogenesis and steroidogenesis), many *in vivo* studies have been performed.[5,6,8,9] Several studies showed that the plant secondary metabolites act either directly on ovarian cells to eliminate the ROS or through action on several enzymes such as catalase, glutathione, superoxide dismutase and glutathione peroxidase.[10-12] On the other hand, plants (infusion, decoction, beverages, crude extracts) showed their implication during the steroidogenesis through their capacity to mimic the biologic effects of endogenous hormones. These plant medicine derivatives can act by binding to their nuclear receptor or regulating the activities of key enzymes of their metabolisms.[6,13]

The present review is an attempt to consummate the available scientific information on various medicinal plants, which have been evaluated for their effect on female reproduction. Among all the female reproductive organs, only the ovary is discussed on this review since it is the site of the folliculogenesis and steroidogenesis.[14] The review also includes known evidences collected for the involvement of plant extracts *in vivo* and *in vitro*. A number of plants and/or secondary metabolites have been discussed in detail and a few others were only

tabulated; a major criterion for this arrangement was the ethnopharmacological relevance of the plant.

Mammalian ovary, folliculogenesis and ovarian follicles

The mammalian ovary is the female gonad which contains germ cells responsible for the perpetuation of the species. Furthermore, it is also the reproductive gland controlling many aspects of female development and physiology.[15] That is why it is important for the reproductive biologists to understand not only the normal functioning of the ovary but also the pathophysiology and genetics of diseases such as infertility.

The ovary consists of many types of differentiated cells, which work together, promoting an ideal environment to perform the endocrine and exocrine functions. Those functions are performed by different factors such as autocrine, paracrine, juxtacrine and endocrine are essential for ovarian folliculogenesis.[16] Folliculogenesis is the result of a complex and closely integrated series of events which start generally soon after conception. This process can be defined as the formation, growth and maturation of follicle, starting with the formation of the oocyte surrounding by the granulosa cells which formed the primordial follicles.[17]

Besides the granulosa cells, the thecal cells are recruited to the oocyte and are directly or indirectly necessary for the oocyte development, physiology and survival. The dynamic of the ovarian folliculogenesis is classified in different stages known as: a) formation of the primordial follicles; b) recruitment into the growing pool to form a primary, secondary, and tertiary follicles; c) lastly ovulation and subsequent formation of a corpus luteum.[18] In most species, the mammalian ovary shows extensive variation mainly in relation to the interstitial tissue of the organ, the so-called interstitial gland, and the degree of gonad regionalisation, which implies the existence of a cortex and a medulla.[15] The internal part consists of fibroelastic connective tissues, nerve and vascular tissues (medula) whereas the external part called the cortex is located at the outer layer and is surrounded by the germinal epithelium, which contains the ovarian follicles and corpora lutea in various stages of development or in regression[16] (figure 1).

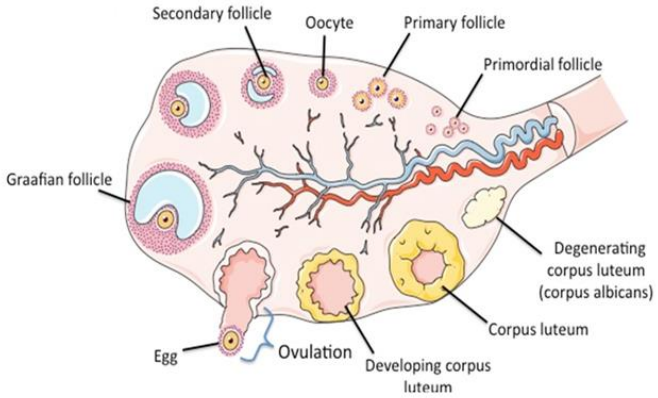


Figure 1. Schematic representation of the ovarian structure. Adapted from http://faculty.southwest.tn.edu/rburkett/A&P2_reproductive_system_lab.htm[19]

The follicle which represent the morphological and functional unit of the mammalian ovary consists of an oocyte surrounded by somatic cells (granulosa and / or theca) organized or demarcaded by the basement membrane.

Before formation of an ovarian follicle, oocytes are present within germ cell clusters. Primordial follicle formation occurs when oocytes that survive the process of germ cell cluster breakdown are individually surrounded with squamous pre-granulosa cells. This process takes place during the latter half of fetal development in humans and in the days immediately following birth in mice.[18,20] In mammals, the population of primordial follicles serves as a resting and finite pool of oocytes available during the female reproductive life span. Germ cell cluster breakdown, primordial follicle formation, and subsequent recruitment remain the least understood steps of folliculogenesis, that is why key regulators of these initial stages of follicle development continue to be identified. Furthermore, despite many unanswered questions during this crucial period, the concept of ovarian cross talk between oocytes and somatic cells is apparent from the formation of primordial follicles onward.[21,22] After differentiation of the primordial germ cells, oogonia undergo mitotic proliferation with incomplete cytokinesis, leaving daughter cells connected by intercellular bridges. The majority of germ cells in a cluster divide synchronously such that a single germ cell cluster contains 2n germ cells.[23] Germ cells subsequently enter meiosis, becoming oocytes. Individual oocytes within these nests lack surrounding somatic cells, and the majority of the oocytes will undergo apoptosis as the germ cell clusters break down to give rise to primordial follicles.

The primordial follicles represents the first category of follicles. After their formation, the granulosa cells stopped multiplying and enter a period of quiescence. Throughout the life of the female, a small group of follicles is stimulated to grow gradually, forming the activation follicular phase. The first sign of activation of primordial follicles is the resumption of proliferation of granulosa cells. Upon activation, a series of events that increases the number of granulosa cells, formation of the zona pellucida and oocyte diameter increased leading to the formation of other categories of preantral follicles, primary and secondary follicles occurs. Once activated, the follicles enter a pre-programmed course of development and maturation which is necessary for successful ovulation and fertilization or alternatively are lost through the process of atresia.[24] The second category of follicles is characterized by the organization of granulosa cells in several layers and formation of a cavity filled with follicular fluid called antrum. This follicular fluid consists of water, electrolytes, serum proteins and high concentrations of steroid hormones secreted by granulosa cells.[25] However, throughout the life of the female, only a small group of follicles, approximately 0.1 %, reached ovulation[26], thus reducing the reproductive potential of the female. In several pathological conditions, the woman can suffer of premature ovarian failure (POF) caused by different factors: endocrine, paracrine, genetic and metabolic factors such as high production of ROS.[27,28]

ROS production in the ovary

Oxidative metabolism is indispensable for energy production of ovarian follicle, which in turn results in generation of ROS (oxygen hydroxide, superoxide ion, heavy metals and free radicals). Although a critical amount of ROS is essential for their physiological activities, excessive amount of them causes oxidative stress[29], damage to mitochondria and also to cellular structures such as the membrane lipids, damage to nucleic acids and proteins.[27] It does become necessary to use antioxidants to counteract this overproduction of ROS[30].

Prevention of oxidative stress is vital in order to maintain normal reproductive function.[31] Sources of ROS during ART procedures could either be endogenously from gametes or via exogenous environmental factors.[32] However, unless measures are taken to curb ROS production, both the endogenous and exogenous sources of ROS will ultimately lead to the development of oxidative stress, which would then have negative impact on follicles development, oocyte maturation, fertilization rates and pregnancy outcome. Valorization of natural compounds of plants could improve and be an alternative to reduce the cost of ART.

Phytotherapy

Phytotherapy can be defined as the use of medicinal plants in the prevention, relief or cure of diseases. A plant can be considered as medicinal when the whole plant or at least one of its parts has one or more medical properties[33]. Medicinal plants are used by the people to treat several diseases, including to solve infertility problems. In this context, some plants are rich in compounds which exhibit regulator effect on reproductive function acting directly or indirectly on the hypothalamic-pituitary-ovarian axis by induction or inhibition of ovulation and steroidogenesis disrupting hormonal functioning of the hypothalamus and pituitary gland[5]. Their use can bring direct answers to some health problems such as reproductive disorders. The use of medicinal plants (figure 2) in response to reproductive problems can be seen as an alternative to manufactured drugs, especially in developing countries where they are expensive and/or inaccessible[34].

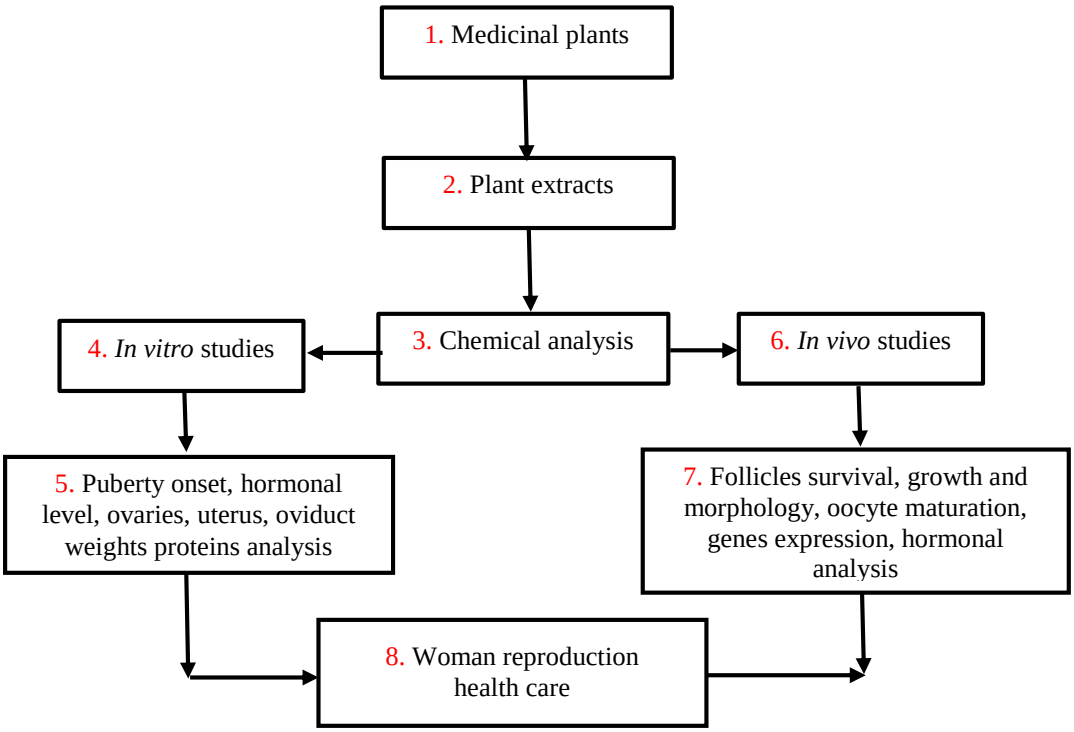


Figure 2: General scheme and sequence (from 1 to 8) of studying the *in vitro* and *in vivo* effects of plant extracts

Several studies have shown the beneficial implication of natural compounds on the woman reproduction acting directly on the reproductive organs or indirectly regulated physiological process. For example, studies by Telefo et al[35] showed that the aqueous extract of the mixture of *Aloe buettneri*, *Dicliptera verticillata*, *Hibiscus macranthus* and *Justicia insularis*, is used in traditional medicine to normalize the menstrual cycle increasing female

fertility. In addition, *Acanthus montanus, Aloe vera, Carica papaya, Citrus aurantifolia, Elaeis guineensis,* and *Panax quiquefolius, Eremomastax speciosa* are used in Nkam (Littoral region in Cameroon) with the same purpose.[36] *Asystasia vogeliana, Crinum distichum, Crinum jagus, Crassocephalum biafrae, Scoparia dulcis, Solanum torvum, Aframomum letestuanum, Aloe buettneri and Eremomastax speciosa* make part of the cast of plants most widely used to treat diseases of the reproductive system.[37] Generally, medicinal plants used for the improvement of the reproductive functions have more than one property. Table 1 illustrates some traditional medicinal plants used in the treatment of female reproductive disorders.

Table 1: Medicinal plants and their in vivo therapeutic utilization on female reproductive function

Plants	Family	Major phytochemical compounds	Used parts	Therapeutic utilization	References
Moringa oleifera	Moringaceae	Lutein, carotene xanthins, kaempferol, quercetin	Leaves	Sexual libido	Cajuday and Pocsidio[38], 2010
Adiantum concinnum	Adiantaceae	No report	Leaves and stems	Menstrual regulation	Bussmann and Glenn[39], 2010
Petroselinum crispum	Apiaceae	Flavonoids	Whole plant	Menstrual regulation	Nielsen[40] et al., 1999; Bussmann and Glenn[39], 2010
Musa sapientium	Musaceae	No report	Leaves	Menstrual regulation	Chifundera[41], 1998
Scabiosa atropurpurea	Dipsacaceae	No report	Leaves	Menstrual regulation	Bussmann and Glenn, 2010[39]
Cassia alata	Fabaceae	Tannins, saponins, flavonoids, steroids, terpenoids, alkaloids	Root	Infertility	Ramaraj[42] et al., 2014; Koch[43] et al., 2015
Ximenia americana	Olacaceae	Saponins, glycosides anthraquinones	Whole plant	Menstrual regulation	James[44] et al., 2007, Bussmann and Glenn[39], 2010
Eremomastax speciosa	Acanthaceae	Flavonoids,alkaloids, saponins, tannins	Leaves	Infertility	Adjanohoun[45] et al., 1996; Priso[36] et al., 2006

Justicia insularis	Acanthaceae	Flavonoids, alkaloids, glycosides	Leaves and stem	Infertility, menstruation unrest	Adjanohoun[45] et al., 1996; Telefo[5] et al.,1998
Crinum distichum	Amaryllidaceae	No report	Whole plant	Amenorrhea Infertility	Priso[36] et al., 2006
Ageratum conyzoides	Asteraceae	Flavonoids, alkaloids, benzofuranes, terpenes	Leaves	Infertility, infections of the genital device	Adewole[46], 2002
Senecio biafrae	Asteraceae	Dihydroisocoumarins, terpenoids,sesquiterpens, amino acids, mineral salts	Leaves and stem	Infertility	Tabopda[47] et al., 2009; Tacham[37], 2000
Emilia coccinia	Asteraceae	No report	Whole plant	Dysmenorrhea	Adjanohoun[45] et al., 1996
Zehnaria scabra	Cucurbitaceae	No report	Leaves and stem	Infertility, dysmenorrhea	Adjanohoun[45] et al., 1996
Euphorbia tirucalli	Euphorbiaceae	No report	Stem	Gonorrhea	Arbonnier[48], 2002
Jatropha curcas	Euphorbiaceae	No report	Leaves and stem	Infertility	Igoli[49] et al., 2002
Aloe buettneri	Liliaceae	Glycosides,quinines, coumarins,anthraquinonic derivatives	Leaves	Infertility, painful menstruations, dysmenorrhoea	Telefo[6] et al., 2004; Tacham[37], 2000
Paulinia pinnata	Sapindaceae	No report	Leaves	Infertility, amenorrhea, gonorrhea	Arbonnier[48], 2002

Solanum torvum	*Solanaceae*	Flavonoids, alkaloids, saponins, glycosides, tannins	Fruit	Infertility, genital infections	Tacham[37], 2000
Ampelocissus Pentaphylla	Vitaceae	No report	Leaves	Infertility	Tacham[37], 2000
Pelargonium odoratisimum	Geraniaceae	No report	Whole plant	Inflammation of the ovaries and womb	Bussmann and Glenn[39], 2010
Pelargonium roseum	Geraniaceae	No report	Flowers and leaves	Hemorrhages, uterus pain	Bussmann and Glenn[39], 2010
Krameria lappacea	Krameriaceae	No report	Leaves and root	Inflammation of the ovaries	Bussmann and Glenn[39], 2010

As can be seen from Table 1, various medicinal, plants belonging to different families showed therapeutic effects. Traditionally, rural women have used plant medicines rather than modern medicine for their personal ailments due to lack of modern facilities in the regions. Bannu region in Pakistan was ranked first having large number of gynaecological plant to medical treatment of female reproductive system: uterus, vagina, and ovaries.[50] Moreover, many women in Costa Rica consider menopause as a natural phenomenon and treat the symptoms with herbs. Interestingly, Latina women in the United States also tend not to use hormone therapy (estrogenic and/or progesteronic compounds) opting for natural remedies for menopause such as diet, exercise and herbal remedies.[51] More detailed scientific studies are desperately needed to evaluate the efficacy and safety of the remedies employed traditionally.

Secondary metabolites of plants: description and medicinal properties

Description

Secondary metabolites are structurally diverse; their classification is mainly derived from their biosynthetic pathways. In pharmacognosy, secondary metabolites are classifying in: a) phenolic; b) alkaloids and c) terpenoids compounds[52], as described below.

a) Phenolic compounds

Phenolic compounds are among the most widespread class of secondary metabolites in nature. This class of compounds are synthesized from a common precursor: the amino acids phenylalanine or tyrosine. Phenolic compounds consist of flavonoids, tannins, coumarins, quinones and anthocyanins and are regarded as the widest spread phytochemicals. Phenolic compounds may assume a wide range of structures from simple ones containing one aromatic ring only, to very complex polymeric forms.[53] The term flavonoid is a collective name for plant pigments, mostly derived from benzo-δ-pyrone.[54] They are widely distributed in plants contain free hydroxyl groups attached to the aromatic rings (Table 2). Flavonoids such as rutin, present in certain buckwheat (*Fagopyrum esculentum*) species are known to inhibit lipid oxidation by radicals scavenging.[55]

b) Alkaloids compounds

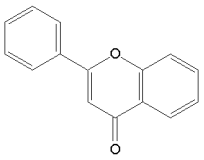
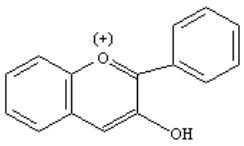
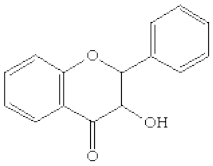
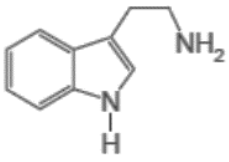
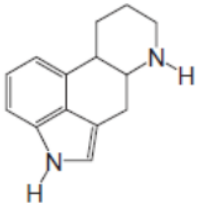
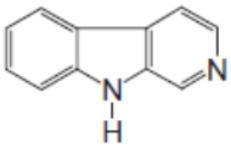
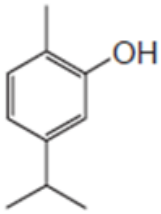
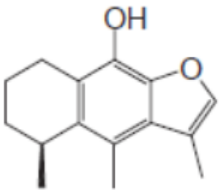
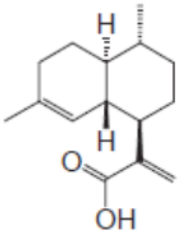
Alkaloids are nitrogenated compounds synthesized by living organisms. In general, they contains heterocyclic rings (Table 2) and due the presence of one or more nitrogen atoms, they present basic properties. The name alkaloid is directly related to the fact that nearly all alkaloids are basic (alkaline) compounds. Derived from amino acids, in general, they are pharmacologically active. Alkaloids constitutes a very large group of secondary metabolites,

with more than 12,000 substances isolated. A huge variety of structural formulas, coming from different biosynthetic pathways and presenting diverse pharmacological poperties.[56]

c) Terpenoids compounds

The terpenoids comprising monoterpenes, sesquiternes, sesterpenes and triterpenes, besides steroids, saponins and cardiac glycosides. They are considered be the phytochemicals having the most diverse chemical structures.[57] Terpenoids are the largest and most diverse family of natural products, ranging in structure from linear to polycyclic molecules and in size from the five carbon hemiterpenes to natural rubber, comprising thousands of isoprene units (Table 2). The monoterpenes and sesquiterpenes are common in essential oils produced by plants.[58]

Table 2: Basic structures of some pharmacologically plant derived flavonoids, alkaloids and terpenoids

Class	Examples			References
Flavonoids				Kumar and Abhay[59], 2013
	Flavone	Anthocyanidin	Dihydroflavone	
Alkaloids				Sarker and Nahar[60], 2007
	Tryptamine	Ergoline	β-Carboline	
Terpenoids				Sarker and Nahar[59], 2007
	Carvacrol	Cacalol	Artemisinic acid	

Medicinal properties

Crude extracts or secondary metabolites from medicinal plants can act as antioxidant agents generally through their ability to scavenge ROS or as a regulator of ovarian hormonal production. These properties can be responsible for their medicinal functions.

Gouveia[61] et al. (2015) identified and quantified five substances from *Amburana cearensis*, namely: protocatechuic acid (PCA), epicatechin, p-coumaric acid, gallic acid and kaempferol, which were identified by High Performance Liquid Chromatography (HPLC) from the crude ethanolic extract of *A. Cearensis*. Gallic acid is a well known antioxidant compound, was the main compound found in *A. cearensis.*[62] Gallic acid prevents *in vivo* and *in vitro* DNA oxidative increasing the activities of antioxidant enzymes (superoxide dismutase, GPx and glutathion-S-transferase-π) and decreasing the intracellular ROS concentrations.[63] Another compound found in the *A. cearensis* extract was the PCA, commonly found in several vegetables and fruits.[64] PCA acts *in vitro* by increasing the activities of antioxidant enzymes such as superoxide dismutase, scavenging ROS or inhibiting their formation, and consequently reducing oxidative stress damage.[64-66] One of the prominent and most useful properties of the flavonoids is their ability to scavenge ROS[67]. They are considered more efficient antioxidants than vitamins C and E.[68] Coumarins, another group of phenolic compound (1,2-benzopyrone) are natural antioxidant compounds widely distributed in plants.[69,70] Studies have reported that coumarins inhibit lipid peroxidation, decreasing the injury caused by oxidative stress and decreasing the levels of ROS in different types of cells.[71,72] Likewise, anethole a constituent of essential oil (terpenoids) from *Croton-zehntneri*, a medicinal plant popularly known as "canela de cunhã" or "canelinha" in the Northeast of Brazil, decreased the levels of ROS both *in vivo*[73,74] using mice model, and *in vitro* during culture of cell lines isolated from the peripheral blood of male patient with acute myeloblastic leukaemia[75]. Sá[76] et al. (2015) showed that addition of anethole (300 and 2000 μg/mL) to the *in vitro* culture medium was able to improve the development of goat preantral follicles by reducing concentrations of ROS and increasing the percentage of oocytes able to resume meiosis. In addition to their antioxidant activity, compounds from plant can have a important role during the steroidogenesis.

Many compounds (flavonoids, lignans, coumestans) derived from plants have hability to mimic the biologic effects of endogenous hormones by binding to their nuclear receptor or regulating the activities of key enzymes of their metabolisms such as cytochrome P450 aromatase, and 17β-hydroxysteroid deshydrogenase which is a key enzyme of the steroidogenesis.[13] The estrogenic effects of some compounds are often related to the stimulation of the hypothalamus-pituitary complex increasing the Follicle Stimulating Hormone (FSH),

which will thereafter induce ovarian steroidogenesis. Flavanoids with estrogenic potential have been reported to inhibit aromatase activity in various tissues.[77]

The best-described property of almost plants is their capacity to act as antioxidants. For instance, flavones and catechins seem to be the most powerful flavonoids for protecting the body against ROS. Follicular cells are continuously threatened by the damage caused by free radicals and ROS, which are produced during normal oxygen metabolism or are induced by exogenous damage.[78] The mechanisms and the sequence of events by which free radicals interfere with cellular functions are not fully understood, but one of the most important events seems to be the lipid peroxidation, which results in cellular membrane damage. This cellular damage causes a shift in the net charge of the cell, changing the osmotic pressure, leading to swelling and eventually cell death.[79] The ROS produced during the metabolism are made inactive according the following equation, where $R^{\bullet}$ is a free radical and $O^{\bullet}$ is an oxygen free radical.

$$\text{Flavonoid (OH)} + R^{\bullet} \longrightarrow \text{flavonoid } (O^{\bullet}) + RH$$

Nowadays, medicinal plants are widely used around the world as an alternative to pharmaceutical drugs. Although herbal products are considered to have fewer adverse effects compared with synthetic drugs, they are not completely free of indesejable effects. The volatile terpenoids camphor, a compound of the essential oil of *Artemisia kopetdaghensis* (*A. kopetdaghensis*) crosses the placenta and may lead to abortion.[80] In another study, Linjawi[81], (2009) reported that camphor induces significant structural changes on uterus of pregnant rats. Therefore, it is rational to assume that camphor is involved in the abortifacient effect of *A. kopetdaghensis.* Results from Oliaee[82] et al. (2014) using female rats as animal model showed that continuous consumption of 800 μg/mL of *A. kopetdaghensis* in pregnancy may increase the risk of abortion and also may have toxic effect on some cells of body. Therefore, its continuous use is not recommended in pregnancy.

Implication of plant extracts or compounds in ovarian cells cultured *in vitro*

With the aim to elucidate their properties, plant extracts or its secondary metabolites have been used in the culture of various types of cells including follicles and granulosa cells. Hsia[83] et al. (2007) demonstrated that the partitioned fractions of *Coix lachryma-jobi* (Adlay), a traditional Chinese medicine used for the dysfunction of endocrine system extracts possess hypogonadal effect *in vitro* conditions. This plant shows a great capacity to reduce the production of progesterone (P4) and estradiol.(E2) by decreasing the activity of cholesterol side-chain cleavage enzyme (P450scc) and 3beta-Hydroxysteroid dehydrogenase (3β-HSD). In

contrary, the use of leaves mixture *of Aloe buettneri, Justicia insularis, Dicliptera verticillata and Hibiscus macranthus* (ADHJ) *in vitro* attest the direct effects of some chemical components on rat ovarian steroidogenesis. Indeed, alkaloids, coumarins glycosides, flavonoids and quinones from ADHJ are more effective when the plant extract (130 g/ml) is combined to 0.1 IU/mL of human chorionic gonadotropin (hCG) during 2 h of incubation. In these conditions, estradiol production increased by 13-fold compared to the medium without hCG and the plant extract; and by 5-fold compared to the medium containing only the plant extract (130 g/mL) or hCG (0.1 IU/mL).[6]

Studies with quercetin, a flavonoid present in several plants, as well as nonsteroidal compounds known as phytoestrogens[84] affects porcine granulosa cell function by interfering with steroidogenic activity and redox status as well as by inhibiting vascular endothelial growth factor output[85]. This phytoestrogen represents a potential modulator of ovarian functions through inhibition of steroidogenic enzymes.[77,86]

Suppressive action of phytoestrogen on cytochrome P450 (enzyme that catalyzed the conversion of cholesterol to pregnenolone) represents a rate-limiting step in the steroidogenic pathway. Several studies[87,88] showed that phytoestrogen induced decrease of P4 production in granulosa cells. This decrease is due to the inhibition of 3β-hydroxysteroid enzyme. Furthermore, Santini[89] et al. (2007) revealed the inhibitory effect of quercetin on aromatase activity. It has been suggested that the action of phytoestrogen on aromatase activity could be mediated by nitric oxide (NO). In fact, this free radical seems to represent an autocrine regulator of granulosa cells E2 production.[90] However, molecular studies should be done to better understand the mechanism of action of phytoestrogen on steroidogenic enzymes.

Implication of plants extract or compounds on oocyte maturation

During *in vitro* oocyte culture, the levels of antioxidants are lower than *in vivo* because the oocytes are divorced from the donor body and do not benefit from the maternal antioxidant protection.[90] The addition of an antioxidant to the medium, therefore, may be important for *in vitro* oocyte maturation.

Reports from Rajabi-Toustani[91] et al. (2013) shows that supplementation of appropriate concentrations of *Papaver rhoeas* extract (50 µg/mL) in maturation medium (bicarbonate-buffered TCM 199 supplemented with 10% FBS, 0.2 mM sodium pyruvate 0.1 IU/mL hMG, 100 IU/mL penicillin and 100 µ/mL streptomycin) improve the sheep oocyte maturation rate. Similar results have been obtained when maturation medium of mouse oocyte was supplemented with 5 µg/mL of *Crocus sativus*[92] or when supplemented with 20 µg/mL of

Phoenix dactylifera pollen grain[93]. Improvement maturation rate of oocytes treated with *P. rhoeas* extract may be partly due to increase of intracellular glutathione (GSH) levels in oocytes[91] or super oxide dismutase (SOD) activity.[94] Anthocyanins protect cells against free radicals by gamma-glutamylcysteine synthetase (γ-GCS) activation, while of γ-GCS elevates GSH levels in medium[95]. Increased GSH levels through oocyte maturation are associated with improvement in subsequent embryo development.[96] On the other hand, the reduction on *in vitro* maturated oocytes to metaphase II stage might be due to deleterious effects of excessive concentrations (200 µg/mL of *P. rhoeas* extract), because some flavonoids displayed toxic effects[91,97] by changing the cell membrane structure and consequently damage cell polarization[98]. Locklear[99] et al. (2010) demonstrated that extract of *Justicia pectoralis* acts as an E2 and P4 agonists on the cellular membrane and inhibits the activity of the cyclooxygenase 2 (COX-2) enzyme *in vitro*. The COX-2, is the rate-limiting enzyme in the biosynthesis of prostaglandins (PGs) which are considered to participate in follicular rupture during ovulation.[100] *J. pectoralis* is a medicinal plant commonly used by women in Costa Rica to treat symptoms associated with premenstrual syndrome (common forms of hormonal imbalance affecting women) and menopause. The Table 3 below shows some *in vitro* implications of medicinal plants from different species on folliculogenesis and steroidogenesis process.

Table 3: *In vitro* implication of some medicinal plants on folliculogenesis and steroidogenesis in different species.

Plants	Chemicals compounds	Used parts	Effects	Species	References
Amburana cearensis	Protocatechuic acid, epicatechin, p-coumaric acid gallic acid, kaempferolin	Leaves	Maintained follicular survival and promoted the development of isolated secondary follicles.	Ovine	Barberino[11] et al., 2015
Moringa oleifera	β-carotene, protein, vitamin C, calcium, potassium	Leaves	Improved the oocyte maturation rate	Ovine	Ibrahim[101] et al., 2015
Croton zehntneri	Anethole	Leaves	Improved the development and oocyte maturation rate of isolated secondary preantral follicles.	Caprine	Sá[76] et al., 2015
DPP	Flavonoids, phenolic acid, diterpenes	Grain	Improved the oocyte maturation rate	Mice	Abdollahi[93] et al., 2015
Yucca shidigera	Sarsapogenin, milagenin, markogenin, samogenin, gitogenin, neogitogenin	Bark	Reduced ovarian cell proliferation, promoted ovarian cell apoptosis, stimulated P4 and inhibited testosterone release.	Swine	Štochmaľová[102] et al., 2014
Gundelia Tournefortii	Phenolic compounds	Leaves	Improved oocyte maturation rate	Murine	Abedi[103] et al., 2014

Crocus sativus	Crocin, crocetin, dimethyl crocetin, safranal, flavonoids.	Stigmas	Improved the IVM, IVF, and early embryo development	Murine	Maleki[104] et al., 2012
Coix lachryma-jobi	Coixenolides, coixans A, B, and C	Seed	Decreased P4 and E2 levels.	Murine	Hsia[83] et al., 2007
ADHJ	Alkaloids, flavonoids, glycosides, coumarins and quinones	Leaves mixture	Induced E2 synthesis	Murine	Telefo[6] et al., 2004

IVM: *in vitro* maturation, IVF: *in vitro* fertilization, ADHJ: mixture of *Aloe buettneri, Dicliptera verticillata, Hibiscus macranthus* and *Justicia insularis. DPP*: *Phoenix dactylifera* pollen grain

Conflicts of interest

The authors declare no conflicts of interest.

Final considerations

Female reproductive problems continue to be a major health challenge worldwide. An impressive number of plant species is traditionally used to remedy such disorder. Those plants mainly constituted of secondary metabolites have been used over decades for the treatment of diseases which affect woman reproduction leading to infertility. These substances widely distributed over the world are constituted of compounds whose concentrations and compositions vary among plants and between the same genus. Several factors affect the plants composition among which the season, site and time of harvest. With the development of technology, an interest of plant is reported due to their antioxidant capacity and their ability to mimic the effect of steroidogenic enzymes. But little remain unknown about their implication *in vitro* which represents alternative studies of the *in vivo* studies. Finally, further studies should be performed to better understand the mechanism of action of plant and / or secondary metabolites. The discovery may also help to reduce the cost and improve the results of treatments normaly applied

Conflicts of interest

The authors declare no conflicts of interest.

REFERENCES

1. Zegers-Hochschild F, Adamson GD, De Mouzon J, Ishihara O, Mansour R, Nygren K, et al. The International Committee for Monitoring Assisted Reproductive Technology (ICMART) and the World Health Organization (WHO) Revised Glossary on ART Terminology. Hum Reprod. 2009; 24: 2683-2687.

2. Daar AS, Merali Z. Infertility and social suffering: the case of ART in developing countries. In: Vayena, E, Rowe, PJ, riffin, P.D. (Eds.), Current Practices and Controversies in Assisted Reproduction. Report of a meeting on "Medical, Ethical and Social Aspects of Assisted Reproduction". World Health Organization, Geneva, Switzerland, 2002; 15-21.

3. Larsen SH, Wagner G, Heitmann BL. Sexual function and obesity. Int J Obes. 2007; 31: 1189-1198.

4. Breart G, De Mouzon J. AMP vigilance. Notice of the Nat Acad Med. 1995; 179: 1759-1764

5. Telefo PB, Moundipa PF, Tchana AF, Tchouanguep DC, Mbiapo FT. Effects of aqueous extract of *Aloe buettneri, Dicliptera verticillata, Hibiscus macranthus,* and *Justicia insularis* on some biochemical and physiological parameters of reproduction in immature female rat. J Ethnopharmacol. 1998; 63: 193-200.

6. Telefo PB, Moundipa PF, Tchouanguep FM. Inductive effects of the leaf mixture extract of *Aloe buettneri, Justicia insularis, Dicliptera verticillata* and *Hibicus marcranthus* on *in vitro* production of oestradiol. J Ethnopharmacol. 2004; 90: 225-230

7. Leal L, Nobre JHV, Cunha GMA, Moraes MO, Pessoa C, Oliveira RA, et al. Amburoside A, a glucoside from *Amburana cearensis*, protects mesencephalic cells against 6-hydroxydopamine-induced neurotoxicity. Neurosci Lett. 2005; 388: 86-90.

8. Jha U, Asad M, Asdaq BMS, Das KA, Satya PSV. Fertility inducing effect of aerial parts of Coccinia cordifoliaL. in female rats. J Ethnopharmacol. 2010; 127: 561-564.

9. Moundipa FP, Kamtchouing P, Koueta N, Mbiapo F, Tantchou J. Effects of aqueous extract of *Hibiscus macranthus* and *Basela alba* Linn. In: Immature Rat Testis Function. Andrology in the Nineties. International Symposium on Male Infertility and Assisted Reproduction, 1993; 21-24

10. Bors W, Saran M, Michel C. Radical intermediates involved in the bleaching of the carotenoid crocin. Hydroxyl radicals, superoxide anions and hydrated electrons. Int J Radiat Biol Relat Stud Phys Chem Med.1982; 41: 493-501.

11. Barberino RS, Barros VRP, Menezes VG, Santos LP, Araújo VR, Queiroz MAA et al. Almeida JRGS, Palheta RCJR, Matos MHT. *Amburana cearensis* leaf extract maintains survival and promotes *in vitro* development of ovine secondary follicles. Zygote. 2015; 1-9

12. Jung-Taek K, Joon HM, Ji-Yei C. Sol JP, Su JK, Islam MS, et al. Byeong CL. Effect of Antioxidant Flavonoids (Quercetin and Taxifolin) on i*n vitro* Maturation of Porcine Oocytes. Asian Australas J Anim Sci. 2016; 29: 352-358

13. Kurzer MS, Xu X. Dietary phytoestrogens. Annu Rev Nutr. 1997; 17: 353-381.

14. Hutt Karla J, David F Albertini. An oocentric view of folliculogenesis and embryogenesis. Reprod BioMed Online. 2007; 14: 758-764

15. Jiménez, Ovarian Organogenesis in Mammals: Mice Cannot Tell Us Everything. Sex Dev. 2009; 3:291-301

16. Mohammadpour AA. Comparative Histomorphological Study of Ovary and Ovarian Follicles in Iranian Lori-Bakhtiari Sheep and Native Goat. Pak J Biol Sci. 2007; 10: 673-675.

17. Juengel JL, Sawyer HR, Smith PR, Quirke LD, Heath DA, Lun S, et al. Origins of follicular cells and ontogeny of steroidogenesis in ovine fetal ovaries. Mol Cell Endocrinol. 2002; 191: 1-10

18. Edson MA, NagarajaAK, Matzuk MM. The Mammalian Ovary from Genesis to Revelation. Endocr. Rev. 2009 30:624-712

19. http://faculty.southwest.tn.edu/rburkett/A&P2_reproductive_system_lab.htm19

20. Hirshfield AN. Development of follicles in the mammalian ovary international
Int Rev Cytol. 1991;124: 43-101.

21. Eppig JJ. Oocyte control of ovarian follicular development and function in mammals. Reproduction, 2001; 122: 829-838

22. Matzuk MM, Burns KH, Viveiros MM, Eppig JJ . Intercellular communication in the mammalian ovary: oocytes carry the conversation. Science, 2002; 296: 2178-2180

23. Pepling ME, Spradling AC. Female mouse germ cells form synchronously dividing cysts. Devel. 1998; 125: 3323-3328

24. Mc Gee EA, Hsueh AJ, Initial and cyclic recruitment of ovarian follicles. Endocr Rev. 2000; 21: 200-214

25. Barnett KR, Schilling C, Greenfeld CR, Tomic D, Flaws JA. Ovarian follicle development and transgenic mouse models. Hum Reprod. 2006;12:537-55

26. Nuttinck F, Mermillod P, Massip A, Dessy F. Characterization of *in vitro* growth of bovine preantral ovarian follicles - a preliminary study. Ther. 1993; 39: 811-821.

27. Costa RAP, Romagna CD, Pereira JL, Souza-pinto NC. The role of mitochondrial DNA damage in the citotoxicity of reactive oxygen species. J Bioenerg Biomembr. 2011, 43: 25-29

28. Şükür YE, İçten BK, Batuhan Ö. Ovarian aging and premature ovarian failure. J Turk Ger Gynecol Assoc. 2014: 15; 190-196

29. Engel RH, Evens AM. Oxidative stress and apoptosis: a new treatment paradigm in cancer Fro in Biosc. 2006; 11: 300-312

30. Silva GM, Araújo VR, Duarte ABG.; Chaves RN. Silva CMG, Lobo CH et al. Influences of FSH and EGF on primordial follicles during *in vitro* culture of caprine ovarian cortical tissue. Ther. 2004; 61: 1691-704

31. Gupta S, Malhotra, N, Sharma D, Chandra A, Agarwal A. Oxidative stress and its role in female infertility and assisted reproduction: clinical implications. Inter J Fertil Steril. 2009; 2: 147-164

32. Du Plessis SS, Makker K, Desai NR, Agarwal A. Impact of oxidative stress on IVF. Expert Rev Obstet Gynecol. 2008; 539-554.

33. Akoka G, Akoka A. Medecine. Medical Encyclopedia. 1972; 3: 692p.

34. Rates SMK. Plants as source of drugs. Toxicon. 2001; 39: 603-613

35. Telefo PB, Moundipa PF, Tchouanguep, FM. Influence of aqueous extract of Aloe leaves buettneri, Dicliptera verticillata, Hibiscus macranthus, Justicia insularis on fertility and some biochemical parameters of reproduction in rats. J Acad Sci Cam. 2001; 3: 144-150

36. Priso RJ, Din N, Konglong SA, Amougou A. Inventory of some medicinal plants for the conception of pregnancy in some localities of the Department of Nkam. Book program and abstracts - XIII Annual Conference of Biosciences, 2006; 108p.

37. Tacham W. An ethnobotanical survey of plants used to treat deseases of the reproductive system in Foreke-Dschang and Fongo-Tongo in the Menoua division. Plant Master's Dissertation. Université de Dschang, Dschang-Cameroun, 2000; 22-24.

38. Cajuday LA, Pocsidio GL. Effects of *Moringa oleifera* Lam. (Moringaceae) on the reproduction of male mice (*Mus musculus*). J Med Plants Res. 2010; 12: 1115-2112

39. Bussmann RW, Glenn A. Medicinal plants used in Northern Peru for reproductive problems and female health. J Ethnobiol Ethnomed. 2010; 6(30): 1-12

40. Nielsen1 SE. Young JF, Daneshvar1 B., Lauridsen ST., Knuthsen P, Sandstro B, Dragsted LO. Effect of parsley (*Petroselinum crispum*) intake on urinary apigenin excretion, blood

antioxidant enzymes and biomarkers for oxidative stress in human subjects. Br J of Nutr.1999; 81: 447-455

41. Chifundera K. Livestock diseases and the traditional medicine in the bushi area, kivu province, democratic republic of Congo. African Study Monographs, 1988; 19: 13-33

42. Ramaraj E, Thamburaj S, Samiraj R, Subban P . Studies on the antibacterial and nucleic acid degradation property of *Cassia alata*. Int J. Drug Dev Res. 2014; 6 (1): 44-43. Koch M, Kehop DA, Kinminja B, Sabak M, Wavimbukie G, Barrows KM et al. An ethnobotanical survey of medicinal plants used in the East Sepik province of Papua New Guinea. J Ethnobiol Ethnomed. 2015; 11:79

44. James, DB.; Abu, EA.; Wurochekke, AU.; Orji, GN. Phytochemical and Antimicrobial Investigation of the Aqueous and Methanolic Extracts of Ximenia americana. J Med. Sci. 2007; 2: 284-288.

45. Adjanohoun JE, Aboubakar N, Dramane K, Ebot ME, Ekpere JA, Enow-Orock EG. Traditional Medicine and Pharmacopoeia. Contribution to Ethnobotanical Floristic studies in Cameroon, 1996; CNPMS Porto-Novo Benin, 22p.

46. Adewole LO. Ageratum conyzoides L. (Asteraceae). Fitoterapia, 2002; 73: 1-16

47. Tabopda TK, Fotso GW, Ngoupayo J, Mitaine-Offer AC, Ngadjui BT, Lacaille- Dubois MA. Antimicrobial dihydroisocoumarins from Crassocephalum biafrae. Planta Med. 2009; 75: 1258-1261

48. Arbonnier M. Shrubs and lianas of the drylands of West Africa, 2 (Eds.). CIRAD-MNHN, 2002; 549p.

49. Igoli JO,Tornyiin TA, Usman SS, Oluma HOA, Igoli NP.Folkmedicines of the lower Benue valley of Nigeria. In: Singh, V.K., Govil, J.N., Hashmi, S., Singh, G. (Eds.), Recent Progress in Medicinal Plants, 7 Ethnomedicine and Pharmacognosy II. Scientific and Technological Publications, 2002; 327-38.

50. Adnan M, Tariq A, Mussarat S, Begum S, AbdEIsalam NM, Ullah R. Ethnogynaecological Assessment of Medicinal Plants in Pashtun's Tribal Society. BioMed Res Int. 2015; 9p

51. Doyle BJ, Frasor J, Bellows LE, Locklear TD, Perez A, Laurito JG et al. Estrogenic effects of herbal medicines from Costa Rica used for the management of menopausal symptoms. Menopause. 2009;16: 748-755.

52. Harborne JB, Classes and functions of secondary products, in: N.J. Walton, D.E. Brown (Eds.), Chemicals from Plants, Perspectives on Secondary Plant Products, Imperial College Press, 1999; 1-25

53. Trease GE, Evans WC. Pharmacognosy, 11th edn. Baillière Tindall, 1996; 75-76

54. Hassig A, Liang WX, Schwabl H, Stampfli K. Flavonoids and tannins: plant-based antioxidants with vitamin character. Med Hyp. 1999; 479-481

55. Jiang P, Burczynski F, Campbell C, Pierce G, Austria JA, Briggs CJ. Rutin and flavonoid contents in three buckwheat species *Fagopyrum esculentum, F. tataricum,* and *F. homotropicum* and their protective effects against lipid peroxidation. Food Res Int. 2007; 40: 356-364.

56. Bourgaud F, Gravot A, Milesi S, Gontier E. Production of plant secondary metabolites: a historical perspective. Plant Sci. 2001; 16: 839 - 851.

57. Saxena M, Saxena J, Nema R, Dharmendra Singh D, Gupta A. Phytochemistry of Medicinal Plants. J Pharmacogn Phytochem. 2013; 1: 168-182

58. Dudareva N, Pichersky E, Gershenzon. J. Biochemistry of Plant Volatiles. Plant Physiol. 2004;135: 1893-1902

59. Kumar S, Pandey AK. Chemistry and Biological Activities of Flavonoids: An Overview. Sci World J. 2013

60. Sarker SD, Nahar L. Chemistry for Pharmacy Students General, Organic and Natural Product Chemistry. England: John Wiley and Sons. 200; 7pp 283-359.

61. Gouveia BB, Barros VRP, Santos LP, Gonçalves RJS, Matos MHT. Immunohistochemical localization of fibroblast growth factor-2 in the sheep ovary and its effects on pre-antral follicle apoptosis and development *in vitro.* Reprod Domest Anim. 2014; 49: 222-228.

62. Tang HR, Covington AD, Hancock RA. Structure-activity relationships in the hydrophobic interactions of polyphenols with cellulose and collagen. Biopolymers. 2003 70: 403-413.

63. Ferk F, Chakraborty A, Jager W, Kundi M, Bichler J, Michle M, et al. Potent protection of gallic acid against DNA oxidation: results of human and animal experiments. Mutat Res. 2004; 715: 61-71.

64. Guan S, Ge D, Liu TQ, Ma X, Cui ZF. Protocatechuic acid promotes cell proliferation and reduces basal apoptosis in cultured neural stem cells. Toxicol *in Vitro*. 2009; 23: 201-208.

65. An LJ, Guan S, Shi GF, Bao YM, Duan YL, Jiang B. Protocatechuic acid from Alpiniaoxyphylla against MPP^+ induced neurotoxicity in PC12 cells. Food Chem Toxicol. 2006; 44: 436-443.

66. Shi GF, An LJ, Jiang B, Guan S, Bao YM. Alpiniaprotocatechuic acid protects against oxidative damage *in vitro* and reduces oxidative stress *in vivo*. Neurosci Lett. 2006; 403: 206-210

67. Wang PF, Zheng RL. Inhibitions of the autoxidation of linoleic acid by flavonoids in micelles. Chem Phys Lipids. 1992; 63: 37-40.

68. Gao Z, Huang K, Xu, H. Protective effects of flavonoids in the roots of *Scutellaria baicalensis Georgi* against hydrogen peroxide-induced oxidative stress in HS-SY5Y cells. Pharmacol Res. 2001;4: 173-178.

69. Egan D, O'Kennedy R, Moran E, Cox D, Prosser E, Thornes RD. The pharmacology, metabolism, analysis and application of coumarin, and coumarin related compounds. Drug Metab Rev. 1990; 22: 503-529

70. Lake BG.. Coumarin metabolism, toxicity and carcinogenicity: relevance for human risk assessment. Food Chem Toxicol. 1999; 37: 423-453

71. Martin-Aragón S, Benedi JM, Villar AM. Effects of the antioxidant (6,7-ihydroxycoumarin) esculetin on the glutathine system and lipid peroxidation in mice. Gerontol. 1998; 44:21-25

72. Kaneko T, Baba N, Matsuo M. Protection of coumarins against linoleic acid hydroperoxide-induced cytotoxicity. Chem Biol Interact. 2000; 142: 239-254

73. Leal-Cardoso JH, Fonteles MC. Pharmacological effects of essential oils plants of the Northeast of Brazil. An Acad Bra Ciênc. 1999; 71: 207-213

74. Freire RS, Morais SM, Catunda-Junior FE, Pinheiro DC. Synthesis and antioxidant, anti-inflammatory and gastroprotector activities of anethole and related compounds. Bioorg Med Chem. 2005; 13:4353-4358

75. Chainy GB, Manna SK, Chaturvedi MM, Aggarwal BB. Anethole blocks both early and late cellular responses transduced by tumor necrosis factor: effect on NF kappa B, AP-1, JNK, MAPKK and apoptosis. Oncogene. 2000; 19:2943-2950

76. Sá NAR, Araújo VR, Correia HHV, Ferreira ACA, Guerreiro DD, Sampaio AM, et al. Anethole improves the *in vitro* development of isolated caprine secondary follicles, Ther. 2016

77. Pelissero C, Lenczowski MJP, Chinzi D, Davail CB, Sumpter JP, Fostier A. Effects of flavonoids on aromatase activity, an *in vitro* study. J Steroid Biochem Mol Biol. 1996; 57: 215-223

78. Grace PA. Ischaemia-reperfusion injury. Br J Surg. 1994; 81:637-47

79. Robert JN, Els VN, Danny EC, Petra GB, Klaske VN, Paul AM. Flavonoids: a review of probable mechanisms of action and potential applications. Am J Clin Nutr. 2001; 74:418 -25.

80. Rabl W, Katzgraber F., Steinlechner M. Camphor ingestion for abortion (case report). Forensic Sci Int. 1997; 89: 137-140

81. Linjawi SA. Effect of camphor on uterus histology of pregnant rats. Journal of King Abdulaziz University, 2009;16: 77-90

82. Oliaee D, Mohammad TB, Naiime O, Ahmad G. Evaluation of Cytotoxicity and Antifertility Effect of Artemisia kopetdaghensis. Adv Pharmacol Sci. 2014; 5 p.

83. Hsia M, Chih-Lan Y., Yueh-Hsiung K. , Paulus S. Wang.; Wenchang C. Hutt KJ, David FA. An oocentric view of folliculogenesis and embryogenesis. Reprod BioMed Online. 2007; 14:758-764

84. Moutsatsou P. The spectrum of phytoestrogens in nature: our knowledge is expanding. *Hormones*. 2007; 6:173-193

85. Dusza L, Ciereszko R, Skarzyński DJ, Nogowski L, Opałka M, Barbara Kamińska et al. Mechanism of phytoestrogen action in reproductive processes of mammals and birds. Reprod Biol. 2006; 6: 151-174

86. Lephart ED. Modulation of Aromatase by Phytoestrogen. Enzyme Res. 2015; 11p

87. Krazeisen A, Breitling R, Oller GM, Adamski J. Phytoestrogens inhibit human 17β-hydroxysteroid dehydrogenase type 5. Mol Cell Endocrinol. 2001; 171: 151-162.

88.Whitehead SA, Lacey M. Phytoestrogens inhibit aromatase but not 17β-hydroxysteroid dehydrogenase (HSD) type 1 in human granulosa-luteal cells: evidence for FSH induction of 17β-HSD, Hum Reprod. 2003;. 18: 487-494

89. Santini SE, Basini G, Bussolati S, Grasselli F. The Phytoestrogen Quercetin Impairs Steroidogenesis and Angiogenesis in Swine Granulosa Cells *In Vitro*. J Biomed Biotechnol. 2007; 6: 173-193

90. Voorhis VBJ, Dunn MS, Snyder GD. Nitric oxide: an autocrine regulator of human granulosa-luteal cell steroidogenesis. Endocr. 1994; 135, 1799-1806.

91. Rajabi-Toustani R, Motamedi-Mojdehi R, Mehr M, Motamedi-Mojdehi R. Effect of Papaver rhoeas L. extract on *in vitro* maturation of sheep oocytes. Small Rumin. Res. 2013; 114: 146-151

92. Tavana S, Eimani H, Azarnia M, Shahverdi A, Eftekhari-Yazdi P. Effects of Saffron (*Crocus sativus* L.) aqueous extract on *in vitro* maturation fertilization and embryo development of mouse oocytes. Cell J. 2012; 13: 259-264.

93. Abdollahi FS, Baharara J, Nejad Shahrokhabadi K, Namvar F, Amini E. Effect of Phoenix dactylifera pollen grain on maturation of preantral follicles in NMRI mice. J Herbmed Pharmacol. 2015; 4: 93-97

94. Moghadam FD, Baharara J, Balanezhad SZ, Jalali M, Amini E. Effect of *Holothuria leucospilota* extracted saponin on maturation of mice oocyte and granulosa cells. Res Pharma Sci. 2016;130 -137.

95. Myhrstad MC, Carlsen H, Nordström O, Blomhoff R, Moskaug J. Flavonoids increase the intracellular glutathione level by transactivation of the gamma-glutamylcysteine synthetase catalytical subunit promoter. Free Radic Bio Med. 2002; 32: 386-393

96. De Matos DG, Furnus CC. The importance of having high glu-tathione (GSH) level after bovine in vitro maturation on embryodevelopment: effect of beta-mercaptoethanol, cysteine and cystine.Ther. 2000; 53:761-771.

97. Wu Z, Yang Y, Chen Y, Xia G, Zhang R. Effects of subcutaneous administration of daidzein on blastocyst implantation in rats. Food Chem Toxicol. 2005; 43: 167–172

98. Sprong RC, Winkelhuyzen-Janssen AM, Aarsman CJ, Van Oirschot JF, Van der Bruggen T, Van Asbeck BS. Low-dose N-acetylcysteine protects rats against endotoxin-mediated oxidativestress, but high-dose increases mortality. Am J Respir Crit Care Med. 1998; 157: 1283-1293.

99. Locklear TD, Yue H, Jonna F, Brian JD, Alice P, Jorge GL, Mahady. Estrogenic and Progestagenic effects of extracts of *Justicia pectoralis*, an herbal medicine from Costa Rica used for the treatment of Menopause and PMS. Maturitas. 2010; 66: 315-322.

100. Lim H, Bibhash C. Paria BC, Das SK, Dinchuk RL, Trzaskos JM, Sudhansu KD. Multiple Female Reproductive Failures in Cyclooxygenase 2–Deficient Mice. Cell. 1997; 91: 197-208.

101. Ibrahim AH, Barakata B, Wagdy KB, Khalilb, Ahmad R, Al-Himaidi. *Moringa oleifera* extract modulates the expression of fertility relatedgenes and elevation of calcium ions in sheep oocytes. Small Rumin Res. 2015; 130: 67-75

102. Štochmaľová A, Kadasi A, Alexa R., Grossman R., Sirotkin A. The effect of yucca on proliferation, apoptosis, and steroidogenesis of porcine ovarian granulosa cells. Potravinarstvo, 2014; 8: 1-5.

103. Abedi A, Rouhi L, Pirbalouti AG. Effect of *Gundelia tournefortii* leaves extract on in immature mouse oocytes. J Herbal Drugs. 2014; 5: 84-89

104. Maleki EM, Eimani H, Bigdeli MR , Ebrahimi B, Shahverdi AH, Narenji AG. Et al. A comparative study of saffron aqueous extract and its active ingredient, crocin on the in vitro maturation, in vitro fertilization, and in vitro culture of mouse oocytes. Taiwan J Obstet Gynecol. 2014; 53: 21-25.

7 CAPÍTULO 2

Justicia insularis melhora a sobrevivência *in vitro* e o desenvolvimento de folículos pré-antrais ovinos inclusos no tecido ovariano

Justicia insularis improves the *in vitro* survival and development of ovine preantral follicles enclosed in ovarian tissue

Periódico: Publicado no Journal of Pharmacy and Pharmacology, 2017. Pages 668-680. Qualis B1

RESUMO

Objetivos: Avaliar o efeito da adição do extrato de J. insularis e FSH na sobrevivência, ativação e produção de ROS após o cultivo *in vitro* de folículos pré-antrais ovinos envoltos em tecido ovariano. os fragmentos ovarianos foram fixados (controle não cultivado) ou cultivados *in vitro* em α-MEM^{+} (controle cultivado), α-MEM^{+} suplementado com FSH 50 ng / mL, ou em α-MEM $^{+}$ suplementado com *J. insularis* (JUS0,3; 1,25 ou 5 mg / mL) durante 1 ou 7 dias, a 39%, 5% de CO_2. No segundo experimento, os fragmentos foram fixados ou cultivados em α-MEM^{+} suplementado com anetol 300 µg / mL + FSH 50 ng / mL ou em α-MEM^{+} suplementado com anetol 300 µg / mL + 0,3 mg / mL JUS. Principais achados JUS0.3 foi o único tratamento que manteve a porcentagem de folículos morfologicamente normais semelhantes ao controle não-cultivado, mesmo após 7 dias de cultuivo. Após 7 dias de cultura, uma porcentagem maior ($P < 0,05$) de folículos em desenvolvimento foi observada no tratamento com JUS5 em comparação com os outros tratamentos, exceto JUS1.25. No segundo experimento, o FSH manteve a porcentagem de folículos normais e promoveu a ativação de folículos primordiais. Uma redução ($P < 0,05$) da densidade celular do estroma foi observada em MEM $^{+}$ + ANE suplementado com JUS ou FSH. Conclusões: *J. insularis* de maneira concentração-dependente manteve os níveis de ROS e melhorou a sobrevivência folicular *in vitro* e a ativação de folículos primordiais de ovinos.

Palavras-chave: Planta medicinal; antioxidante; foliculogênese in vitro; folículos pré-antrais

Justicia insularis Improves the *in vitro* Survival and Development of Ovine Preantral Follicles Enclosed in Ovarian Tissue

Gildas Mbemya Tetaping[1], Denise Damasceno Guerreiro[1], Nathalie Jiatsa Donfack[1], Luciana Mascena Silva[1], Luis Alberto Vieira[1], Geovania Francisca Canafistula de Sousa[1], Benner Geraldo Alves[1], Aryele Pinto Izaguirry[2], Francielli Weber Santos[2], Phelix Bruno Telefo[3], Otília Deusdênia Loiola Pessoa[4], Johan Smitz[5], José Ricardo de Figueiredo[1], Ana Paula Ribeiro Rodrigues[1,*]

[1]Laboratory of Manipulation of Oocyte and Ovarian Preantral Follicles (LAMOFOPA), Faculty of Veterinary (FAVET), State University of Ceará, Fortaleza, Brazil

[2]Laboratory of Reproduction Biotechnology (Biotech), State of University of Pampa, Uruguaiana, RS, Brazil

[3]Laboratory of Biochemistry of Medicinal Plants, Food and Nutritional Sciences (LABPMAN), Faculty of Science, University of Dschang, Cameroon

[4]Laboratory of Phytochemical Analysis of Medicinal Plants (LAFIPLAN), Federal University of Ceará, Fortaleza, Brazil

[5]Follicle Biology Laboratory, Center for Reproductive Medicine, UZ Brussel, Brussels, Belgium

[*]Correspondence

E-mail: aprrodriguespapers@gmail.com (A.P.R. Rodrigues).

Prof. Dr. Ana Paula Ribeiro Rodrigues, Laboratory of Manipulation of Oocyte and Ovarian Preantral Follicles, Faculty of Veterinary, State University of Ceará, Av. Dr. Silas Munguba, 1700-Fortaleza-CE- 60.714-903. Phone: + 55 8531019852, Fax: + 55 8531019840.

Abstract

Objectives Evaluate the addition effect of *J. insularis* extract and FSH on the survival, activation and ROS production after *in vitro* culture of ovine preantral follicles enclosed in ovarian tissue. **Methods** In the first experiment, ovarian fragments were fixed (non-cultured control) or *in vitro* cultured in α-MEM$^+$ (cultured control), α-MEM$^+$ supplemented with FSH 50 ng/mL, or in α-MEM$^+$ supplemented with *J. insularis* (JUS0.3; 1.25 or 5 mg/mL) for 1 or 7 days, at 39˚C, 5% CO_2. In the second experiment, fragments were fixed or cultured in α-MEM$^+$ supplemented with anethole 300 µg/mL + FSH 50 ng/mL or in α-MEM$^+$ supplemented with anethole 300 µg/mL + 0.3 mg/mL JUS.

Key findings JUS0.3 was the only treatment that maintained the percentage of morphologically normal follicles similar to non-cultured control even after 7 days of culture. After 7 days of culture, a higher ($P < 0.05$) percentage of developing follicles was observed in JUS5 treatment compared to the other treatments except JUS1.25. In the second experiment, FSH maintained the percentage of normal follicles and promoted activation of primordial follicles. A reduction ($P < 0.05$) of stromal cell density was observed in MEM$^+$+ANE supplemented with JUS or FSH.

Conclusions: *J. insularis* in a concentration-dependent manner maintained the levels of ROS and improved *in vitro* follicular survival and activation of ovine primordial follicles.

Key words: Medicinal plant; antioxidant; *in vitro* folliculogenesis; preantral follicles

1. Introduction

The *in vitro* follicle culture studies have been performed either using primordial follicles enclosed in ovarian slices (*in situ* culture) or in the isolated form [1]. Such follicle culture systems have been used respectively to investigate *in vitro* early and late folliculogenesis at preantral follicle stage [2]. Culture systems for primordial follicles are important tools for studying the mechanism of oocyte development, and are a potential source of oocytes that can be used for *in vitro* embryo production. The factors that control primordial follicle activation and further growth of primary follicles are not well understood [2]. However, endocrine hormones, like follicle stimulating hormone (FSH), are known to regulate the production of several growth factors that play a critical role in primordial follicle activation and growth [3].

Some *in vitro* studies have demonstrated that the addition of FSH to the culture medium is important to maintain viability and to promote ovine follicular activation and further growth *in vitro* [3-5]. In addition, Magalhães et al. [6] showed that the addition of 50 ng/mL recombinant bovine FSH (rFSH) during *in vitro* culture of goat preantral follicles maintained viability, activation and follicular growth.

Despite the advances made in this field, the success of *in vitro* culture of preantral follicles is still very limited and the majority of reports is restricted to investigative studies to elucidate how early or preantral folliculogenesis works. As assisted reproductive technique (ARTs), to date, this tool has been limited to advances in mice, the only species to have been reported the birth of animals from embryos originated from completely developed preantral follicles *in vitro* [7, 8]. In large animals such as sheep [9] or even in human [10] the complete development *in vitro* of preantral follicles from a primordial to an ovulatory follicle has been hampered by growth arrest at the primary follicle stage [9,10]. One reason for this shortcoming is that the requirements for *in vitro* growth are not well characterized due to lack of knowledge regarding activation of primordial and development of primary follicles compared to development of follicles in later stages [11]. In addition, it is known that during *in vitro* culture of preantral follicles, there is an increase production of reactive oxygen species: ROS [12], which can affect growth, survival and consequently can lead to cell death [13]. In this context, at the present time, there is an increase interest in natural products (medicinal plants) that prevent oxidative damages caused by the ROS and as a result may contribute to promote the activation, survival, growth of preantral follicles *in vitro* enclosed in ovarian tissue. Among these potential natural products it is important to higligh *J. insularis* and anethole.

J. insularis T. Anders (family Acanthaceae), is an herbaceous and perennial plant, widely distributed in tropical area of Africa [14]. In ghomala'a (traditional language spoken in

Western Cameroon), *J. insularis* is called “kwe mchie” [15]. Traditionally, in Senegal, the leaf decoction of *J. insularis* is given to women during the last month of pregnancy to reduce labour pains. In Cameroon and specifically in the Western region, their leaves are used in association with the leaves of three others medicinal plants (*Aloe buettneri, Hibiscus macranthus and Dicliptera verticillata*), to treat dysmenorrhoea and some cases of women infertility [15,16]. The *in vivo* foliculogenic effect of their leaves have been related to their composition. Besides alkaloids, glycosides, polyphenols and triterpenoids, studies undertaken by by Telefo et al. [14] and Goka et al. [17] revealed the presence of flavonoids in their leaves which can acts as a natural antioxidant. A mix of aqueous extract of *J. insularis* and others medicinal plants (*Aloe buettneri, Hibiscus macranthus* and *Dicliptera verticillata*) has also been proven, in a series of studies to induce ovarian steroidogenesis and folliculogenesis in female rats [17-19].

Anethole, other natural compound originated from *Croton zehntneri* Pax & K. Hoffm (family Euphorbiaceae), a plant locally known as “*canela* de *cunhã*” or “*canelinha*” in the Northeast of Brazil [20] has also showed antioxidant activity due its capacity to decrease the concentrations of ROS both *in vivo* [21] and *in vitro* [22]. Recently, Sá et al. [23] demonstrated that anethole reduced the levels of ROS, proving its antioxidant activity on goat isolated preantreal follicles (secondary stage) cultured *in vitro*.

Despite the importance of the aforementioned natural products for ARTs, to the best of our knowledge, there is no study investigating the effect of the aqueous extract of *J. insularis* on *in vitro* folliculogenesis. In addition the effect of anethole on the *in vitro* culture of primordial follicle enclosed in ovarian tissue is not known. Therefore this study was conducted to 1) investigate the addition effect of FSH and *J. insularis* extract on the survival, activation, growth and ROS generation after *in vitro* culture of ovine preantral follicles enclosed in ovarian tissue 2) compare the efficiency of FSH and *J. insularis* on stromal cell density and all parameters above mentioned in culture medium containing anethole.

2. Materials and Methods

This study was approved and performed under the guidelines of the Ethics Committee for Animal Use of the State University of Ceará (N° 6004720/2015). Unless mentioned otherwise, the culture media, anethole and other chemicals used in the present study were purchased from Sigma Chemical Co. (St. Louis, USA).

2.1. Source of ovaries

Ovaries (n = 22) were collected at a local slaughterhouse from 11 adults (1-3 years old) mixed-breed sheep (*Ovis aries*). 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 [5].

2.2. Plants materials to extracts preparation and culture medium

The fresh leaves of *J. insularis* previously identified in the National Herbarium of Cameroon under voucher specimen code 34997 [17] were collected in Western Cameroon (Batoufam subdivision, Upper-Plateau division, 5° 21′ Nord 10° 24′ East, Altitude 1515 m). The fresh leaves were then dried at room temperature in the shade. Subsequently, the plant extract decoction was prepared according to the protocol [16]. Finally, the plant decoction was lyophilized and kept in the freezer at -20 ºC. The lyophilized extract was then diluted in the distilled water to obtain the desired concentrations (0.3 mg/mL, 1.25 mg/mL and 5 mg/mL).

The basic culture medium consisted of α-MEM (pH 7.2 - 7.4) supplemented with 1.25 mg/mL bovine serum albumin, 10 µg/mL insulin, 5.5 mg/mL transferrin, 5 ng/mL selenium, 2 mM glutamine, 2 mM hypoxanthine and antibiotics (100 µg/mL penicillin-streptomycin) which was referred to as α-MEM$^+$ [24]. In a first experiment, to test the effect of *J. insularis* on the culture of ovine preantral follicles, the α-MEM$^+$ was supplemented with FSH or different concentrations of *J. insularis*, mentioned above. In a second experiment, the α-MEM$^+$ was supplemented with anethole + FSH or with anethole + *J. insularis*.

The concentrations of *J. insularis* were defined based on a concentration curve done according to *in vivo* [16] and *in vitro* [25]. Briefly, the best concentration *in vivo* (5 mg/mL) was divided per 4 to obtain 1.25 mg/mL which subsequently was divided by the same factor (4) to obtain 0.3 mg/mL. This latest concentration was close to the best concentration of *Amburana Cearensis*, other medicinal plant tested in *in vitro* culture of sheep preantral follicles [25]. While the concentration of anethole used was chosen based on previous studies performed in our laboratory on *vitro* culture of goat preantral follilces [23].

2.3. Experimental design

As briefly mentioned, this work was divided into two non-simultaneous experiments.

In the first one, sheep ovarian cortex from each ovarian pair (n= 5) was cut using a tissue slicer (Thomas Scientific, USA) into 22 fragments (approximately 3 x 3 x 0.5 mm). One fragment was taken randomly and immediately fixed for histological analysis and identified as non-cultured control, the remaining fragments were *in vitro* cultured in 1 mL of α-MEM$^+$; α-MEM$^+$ supplemented with FSH 50 ng/mL, or with different concentrations (0.3; 1.25 or 5 mg/mL) of lyophilized plant extract *J. insularis* for 1 or 7 days at 39 °C in 5% CO_2 in air. These treatments were referred to as: MEM$^+$ (cultured control), FSH, JUS0.3, JUS1.25 and JUS5, respectively. The culture medium was equilibrated at least 3 h prior to use. Every two days,

whole culture medium was replaced. Based on the histological analysis, the best treatments (higher percentage of morphological normal follicles) were selected to next experiment.

In the second experiment, ovarian fragments were obtained as previously described in experiment 1. The fragments from each ovarian pair (n= 6) were either fixed for histological analysis (non-cultured control) or *in vitro* cultured in 1 mL of α-MEM^{+} + anethole 300 µg/mL + FSH 50 ng/mL or α-MEM^{+} + anethole 300 µg/mL + *J. insularis* 0.3 mg/mL, corresponding to the following treatments: MEM^{+}+ANE+FSH and MEM^{+}+JUS+ANE, respectively.

2.4. Morphological analysis and evaluation of follicular growth in vitro

Before (non-cultured control) and after 1 or 7 days of culture, the ovarian fragments were fixed individually in 4 % buffered paraformaldehyde for 2 h. Subsequently, fragments were dehydrated in a graded concentrations of ethanol. After paraffin embedding (Synth, São Paulo, Brazil), the ovarian fragments were cut into 7 µm sections and mounted on glass slides and stained by periodic acid schiff-hematoxylin. Follicle stage and survival were assessed microscopically on serial sections.

The developmental stages of follicles are *primordial* (one layer of flattened pregranulosa 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) [26]. These follicles were still classified individually as histologically normal when an intact oocyte was present, surrounded by granulosa cells which are well organized in one or more layers and that have 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 [26]. Were evaluated 150 follicles for each treatment (30 follicles per each five repetitions) in both experiments.

To evaluate follicular activation, the percentages of healthy primordial and growing follicles were calculated before (non-cultured control) and after culture in each treatment. 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 [26].

2.5. Ovarian stromal cell density

Ovarian stroma density was evaluated by calculating the stromal cell per 100 µm^{2}. For each treatment, ten fields per slide were assessed and the mean number of stromal cell per field

was calculated in experiment 2 [27]. All evaluations and measurements were performed by a single operator.

2.6. Reactive oxygen species levels

The ROS levels were determined by a spectrofluorimetric method [28], using 2',7'-dihydrodichlorofluorescein diacetate (DCHF-DA) assay. Sample aliquot (50 µL) was incubated with 5 µL of DCHF-DA (1 mM). The oxidation of DCHF-DA to fluorescent dichlorofluorescein was measured for the detection of ROS. The DCF fluorescence intensity emission was recorded at 520 nm (with 480 nm excitation) 2 h after the addition of DCHF-DA to the medium. The correlation between the follicular viability and ROS levels was done to better understand the effect of ROS by the cells during *in vitro* culture of preantral follicles.

2.7. Statistical analysis

Statistical analyses were carried out using the Sigma Plot 11.0 software (Systat Software Inc, San Jose, California, USA). Data that were not normally distributed (Shapiro-Wilk test) were submitted to logarithmic transformation. The percentage of morphologically normal and growing preantral follicles among treatments and days of culture were compared by Fisher's exact or chi-square tests. The Mann-Whitney test was performed to analyze the levels of reactive oxygen species and stromal cell density among treatments and days of culture. Spearman correlation test was used to assess the association between normal preantral follicles and reactive oxygen species. In addition, the association between stromal cell density and percentage of normal preantral follicles was evaluated by linear regression analysis. Data were presented as mean (± standard error of the mean) and percentage, unless otherwise indicated. Statistical significance was defined as $P < 0.05$ and probability values > 0.05 and ≤ 0.1 indicated that a difference approached significance.

3. Results

3.1. Sheep preantral follicles morphology and development

In the first experiment, a total of 1,374 preantral follicles were analyzed. Morphologically normal or degenerated follicles were observed in the non-cultured control as well as in the *in vitro* cultured ovarian tissues (Fig. 1).

The percentage of morphologically normal and follicular growth before and after *in vitro* culture is shown in table 1 (experiment 1). JUS0.3 was the only treatment that maintained the percentage of morphologically normal follicles similar to non-cultured control even after 7 days of culture. In addition, this treatment showed a higher percentage ($P < 0.05$) of normal follicles than the other treatments regardless to the culture time. However, at day 7, *J. insularis* significantly reduced ($P < 0.05$) the percentage of morphologically normal follicles in a concentration-dependent manner. On the other hand, after 7 days of culture, a significantly higher percentage of developing follicles was observed in the JUS5 treatment compared to the other treatments except JUS1.25.

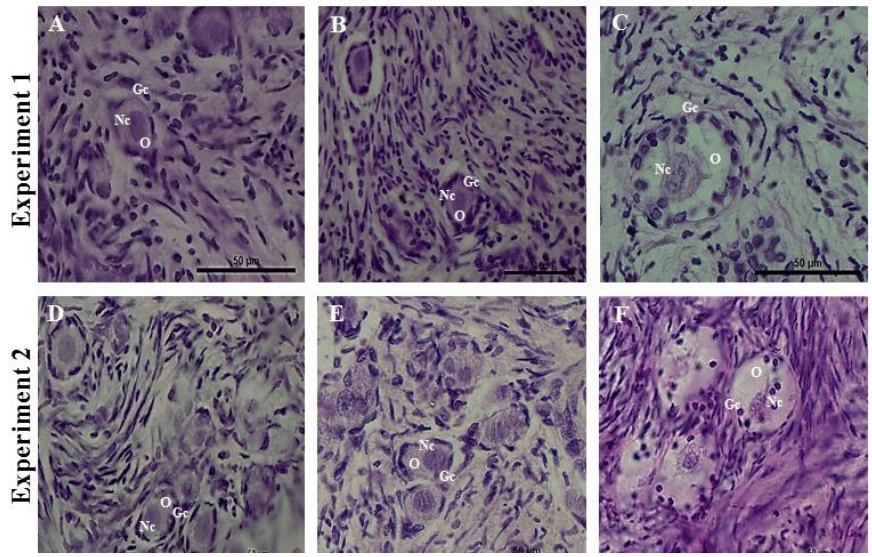


Fig. 1 Representative images of the morphology of ovine preantral follicles before and after *in vitro* culture. On the top panels we can see follicles from experiment 1 and on the bottom panels, follicles from experiment 2, after staining with periodic acid schiff-hematoxylin. Normal follicles are shown in non-cultured control (A), JUS0.3 (B), FSH (D) and FSH+ANE (E), while degenerated follicles are represented in JUS5 (C) and JUS+ANE (F) after 7 days of culture. Note the retracted oocyte with a pyknotic nucleus, disorganized granulosa cells (C and F). O: oocyte; Nc: oocyte nucleus; Gc: granulosa cells (400 x), bar 50 µm.

Table 1 Percentage of morphologically normal and growing preantral follicles before (non-cultured control) and after *in vitro* culture for 1 or 7 days in different treatments, in experiment 1.

	Follicular morphology (%)		**Follicular development (%)**			
			Primordial		**Developing**	
Non-cultured control	84.6 (127/150)		86.6 (110/127)		13.4 (17/127)	
	Day 1	**Day 7**	**Day 1**	**Day 7**	**Day 1**	**Day 7**
MEM^+ (cultured control)	82.5 (109/132)^{aA}	62.5 (70/112)^{*bA}	88.9 (97/109)^{aA}	85.7 (60/70)^{aA}	11.1 (12/109)^{aA}	14.3 (10/70)^{aA}
FSH	79.8 (99/124)^{aA}	65.2 (62/95)^{*bA}	75.7 (75/99)^{*aB}	87.1 (54/62)^{aA}	24.3 (24/99) ^{*aB}	12.9 (8/62)^{aA}
JUS0.3	91.3 (137/150)^{aB}	89.5 (111/124)^{aB}	77.4 (106/137)^{aB}	84.7 (94/111)^{aA}	22.6 (31/137)^{aB}	15.3 (17/111)^{aA}
JUS1.25	58.1 (57/98)^{*aC}	47.3 (71/150)^{*aC}	85.9 (49/57)^{aAB}	74.6 (53/71)^{*aAB}	14.1 (8/57) ^{aAB}	25.4 (18/71)^{*aAB}
JUS5	66.1 (52/111)^{*aC}	21.9 (29/132)^{*bD}	82.5 (66/80)^{aAB}	65.5 (19/29)^{*aB}	17.5 (14/80) ^{aAB}	34.5 (10/29)^{*aB}

* Differs from non-cultured control ($P < 0.05$)

^{a,b} Within a row and the same parameter evaluated, values without a commom superscript differed ($P < 0.05$)

^{A,B,C,D} Within a column, values without a commom superscript differed ($P < 0.05$)

In the second experiment, a total of 1,286 preantral follicles were analyzed. The percentages of morphologically normal preantral and growing follilces in non-cultured control and after 1 or 7 days of culture in medium containing ANE supplemented with FSH or JUS are shown (table 2). After *in vitro* culture, compared to non-cultured control, the percentage of normal follicles was reduced ($P < 0.05$), except in MEM^++ANE+ FSH treatment on day 1. Both cultured treated groups significantly reduced ($P < 0.05$) the percentage of normal follicles from day 1 to day 7 ($P < 0.05$).

After 1 and 7 days of culture, there was a significant reduction ($P < 0.05$) in the percentage of primordial follicles with concomitant increase ($P < 0.05$) in the percentage of

developing follicles in both treatments compared to non-cultured control, indicating the follicular activation process. Furthermore, with the progression of the culture time, only MEM$^+$+ANE+FSH significantly increased ($P < 0.05$) the percentage of developing follicles. In addition, at day 7 of culture, MEM$^+$+ANE+FSH treatment showed a higher percentage of developing follicles than MEM$^+$+ANE+JUS treatment.

Table 2 Percentage of morphologically normal and growing preantral follicles before (Non-cultured control) and after *in vitro* culture for 1 or 7 days in MEM$^+$+ anethole supplemented with FSH or *J. insularis* (experiment 2).

	Follicular morphology (%)		**Follicular development (%)**			
			Primordial		**Developing**	
Non-cultured control	95.5 (172/180)		69.1 (119/172)		30.9 (53/172)	
	Day 1	**Day 7**	**Day 1**	**Day 7**	**Day 1**	**Day 7**
MEM$^+$ + ANE + FSH	91.4 (139/152)^{aB}	61.1 (80/131)^{*bA}	58.3 (81/139)^{*aA}	18.7 (15/80)^{*bA}	41.7 (58/139)^{*aA}	81.3 (65/80)^{*bA}
MEM$^+$ + ANE + JUS	77.2 (139/180)^{*aA}	16.5 (16/97)^{*bB}	56.8 (79/139)^{*aA}	43.7 (7/16)^{*aB}	43.2 (60/139)^{*aA}	56.3 (9/16)^{*aB}

*Differs from non-cultured control ($P < 0.05$)

^{a,b} Within a row and the same parameter evaluated, values without a commom superscript differed ($P < 0.05$)

^{A,B} Within a column, values without a commom superscript differed ($P < 0.05$)

3.2. Evaluation of stromal cell density and correlation with normal follicular morphology in MEM$^+$ + anethole supplemented with FSH or J. insularis

Stroma cells from non-cultured control, and after 1 or 7 days of culture in two treatments are shown (Fig. 2). Regardless the culture time, a significant reduction was observed ($P < 0.05$) in the percentage of stroma density compared to non-cultured control as well as from day 1 to day 7. When treatments were compared at day 1 of culture, the percentage of stroma density was significantly higher ($P < 0.05$) in MEM$^+$+ANE+JUS treatment, while the opposite was observed at day 7 of culture.

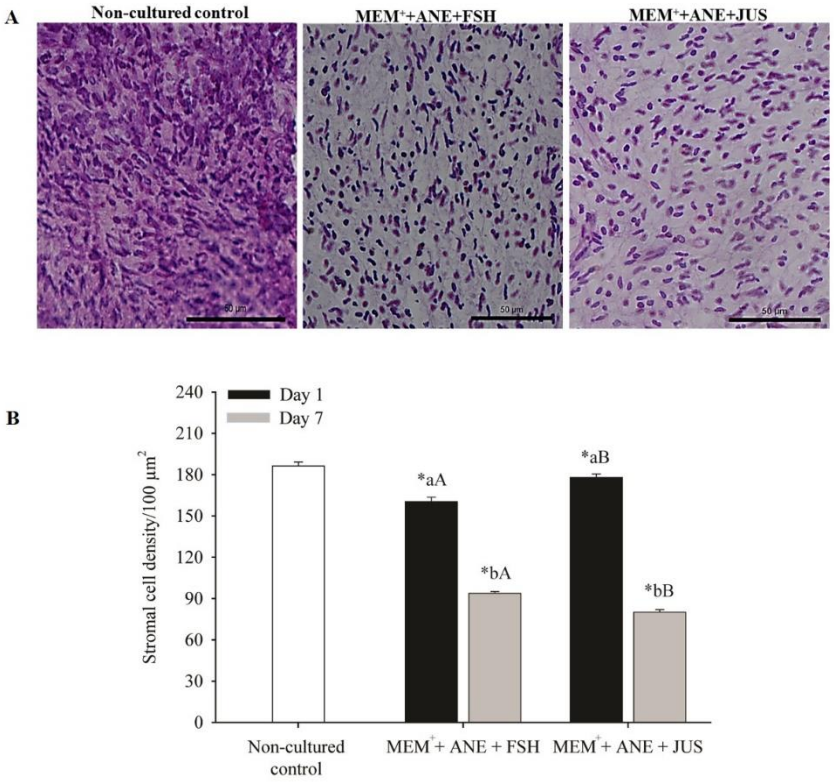


Fig. 2 Stromal cell density (cells/100 µm^2). (A) Representative images (400 x), bar 50 µm and (B) mean (± SEM) of ovarian stroma cell density in non-cultured control and after 7 days of in vitro culture in MEM$^+$+ANE+FSH and MEM$^+$+ANE+JUS treatments.

*Differs from non-cultured control ($P < 0.05$)

^{a,b} Within treatment, values without a commom superscript differed ($P < 0.05$).

^{A,B} Within day, values without a commom superscript differed ($P < 0.05$).

As shown in Fig. 3, increase of the stromal cell density simultaneously results in the increase in the percentage of morphologically normal follicles.

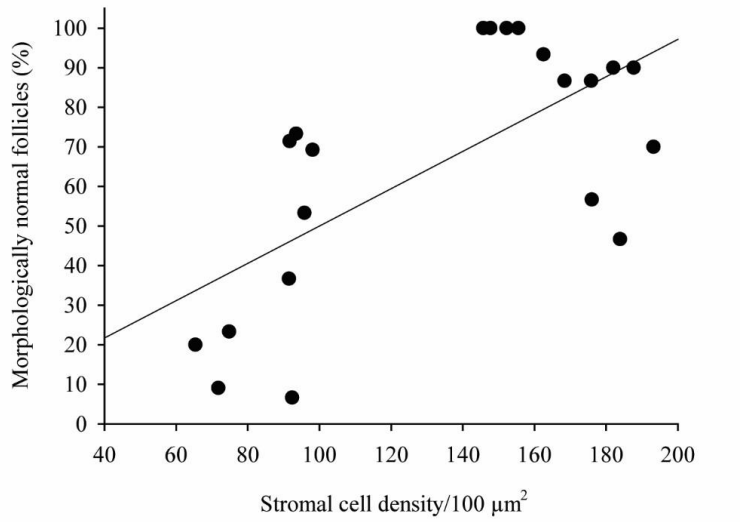


Fig. 3 Relationship of stromal cell density with percentage of normal preantral follicles. The association between variables (black line) was analyzed by linear regression [Normal preantral follicles = 2.887 + (0.471 × stromal cell density); r = 0.66; R^2 = 0.44; P < 0.001]. Each point on the chart represents one ovarian fragment evaluated.

3.3. Levels of reactive oxygen species after in vitro culture

The ROS levels were determined on days 1, 2, 4 and 6 of *in vitro* culture and is shown in Fig. 4 (experiment 1) and 5 (experiment 2).

In the experiment 1, regardless the culture time, addition of either FSH or JUS did not change the ROS levels compared to control treatment. However the control treatment showed a significant higher ($P < 0.05$) ROS levels on day 4 compared to day 1. In addition, only on day 2, the ROS levels were significant higher ($P < 0.05$) in JUS5 than JUS1.25 treatment. Finally a negative correlation was observed between the percentage of normal follicle and the concentration of ROS.

With regard to experiment 2, from day 2 onwards, MEM^++ANE+JUS treatment showed a significant higher ($P < 0.05$) ROS levels than MEM^++ANE+FSH treatment. Contrary to MEM^++ANE+JUS treatment, MEM^++ANE+FSH significantly increased ($P < 0.05$) the ROS levels from day 1 to day 6.

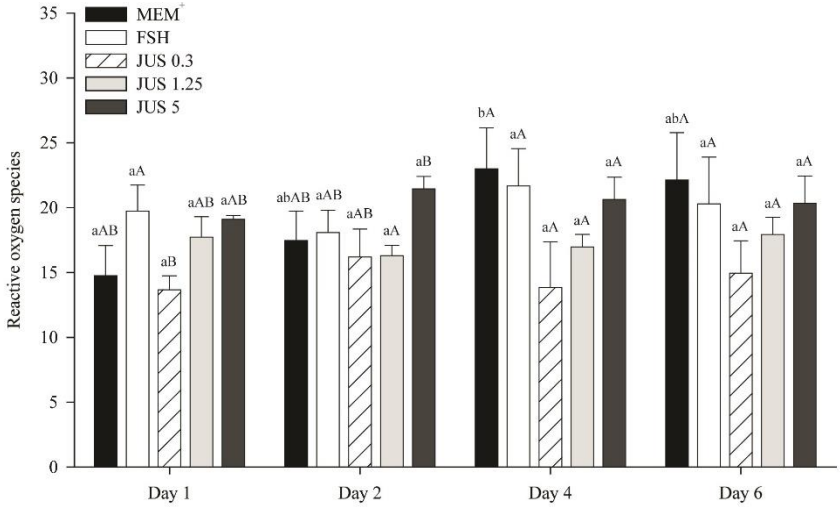


Fig. 4 ROS levels after 1 or 6 days in the absence or presence of FSH or different concentrations of Justicia insularis.

[a,b] Within treatment, values without a commom superscript differed ($P < 0.05$)

[A,B] Within day, values without a commom superscript differed ($P < 0.05$)

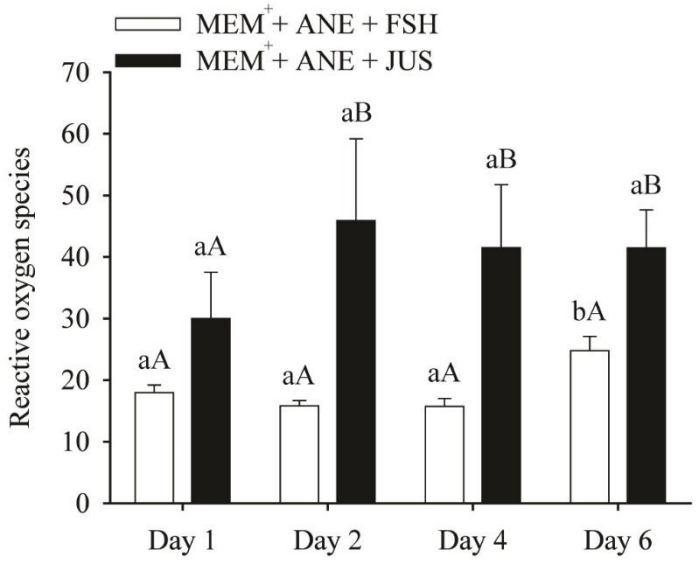


Fig. 5 ROS levels after 1 or 6 days in the presence of FSH + anethole and Justicia insularis + anethole.

[a,b] Within treatment, values without a commom superscript differed ($P < 0.05$)

[A,B] Within day, values without a commom superscript differed ($P < 0.05$)

As shown in Fig. 6, increase of the ROS levels simultaneously result in the decrease in the percentage of morphologically normal follicles.

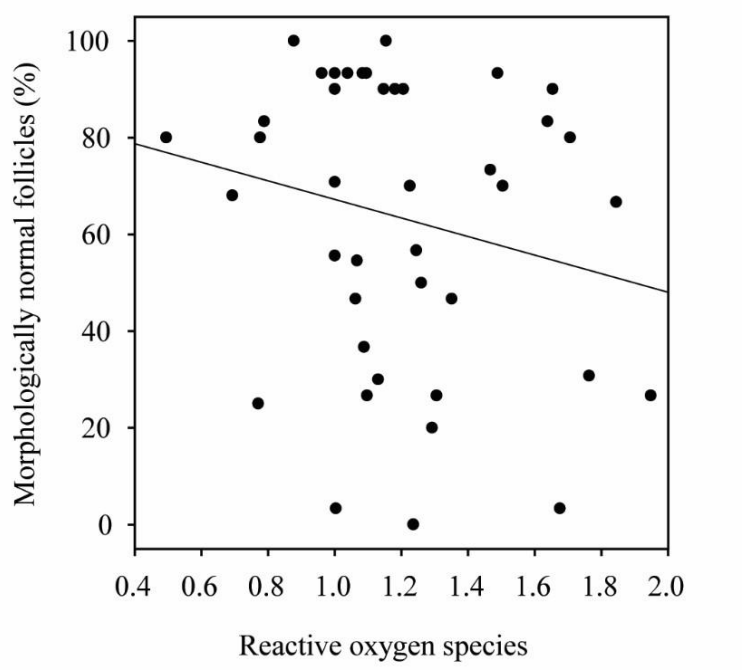


Fig. 6 Correlation between the follicular morphology and the reactive oxygen species. Each point of the graph represent a sample of the medium recovered during the in vitro culture (n=42), r = - 0,25; P = 0,1.

4. Discussion

Although the action of FSH on the development of preantral follicles cultured *in vitro* within the ovarian cortex has already been investigated in different species (human [29], sheep [3], goat [6], bovine [30], buffalo [31], baboon [32]), this is the first study to describe the effects of the aqueous extract of *J. insularis* on *in vitro* early folliculogenesis. *J. insularis* is a plant commonly used in the tropical area of Africa to ameliorate female reproductive disorders [16, 17] and it is known to have a FSH-like and antioxidant effects. Thus, this study demonstrated that adding *J. insularis* in a concentration-dependent manner maintained the levels of ROS and improved both *in vitro* follicular survival and activation of ovine primordial follicles enclosed in ovarian tissue when compared to the cultured control and FSH treatments. On the other hand, addition of *J. insularis* or FSH in a culture medium containing anethole promote beneficial effects on activation of ovine preantral follicles.

In the present study, JUS0.3 treatment maintained the percentage of normal follicles similar to the non-cultured control even after 7 days of culture and showed a significantly higher values than the other treatments. Such effect could be due to the action of FSH-like compounds of the extract of *J. insularis*. Phytochemical analysis of *J. insularis* have revealed the presence of alkaloids, glycosides, polyphenols, triterpenoids and flavonoids [14]. These metabolites are responsible for the induction of ovarian follicle growth and increase in the number of corpora lutea recorded during the fertility assay performed on immature female rats [16]. According to Andrade et al. [4], addition of FSH to the culture medium is important to the maintenance of morphology and activation of sheep primordial follicles, as well as for further follicular growth *in vitro* [33]. In addition, Thomas et al. [34] reported that FSH stimulates the expression of some growth factors, such as Kit Ligand, Bone Morphogenetic Protein-15 and Growth Differentiation Factor-9, which are important for the regulation of early folliculogenesis. The presence of FSH in the culture medium containing anethole also maintained follicular morphology.

J. insularis in high concentration (expeiriment 1) or in low concentration in the presence of anethole (experiment 2) have detrimental effects on the ovarian tissue. This could be due to an excess of antioxidant in the culture medium which can be harmful to preantral follicles. It is well known that oxidative metabolism is indispensable for energy production of follicles during *in vitro* culture [35], which in turn results in generation of ROS. Although a critical amount of ROS is essential for their physiological activities, excessive amount of them generates a contrary effect [12, 35]. Furthermore, some antioxidant substances act as pro-oxidants when used at high concentrations [36]. In this way, some studies have shown that high concentrations of ascorbic acid, an important antioxidant added to the culture medium, can inhibit important physiological processes in the ovary, resulting in follicular degeneration [37] besides promoting oxidative damage to cellular DNA [38].

This study also focused on the features of stroma tissue surrounding the follicles because any damage to this compartment may affect follicular morphology. After 1 and 7 days of culture, a reduction in the percentage of stromal cell density was observed in MEM^{+}+ANE supplemented either with *J. insularis* or with FSH compared to non-cultured control. The *in vitro* culture may have affected the bi-directional contact between germinal and somatic cells which are important to maintain the integrity of the cortex. In addition, in our opinion, cells can die during *in vitro* cultured by various factors such as nutrient deficiency in the medium, ischemia, generation of ROS, toxicity of the supplements. Our results are an in agreement with findings using sheep [39] and goat [27] ovarian tissues. Interestingly, a positive relationship between morphology and stromal cell density was observed. Previous reports have shown that

ovarian stromal cells are responsible for the production of growth factors and peptides which are essential substances for follicular development [2].

Finally, we have evaluated the antioxidant effect of *J. insularis* and FSH in basic culture media in the absence (experiment 1) or presence (experiment 2) of anethole. In the first experiment (Fig. 4), addition of either FSH or JUS did not change the ROS levels compared to control treatment.The maintenance of ROS levels could be due to the capacity of *J. insularis* and FSH to scavenge ROS and their metal chelating properties. Similar results have been found during *in vitro* culture of ovine preantral follicles with *Amburana cearensis* [25]. According to Gouveia et al. [40], the extract of *A. cearensis* contains gallic acid, protocatechuic acid (PCA), epicatechin, *p*-coumaric acid and kaempferol. Gallic acid and PCA are endogenous plant phenols which stimulates the production antioxidant enzymes such as catalase (CAT) and glutathione peroxidase (GPx) [41]. Reports from Telefo et al. [17] and Goka et al. [14] showed that *J. insularis* are constituted of several secondary metabolites among which the phenols and flavonoids, we thus belevied that those phenolic compounds act in a similar way as metabolic of *A. Cearensis* to maintain the ROS produced during *in vitro* culture.

In the present study, from day 1 to day 6, the presence of FSH in culture medium containing anethole increase the ROS levels. To date, it has been shown that FSH stimulates catalase activity in goat granulosa cells modulating intracellular ROS levels [42]. ROS inhibitors, in a concentration-dependent manner decreased oocyte maturation induced by FSH [43]. In our study, we suggest that an adequate FSH concentration (50 ng/mL) in a culture medium which contained anethole contributed to the maintenance of suitable levels of ROS after day 6 of culture, resulting in higher rates of follicle survival and activation. As above mentioned, we also observed that the combination between *J. insularis* and anethole increased the ROS level. This may be due to the high concentration of antioxidant in the culture medium. Indeed, antioxidant compounds in high concentration can be converted in pro-oxidant compounds. Some authors showed that some antioxidants could have oxidative action depending on the concentration [44]. Furthermore, during *in situ* culture, many cells such as granulosa, theca cells produce ROS. Those ROS with the time progression during *in vitro* culture caused injury to the follicular cells and thus affect the follicular morphology [12].

5. Conclusions

J. insularis in a concentration-dependent manner maintained the levels of ROS and improved both *in vitro* follicular survival and activation of ovine primordial follicles enclosed in ovarian tissue. Finally, as well as FSH 50 ng/mL, the addition of *J. insularis* 0.3 mg/mL in a culture medium containing anethole promotes beneficial effects on activation of ovine preantral

follicles kept *in vitro* at least for a short-term. Although the mechanisms of interaction are unclear, these results open a vast field of research regarding interactions between plant extract and FSH in the development of primordial follicles until the antral stage. This study represents a hope for the use of medicinal plants in more complex experiments which aimed at the complete development of preantral follicles *in vitro*. *J. insularis* could be used for futher experiments to evaluate its beneficial effects on isolated preantral follicles development and oocyte maturation.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

The authors would like to thank the National Council for Scientific and Technological Development (CNPq Brazil) for financial support. Gildas Mbemya Tetaping is the recipient of a doctoral scholarship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (PEC-PG/CAPES, Brazil). Ana Paula Ribeiro Rodrigues is recipient of a grant from CNPq Brazil through the projects 473968/2013-4 and 457226/2013-7. Johan Smitz is Especial Visitor Researcher from CAPES (88881.030.433/2013-01).

References

[1] Araújo, V. R., Gastal, M. O., Figueiredo, J. R., and Gastal, E. L. 2014. "*In vitro* culture of bovine preantral follicles: a review." Reprod Biol Endocrinol. 12, 78.

[2] Picton, H. M., Harris, S. E., Muruvi, W., and Chambers, E. L. 2008. "The *in vitro* growth and maturation of follicles." Reproduction 136: 703-715.

[3] Esmaielzadeh, F., Hosseini, S. M., Hajian, M., Nasiri, Z., Chamani, M., Afshar, A.M., et al. 2013. "Role of follicle stimulating hormone in the survival, activation and further growth of *in vitro* cultured sheep primordial follicles." Iran. J. Appl. Anim. Sci. 3: 785-790.

[4] Andrade, E. R., Seneda, M. M., Alfieri, A. A., Oliveira, J. A., Bracarense, A. P. F., Figueiredo, J.R., et al. 2005. "Interactions of indole acetic acid with EGF and FSH in the culture of ovine preantral follicles." Theriogenelogy 64: 1104-1113.

[5] Lima, L. F., Rocha, R. M. P., Alves, A. M. C. V., Saraiva, M. V. A., Araújo, V. R., Lima, I. M. T., et al. 2013. "Dynamized follicle-stimulating hormone affects the development of ovine preantral follicles cultured *in vitro*." Homeopathy 102: 41-48.

[6] Magalhães, D. M, Araújo V. R., Lima-Verde, I. B., Matos, M. H. T., Silva, R. C., Lucci, C. M., et al. 2009. "Impact of pituitary FSH purification on *in vitro* early folliculogenesis in goats." Biocell. 33: 91-97.

[7] Eppig, J. J, and O'Brien, M. J., 1996. "Development *in vitro* of mouse oocytes from primordial follicles." Biol. Reprod. 54: 197-207.

[8] O'Brien, M. J., Pendola, J. K., and Eppig, J. J., 2003. "A revised protocol for *in vitro* development of mouse oocytes from primordial follicles dramatically improves their developmental competence." Biol. Reprod. 68: 1682-1686.

[9] Cecconi, S., Barboni B., Coccia, M., and Mattioli, M., 1999. "*In Vitro* Development of Sheep Preantral Follicles." Biol. Reprod. 60: 594-601.

[10] Mery, L., Lefevre, A., Benchaib, M., Demirci, B., Salle, B., Guerin, J. F., et al. 2007. "Follicular growth *in vitro:* detection of growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) during *in vitro* culture of ovine cortical slices." Mol. Reprod. Dev. 74: 767-774.

[11] Muruvi, W., Picton, H. M., Rodway, R. G., and Joyce, I. M., 2009. "*In vitro* growth and differentiation of primary follicles isolated from cryopreserved sheep ovarian tissue. Anim." Reprod. Sci. 112: 36-50.

[12] Costa, R. A. P., Romagna, C. D., Pereira, J. L., and Souza-pinto, N. C., 2011. "The role of mitochondrial DNA damage in the citotoxicity of reactive oxygen species." J. Bioenerg. Biomembr. 43: 25-29.

[13] Lima-Verde, I. B., Rossetto, R., Matos, M. H. T., Celestino, J. J. H., Bruno, J. B., Silva, C. M. G., et al. 2009. "Androstenedione and follicle stimulating hormone involvement in the viability and development of goat preantral follicles *in vitro*." Anim. Reprod. 7: 80-89.

[14] Goka, M. S. C., Telefo, P. B., Mbemya, G. T., Awouafack, M. D., Lienou, L. L., Yemele D. M., et al. 2016. "Potentialisation of pregnant mare serum gonadotropin inducing effect on ovarian follicles growth by the aqueous extract of *Aloe buettneri, Dicliptera verticillata, Hibiscus macranthus* and *Justicia insularis* leaves in immature rats." Pharmacolology. 7: 328-336.

[15] Telefo, P. B., Lienou, L. L., Yemele, M. D., Lemfack, M. C., Mouokeu, C., Goka, C. S., et al. 2011. "Ethnopharmacological survey of plants used for the treatment of female infertility in Baham, Cameroon". J. Ethnopharmacol. 136: 178-187.

[16] Telefo, P. B., Tagne, S. R., Koona, O. E. S., Yemele, D. M., and Tchouanguep, F. M., 2012. "Effect of the aqueous extract of *Justicia insularis* T. Anders (acanthaceae) on ovarian folliculogenesis and fertility of female rats." Afr. J. Tradit. Complement. Altern. Med. 9: 197-203.

[17] Telefo, P. B., Moundipa, P. F., Tchana, A. N., Tchouanguep, D., and Mbiapo, F. T., 1998. "Effects of an aqueous extract of *Aloe buettneri, Justicia insularis, Hibiscus macranthus, Dicliptera verticillata* on some physiological and biochemical parameters of reproduction in immature female rats." J. Ethnopharmacol. 63: 193-200.

[18] Telefo, P. B., Moundipa, F. P., and Tchouanguep, M. F., 2001. "Influence of the aqueous extract of *Aloe buettneri, Justicia insularis, Hibiscus macranthus* and *Dicliptera verticillata* on fertility and some biochemical parameters of reproduction in rats." J. Cameroon Acad. Sci. 3: 144-150.

[19] Telefo, P. B., Moundipa, P. F., and Tchouanguep, F. M., 2004. "Inductive effects of the leaf mixture extract of *Aloe buettneri, Justicia insularis, Dicliptera verticillata* and *Hibicus marcranthus* on *in vitro* production of oestradiol." J. Ethnopharmacol. 91: 225-230.

[20] Leal-Cardoso, J. H., and Fonteles, M. C., 1999. “Pharmacological effects of essential oils plants of the Northeast of Brazil.” An. Acad. Bra. Ciênc. 71: 207-213.

[21] Freire, R. S., Morais, S. M., Catunda-Junior, F. E., and Pinheiro, D. C., 2005. “Synthesis and antioxidant, anti-inflammatory and gastroprotector activities of anethole and related compounds.” Bioorg. Med. Chem. 13: 4353-4358.

[22] Chainy, G. B., Manna, S. K., Chaturvedi, M. M., and Aggarwal, B. B., 2000. “Anethole blocks both early and late cellular responses transduced by tumor necrosis factor: effect on NF kappa B, AP-1, JNK, MAPKK and apoptosis.” Oncogene 19: 2943-2950.

[23] Sá, N. A. R., Araújo, V. R., Correia, H. H. V., Ferreira, A. C. A., Guerreiro, D. D., Sampaio, A. M., et al. 2016. “Anethole improves the *in vitro* development of isolated caprine secondary follicles.” Theriogenology 89: 226-234.

[24] Bandeira, F. T., Carvalho, A. A., Castro, S. V., Lima, L. F., Viana, D. A., Evangelista J. S. A. M., et al. 2015. “Two methods of vitrification followed by *in vitro* culture of the ovine ovary: Evaluation of the follicular development and ovarian extracellular matrix.” Reprod. Domest. Anim. 50: 177-185.

[25] Barberino, R. S., Barros, V. R. P., Menezes, V. G., Santos, L. P., Araújo, V. R., Queiroz, M. A. A., et al. 2015. “*Amburana cearensis* leaf extract maintains survival and promotes *in vitro* development of ovine secondary follicles.” Zygote 1-9.

[26] Matos, M. H. T., Lima-verde, I. B., and Luque, M. C. A., 2007. “Essential role of follicle stimulating hormone in the maintenance of caprine preantral follicle viability *in vitro*.” Zygote 15: 173-182.

[27] Carvalho, A. A., Faustino, L. R., Castro, S. V., Silva, C. M. G., Campello, C. C., Figueiredo, J. R., et al. 2013. “Tissue thickness may influence the outcome of vitrification of goat ovarian cortex.” Acta Sci. Vet. 41: 1150.

[28] Loetchutinat, S., Kothan, S., Dechsupa, J., Meesungnoen, J., and Jay-Gerin, S. M., 2005. “Spectrofluorometric determination of intracellular levels of reactive oxygen species in drug-sensitive and drug-resistant cancer cells using the 2’,7’- dichlorofluorescein diacetate assay.” Radiat. Phys. Chem. 72: 323-331.

[29] Wright, C. S., Hovatta, O., Margara, R., Trew, G., Winston, R. M., Franks, S., et al. 1999. “Effects of follicle-stimulating hormone and serum substitution on the in-vitro growth of human ovarian follicles.” Hum. Reprod. 14: 1555-1562.

[30] McLaughlin, M., Bromfield, J. J., Albertini, D. F., and Telfer, E. E., 2010. Activin promotes follicular integrity and oogenesis in cultured pre-antral bovine follicles. Mol. Hum. Reprod.16, 644-53.

[31] Tamilmani, G., Varshney, P. K., Dubey, V. P., Pathak, M. C., and Sharma, G. T., 2013. “Influence of FSH on *in vitro* growth, steroidogenesis and DNA synthesis of buffalo (*Bubalus bubalis*) ovarian preantral follicles.” Anim. Reprod. 10: 32-40.

[32] Xu, M., Fazleabas, A. T., Shikanov, A., Jackson, E., Barrett, S. L., Hirshfeld-Cytron, J., et al. 2011. “*In Vitro* Oocyte Maturation and Preantral Follicle Culture from the Luteal-Phase Baboon Ovary Produce Mature Oocytes.” Biol. Reprod. 84: 689-697.

[33] Silva, J. R., Hurk, V. D., Matos, M. H., Santos, R. R., Pessoa, C. Moraes, M. O., et al. 2004. "Influences of FSH and EGF on primordial follicles during *in vitro* culture of caprine ovarian cortical tissue." Theriogenology 61: 1691-1704.

[34] Thomas, F. H., Ethier J. F., Shimasaki S., and Vanderhyden, B. C., 2005. "Follicle-stimulating hormone regulates oocyte growth by modulation of expression of oocyte and granulosa cell factors." Endocrinology 146: 941-949.

[35] Engel, R. H., and Evens, A. M., 2006. "Oxidative stress and apoptosis: a new treatment paradigm in cancer." Fro. in Biosc. 11: 300-312.

[36] Andrade, E. R., Maddox-Hyttel, P., Landim-Alvarenga, D. C., Silva, J. R. V., Alfieri, A. A., Seneda, M. M., et al. "Ultrastructure of sheep primordial follicles cultured in the presence of indol acetic acid, EGF, and FSH." Vet. Med. Int. 1-7.

[37] Murray, A. A., Molinek, M. D., Baker, S. J., Kojima, F. N., Smith, M. F., Hillier, S.G., et al. 2001. "Role of ascorbic acid in promoting follicle integrity and survival in intact mouse ovarian follicles *in vitro*." Reproduction 121: 89-96.

[38] Li, Y., and Schellhorn, H. E., 2007. "New developments and novel therapeutic perspectives for vitamin C." J. Nutr. 137: 2171-2184.

[39] Faustino, L. R., Santos, R. R., Silva, C. M. G., Pinto, L. C., Celestino, J. J. H., Campello, C. C., et al. 2010. "Goat and sheep ovarian tissue cryopreservation: Effects on the morphology and development of primordial follicles and density of stromal cell." Anim. Reprod. Sci. 122: 90-97.

[40] Gouveia, B. B., Barros, V. R. P., Gonçalves, R. J. S., Barberino, R. S., Menezes, V. G., Lins, T. L. B., et al. 2015. "Effect of ovarian tissue transportation in *Amburana cearensis* extract on the morphology and apoptosis of goat preantral follicles." Anim. Reprod. 12: 316-23.

[41] Mansouri, M. T, Farbood, Y., Sameri, M. J., Sarkaki, A., Naghizadeh, B., and Rafeirad, M., 2013. "Neuroprotective effects of oral gallic acid against oxidative stress induced by 6-hydroxydopamine in rats." Food Chem. 138: 1028-1033.

[42] Behl, R., and Pandey, R. S., 2002. "FSH induced stimulation of catalase activity in goat granulosa cells *in vitro*." Anim. Reprod. Sci.70: 215-21.

[43] Chen, Q., Zhang, W., Ran, H., Feng, L., Yan, H., and Um, X., 2014. "PKCδ and θ possibly mediate FSH-induced mouse oocyte maturation via NOX-ROS-TACE cascade signaling pathway." PLoS One. 9: 1-8.

[44] Sadeghipour, M., Terreux, R., and Phipps, J., 2005."Flavonoids and tyrosine nitration: structure activity relationship correlation with enthalpy of formation." Toxicol. in Vitro. 19: 155-165.

8 CAPÍTULO 3

A suplementação in vitro do meio de cultivo com FSH para o crescimento de folículos e oócitos maduros pode ser substituído por extractos de Justicia insularis

Supplementation of in vitro culture medium with FSH to grow follicles and mature oocytes can be replaced by extracts of Justicia insularis

Periódico: Publicado Plos One, 2018. Pages 1-21.
Qualis A1

RESUMO

O presente estudo avaliou o efeito da suplementação *in vitro* de meio de cultivo *com J. insularis* comparado ao FSH em folículos secundários isolados e na maturação *in vitro* de oócitos desses folículos. Folículos secundários foram isolados de ovários ovinos e individualmente cultivados por 18 dias em α-MEM^{+} (Controle), α-MEM + suplementado com 100 ng/mL de hormônio folículo estimulante bovino recombinante (FSH) ou com 0,3, 1,25 ou 2,5 mg/mL de extrato de *J. insularis* (JI0.3, JI1.25 e JI2.5, respectivamente). Meio de cultivo coletado a cada 2 dias foi utilizado para medir os níveis de EROs. No final do período de cultivo, o complexo cumulus oócitos (CCOs) foram coletados e maturados *in vitro*. As paredes foliculares foram usadas para quantificação de mRNA. JI0.3 levou a uma maior ($P < 0,05$) percentagens de folículos intactos do que outros grupos após 18 dias de cultivo. Enquanto o diâmetro folicular permaneceu inalterado desde o dia 6 em diante com JI0.3 e FSH, as percentagens de formação da cavidade antral foram maiores ($P < 0.05$) com JI0.3 no dia 6 do que em todos os outros tratamentos. Não foram observadas diferenças entre os controles e os demais tratamentos quanto aos níveis de ROS e expressão de genes de RNAm. A viabilidade dos oócitos resultantes foi maior ($P <0,05$) em JI0,3 em comparação com o FSH. Curiosamente, no experimento controle, a suplementação de meio de maturação com JI0.3 levou a maiores porcentagens ($P <0,05$) da metáfase II em comparação ao controle. Embora mais validações sejam necessárias, parece que este extrato natural poderia ser usado como uma alternativa barata e facilmente disponível ao FSH comercial.

Palavras-chave: Planta medicinal, hormônio folículo-estimulante, cultura, maturação, ovinos

Supplementation of *in vitro* culture medium with FSH to grow follicles and mature oocytes can be replaced by extracts of *Justicia insularis*

Gildas Tetaping Mbemya[1¶], Jesus Cadenas[1¶], Naiza Arcângela Ribeiro de Sá[1¶], Denise Damasceno Guerreiro[1¶], Nathalie Jiatsa Donfack[1¶], Luis Alberto Vieira[1¶], Francisca Geovania Canafístula de Sousa[1¶], Benner Geraldo Alves[2], Carlos Henrique Lobo[3], Francielli Weber Santos[4], Francisco das Chagas Lima Pinto[5], Otília Deusdênia Loiola *Pessoa*[5&], Johan Smitz[6&], Pierre Comizzoli[7&], José Ricardo Figueiredo[1&], Ana Paula Ribeiro Rodrigues[1*#a]

[1] Laboratory of Manipulation of Oocyte and Ovarian Preantral Follicles (LAMOFOPA), Faculty of Veterinary (FAVET), State University of Ceará, Fortaleza, Brazil

[2] Laboratory of Biology of Reproduction, Federal University of Uberlândia, Minas Gerais, Brazil

[3] Laboratory of Animal Physiology, Department of Animal Science, Federal University of Ceará, Fortaleza, CE, Brazil

[4] Laboratory of Reproduction Biotechnology (Biotech), State of University of Pampa, Uruguaiana, Brazil

[5] Laboratory of Phytochemical Analysis of Medicinal Plants (LAFIPLAN), Federal University of Ceará, Fortaleza, Brazil

[6] Follicle Biology Laboratory, Center for Reproductive Medicine, UZ Brussel, Brussels, Belgium

[#a] Laboratory of Manipulation of Oocyte and Ovarian Preantral Follicles (LAMOFOPA), Faculty of Veterinary (FAVET), State University of Ceará, Fortaleza, Brazil

[*] Corresponding author:

E-mail: aprrodriguespapers@gmail.com (APRR)

[¶]These authors contributed equally to this work.

[&]These authors also contributed equally to this work.

Abstract

The present study evaluated the effect of supplementing *in vitro* culture medium with *J. insularis* compared to FSH on isolated secondary follicles and *in vitro* maturation of oocytes from those follicles. Secondary follicles were isolated from sheep ovaries and individually cultured for 18 days in α-MEM$^+$ (*Control*), α-MEM$^+$ supplemented with 100 ng/mL recombinant bovine follicle stimulating hormone (FSH) or with 0.3, 1.25, or 2.5 mg/mL of *J. insularis* extract (JI0.3, JI1.25, and JI2.5, respectively). Culture medium collected every 2 days was used to measure ROS levels. At the end of the culture period, cumulus oocytes complex (COCs) were collected and matured *in vitro*. Follicular walls were used for mRNA quantitation. JI0.3 led to a higher ($P < 0.05$) percentages of intact follicles than other groups after 18 days of culture. While follicular diameter remained unchanged from Day 6 onwards with JI0.3 and FSH, percentages of antral cavity formation were higher ($P < 0.05$) with JI0.3 at Day 6 than in all other treatments. No differences were observed between controls and treatment groups regarding ROS levels and mRNA expression of genes. Viability of resulting oocytes was higher ($P < 0.05$) in JI0.3 compared to FSH. Interestingly, in control experiment, supplementation of maturation medium with JI0.3 led to higher ($P < 0.05$) percentages of metaphase II compared to controls. Although more validations will be needed, it seems that this natural extract could be used as a cheap and easily available alternative to commercial FSH.

Keywords: Medicinal plant, Follicle-stimulating hormone, Culture, Maturation, Sheep

Introduction

The secondary follicles, the last category of preantral follicles (PF), according to Araújo et al. [1], is an excellent source of potentially fertilizable oocytes; however, the *in vitro* development of these follicles has been a great challenge to produce fully grown and competent oocytes. To date, live birth after the *in vitro* culture of PF has been reported only in mice [2 - 4]. In large mammals, on the other hand, results are still modest and may be summarized in the production of a variable number of embryos in buffaloes [5], sheep [6] and goats [7, -8].

Several variables may affect the outcome of *in vitro* follicle culture such as the culture base media composition [9, 10] and supplementation [11]; the animal model [8]; culture system [1, 12]; follicular category [13, 14] and reactive oxygen species (ROS) production [15], leading to oxidative stress. Many authors have shown that an increase of glutathione peroxidase (GPx) can represent a cellular transcriptional response to ROS [16], such as an activation or silencing of genes encoding antioxidant defense enzymes, growth and progression of meiosis [17]. To date it still is a challenge to reach a good balance between all components of the culture medium, getting the right concentrations and interaction that should exist between the components of the medium.

The addition of supplements (energy substrates, antioxidants, hormones and/or growth factors) to alpha modified minimal essential medium (α-MEM) is necessary to enable acceptable rates of follicular growth, oocyte viability and maturation [18] in caprine [19, 20] and ovine species [9, 21]. In an attempt to improve secondary follicles development, oocyte viability and maturation, natural supplements may be added to the culture media such as *Amburana cearensis* [22, 23], anethole [19] and rutin [24]. Although these studies have not involved investigation related to gene expression, it is known that *GPx* [25], *kit ligand* (KL - [13]), *cyclin B1* (CCNB1 – [26]) and *hyaluronan synthase 2* (HAS2 – [27] genes are expressed in mature oocytes. Therefore, the identification of these genes in oocytes may be a good indicator of an *in vitro* culture system's viability for secondary follicles.

Justicia insularis T. Anders (Acanthaceae) is an herbaceous and perennial plant of 30 - 75 cm high with opposite ascending branches, widely distributed in the tropical area of Africa [28]. Besides alkaloids, glycosides, polyphenols and triterpenoids, studies undertaken by Telefo et al. [29] and Goka et al. [28] revealed the presence of flavonoids in their leaves which acts as natural antioxidant and FSH-like compound. In fact, a literature survey showed that several studies performed with *Justicia* species have revealed the presence of the aforementioned secondary metabolites. It is worth mentioning that major part of these studies was done by phytochemical screening. In the Western region of Cameroon, *J. insularis* is used in association

with the leaves of three others medicinal plants (*Aloe buettneri, Hibiscus macranthus* and *Dicliptera verticillata*), to treat dysmenorrhoea and some cases of women infertility. This aqueous extract mixture has also been proven, in a series of studies to induce ovarian steroidogenesis and folliculogenesis in female rats [29-31]. In a recent study performed by our team, the aqueous extract of *J. insularis* has successfully promoted beneficial effects on morphology, activation and growth of PF (primordial, intermediary, primary and secondary) enclosed in ovarian tissue cultured *in vitro* for 7 days [32]. To date, due to their widely distribution and cost compared to chemical product, there is an increase interest in natural products that prevent oxidative damages, promote follicular growth and oocyte maturation.

Considering the positive results of *J. insularis* on the reproductive function *in vivo* [33] and *in vitro* culture of ovarian tissue [32], previously reported, in a general way, the aim of this study was to investigate the effect of different concentrations of the aqueous extract of *J. insularis* on the behavior of secondary follicles after 18 days of *in vitro* culture. In addition, we evaluate the effect of the extract on *in vitro* maturation of oocytes from *in vivo* grown follicles. Therefore, were analyzed: *a)* follicular morphology; *b)* ability to grow and form the antral cavity; *c)* gene expression (GPx, KL, CCNB1 and HAS2) in the follicular wall; *d)* ROS levels in the follicles culture medium and finally, *e)* the viability and *in vitro* maturation of oocytes from *in vitro* and in *vivo* grown secondary follicles.

Materials and methods

This study was approved and performed according to the recommendations of the Committee of Animal Handling and Ethical Regulation from the State University of Ceará (N° 6004720/2015).

Chemicals and media

Unless mentioned otherwise, the culture media and other chemicals used in the present study were purchased from Sigma Chemical Co. (St. Louis, Mo, USA).

Plants materials and phytochemical investigation from leaves of *J. insularis*

The fresh leaves of *J. insularis* previously identified in the National Herbarium of Cameroon under voucher specimen code 34997 [29] were collected in Western Cameroon (Batoufam subdivision, Upper-Plateau division, 5° 21′ Nord 10° 24′ East, Altitude 1515 m). The fresh leaves were then dried at room temperature in the shade. Subsequently, the plant

extract decoction was prepared according to the protocol described by Telefo et al. [33]; briefly, 100 g of powder was submerged in 1.5 L of boiling distilled water for 30 minutes. After cooling, the extract was filtered and dried in a ventilated oven at 45 °C. Finally, the plant decoction was lyophilized and kept in the freezer at -20 ºC. 22.96 g of the lyophilized extract was then diluted in the distilled water to obtain the desired concentrations.

A phytochemical screening of *J. insularis,* was performed by thin layer chromatography (TLC), proton nuclear magnetic resonance (^{1}H NMR) and liquid chromatography-mass spectrometry (LC-MS), reliable methods used for identification of secondary metabolites.

Firstly, 100 mg of aqueous extract was suspended in MeOH (2 x 10 mL), over sonication for 15 min. The MeOH soluble fraction was evaporation under reduced pressure to yield 1.8 mg of the MeOH fraction (F1), while the insoluble fraction, designed F2, afforded 98.2 mg. 1.0 mg of each fraction (F1 and F2) was solubilized in 1.0 mL of MeOH and H_2O, respectively, and subsequently applied on silica gel (Merck) chromatography plates (6 x 2 cm) with fluorescence indicator (F254). The samples were applied to the plates with the aid of a capillary tube, and for its development a ternary mixture of n-BuOH/AcOH/H_2O (6:2:2) was used as the elution system. The plates were immersed in suitable developers and heating to ~100 °C, when necessary. Developers specific for each class of secondary metabolites were used: Drangendorffi reagent (A) to identify alkaloids; α-naphthol acid solution (B) for glycosylated compounds; cerium sulfate acid/EtOH (C) for flavonoids and terpenoids, and vanillin/EtOH/perchloric solution (D) and EtOH/H_2SO_4 solution (E) as universal developers [34, 35]. After this procedure, a sample of the aqueous extract was fractionated on sephadex LH-20 using MeOH/H_2O 8:2 as solvent of elution to obtain four main fractions; whose ^{1}H NMR and LC-MS spectra were obtained.

Source of ovaries and experimental design

Ovaries from 35 adults (1-3 years old) mixed-breed sheep (*Ovis aries*) were collected at a local abattoir (Guaiúba municipality, Ceará, Brazil) at different times. Immediately postmortem, pairs of ovaries were washed once in 70% alcohol and then twice in minimum essential medium (MEM) plus HEPES (MEM-HEPES). The ovaries were placed into tubes containing 15 mL of MEM-HEPES, supplemented with penicillin (100 μg/mL) and streptomycin (100 μg/mL) and then transported to the laboratory at 4 ºC within 1 h since they were collected [36]. A total of 342 secondary follicles obtained from those ovaries were randomly *in vitro* cultured for 18 days in five different treatments as described below.

Additionally, to ensure that oocytes respond to *in vitro* maturation (IVM), ovaries from others 40 (1-3 years old) mixed-breed sheep were obtained as previously described and transported at 34 ºC during 1 - 2 h. We performed three replicates to obtain a total of 328 complexes oocyte cumulus cells (COCs) from antral follicles which were randomly distributed into five different IVM protocols as described below.

Isolation, selection and in vitro culture of sheep secondary follicles

In the laboratory, ovarian cortical slices (1-2 mm thick) were cut using a surgical blade and placed in MEM-HEPES. Then, secondary follicles (approximately 150-250 μm in diameter) were visualized under a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan) and manually dissected from the slices of ovarian cortex using 26-gauge needles. After isolation, only secondary follicles with a visible oocyte, surrounded by granulosa cells, an intact basement membrane and no antral cavity [36] were selected to *in vitro* culture in different media conditions, corresponding to five different treatments. Therefore, secondary follicles were cultured in α-MEM (M5650, pH 7.2-7.4), supplemented with 3 mg/mL of bovine serum albumin (BSA), ITS (10 μg/mL insulin, 5.5 μg/mL transferrin, 5 ng/mL selenium), 2 mM of glutamine, 2 mM of hypoxanthine, 50 ng/mL of Leukemia Inhibitory Factor (LIF) and 50 ng/mL of Kit Ligant (KL), referred as α-MEM$^+$ [37], considered the *Control*. Others follicles were *in vitro* cultured in α-MEM$^+$ supplemented with 100 ng/mL recombinant bovine follicle stimulating hormone (FSH) or 0.3, 1.25, or 2.5 mg/mL of *J. insularis* (JI0.3, JI1.25, and JI2.5, respectively). The concentration of recombinant bovine FSH and *J. insularis* were chosen based on previous studies performed in our laboratory [8, 32]. The secondary follicles were individually cultured in 100 μL drops of five different (α-MEM$^+$, FSH, JI0.3, JI1.25, and JI2.5) culture medium on Petri dishes (60 ×15 mm; Corning, USA) under mineral oil for 18 days at

39°C in 5% CO_2 in air. Fresh medium was prepared immediately before use and incubated for 2 h prior to use, with 60 µL medium being replaced in each drop every 2 days [24].

After *in vitro* culture, follicular development and ROS levels were analyzed. In addition, oocytes recovery from follicles at the end of the culture period were *in vitro* matured for evaluation of oocyte viability, meiotic stages, meiotic resumption, and genes expression on follicular walls.

Morphological and follicular development (diameter and antrum formation) evaluation

Follicles were classified according to their morphology as *intact* (no rupture of basement membrane), *extruded* (follicles were those that underwent rupture of their basement membrane) or *degenerated* (follicles showed darkened oocyte and/or misshapen granulosa cells). The percentage of morphologically intact follicles and follicular diameter were calculated only in intact follicles. The percentage of extruded follicles was calculated taking into account the total of extruded follicles divided by the total of intact normal follicles. Follicular diameter was calculated as the mean of two perpendicular measures of each follicle every 6 days with the aid of an ocular micrometer attached to a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan; 100 x magnification). The average follicular daily growth was calculated as follows: the diameter of morphologically intact follicles on the last day they were intact minus their diameter at day zero divided by the number of days they remained intact. Antral cavity formation was defined as a visible translucent cavity within the granulosa cell layers [37].

Categories of follicular growth velocity

All follicles in each treatment (Control, FSH, JI0.3, JI1.25 and JI2.5) were divided into three categories according to their daily growth as described previously [38]: (1) null growth (–12.7–0.0 µm day $^{-}$1), follicles that did not grow during the culture, (2) low growth (0.1–12.0 µm day^{-}1), follicles that grew up to 12 µm daily and (3) fast growth (12.1– 46.7 µm day^{-}1), follicles that grew up to 46.7 µm daily.

Reactive oxygen species levels

The ROS levels were determined in the conditioned media by a spectrofluorometric method [39], using 2', 7' dihydrodichlorofluorescein diacetate (DCHF-DA) assay. Sample aliquot (50 µL) of media collected was incubated with 5 µL of DCHF-DA (1 mM). The oxidation of DCHF-DA to fluorescent dichlorofluorescein was measured for the detection of ROS. The DCF fluorescence intensity emission was recorded at 520 nm (with 480 nm excitation) 2 h after the addition of DCHF-DA to the medium.

Quantitative RT-PCR

For evaluation of GPx, KL, CCNB1, HAS2 and GAPDH mRNA expression, total RNA of three pools of 8-10 viable follicular walls (granulosa and theca cells) was extracted using the Trizol® reagent method (Invitrogen, Carlsbad, CA, USA) according to the recommendations of the manufacturer and further purified with PureLink ™ RNA Mini Kit (Anbion®, Carlsbad, CA, USA). After extraction, RNA concentration was determined using the NanoDrop System (Thermo Scientific NanoDrop Products), performed with 2 µL of material. Before the cDNA synthesis, all samples were standardized with the same amount of RNA to minimize qPCR variability. cDNA synthesis was performed according to the instructions of SuperScript III RT-PCR (Invitrogen, Carlsbad, CA, USA) manual using random primers (Invitrogen, Carlsbad, CA, USA) from 1 ng of total RNA. The gene-specific primers used for the amplification of different transcripts are shown in Table 1. All primers set annealed at 60ºC.

The qPCR reaction was performed in quadruplet always using control without cDNA to avoid possible contamination. Evaluations were performed in IQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using analysis of relative quantification. Detection of PCR products was measured by monitoring the increase in fluorescence emitted by the marker Power SYBR ® Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA). For all amplifications, one dissociation curve (melting curve) was done for the verification of unspecific amplifications arising from contamination was held. The qPCR thermal cycle was as follow: 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. Quantification of the transcripts of target genes was calculated from the difference of the values of the Ct values (threshold cycle PCR) in relation to transcripts of the endogenous gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). First, the mean Ct of each sample, both the target gene and the endogenous gene was determined. From each sample, the subtraction of

the mean value of the Ctgene-target to Ctgene-endogene provided the ΔCt. Subsequently, one ΔCt corresponding to a calibrator was chosen, normalizing all values by subtracting the resulting ΔCt chosen, to obtain the ΔΔCt. Finally, the final value of relative quantification was given by $2^{-\Delta\Delta Ct}$, where the calibrator or standard sample chosen was equal to one [40].

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

Target gene	Primer sequence (5´→ 3´)	Orientation	Genbank accession no.
GPX	GCAACCAGTTTGGGCATCAG TAGGGTCGGTCATGAGAGCA	SenseAnti-sense	GI: 004018462 (*Ovis aries*)
KL	AGCGAGATGGTGGAACAACTGTCA GTTCTTCCATGCACTCCACAAGGT	Sense Anti-sense	GI: 16580734 (*Capra hircus*)
CCNB 1	AGCGGATCCAAACCTTTGTAGTG CAATGAGGATGGCTCTCATGTTTC	SenseAnti-sense	GI: 327679 (*Bos taurus*)
HAS 2	CCTCATCATCCAAAGCCTG ACATTTCCGCAAATAGTCTG	SenseAnti-sense	GI: 174079.2 (*Capra hircus*)
GAPDH	ATGCCTCCTGCACCACCA AGTCCCTCCACGATGCCAA	SenseAnti-sense	GI: 298676424 (*Ovis aries*)

GPx: *glutathione peroxidase*; KL: *Kit ligand*; CCNB 1: *Cyclin B1*: HAS 2: *hyaluronan synthase 2*; GAPDH: *glyceraldehyde-3-phosphate dehydrogenase*

In vitro maturation of oocytes from *in vitro* grown secondary follicles

At the end of the culture period (day 18), all morphologically intact and extruded secondary follicles were carefully opened with 26-G needles under a stereomicroscope for oocyte recovery. Only oocytes (≥110 μm) with homogeneous cytoplasm and surrounded by at least one compact layer of cumulus cells were selected for *in vitro* maturation (IVM). The recovery rate was calculated by dividing the number of oocytes (≥110 μm) by the sum of morphologically intact and extruded follicles at day 18 of culture, multiplied by 100. The selected cumulus oocyte complexes (COCs) were washed three times in maturation medium composed of TCM 199 supplemented with 1% BSA, 5 μg/mL LH, 0.5 μg/mL recombinant bovine FSH, 10 ng/mL epidermal growth factor (EGF), 50 ng/mL insulin like growth factor 1 (IGF-1), 1 mM pyruvate, 1 μg/mL estradiol (E2), 100 μM cysteamine and different concentrations of *J. insularis* (JI0.3, JI1.25, or JI2.5). After being washed, the COCs were transferred to 100-μL drops of IVM medium (approximately 10 COCs per drop) under mineral

oil and then incubated for 40 h at 39 °C with 5% CO_2. At the end of the maturation period, oocytes were stained with 10 μM Hoechst 33342 (483 nm) for the assessment of chromatin configuration.

In vitro maturation of oocyte recovery from antral follicles

To evaluate the effect of *J. insularis* on viability and maturation of oocytes grown *in vivo* (from antral follicles), selected COCs which served as a control for IVM of oocytes from secondary follicles grown *in vitro* were recovered from sheep ovary by slicing. The recovered COCs were washed twice with TCM 199 buffered with 25 mM HEPES (TCM 199 - HEPES) and antibiotics. Then, only oocytes with homogeneous cytoplasm and surrounded by at least one compact layer of cumulus cells were selected and randomly distributed into five maturation procedures as follows: TCM 199 (*Control*), TCM 199 supplemented with 0.5 μg/mL FSH or with 0.3, 1.25, or 2.5 mg/mL of *J. insularis* (JI0.3, JI1.25, and JI2.5, respectively). Furthermore, groups of 20-35 oocytes were cultured in 200 - 350 μL (10 μL per COCs) maturation medium, for 24 hours in the same conditions mentioned above. At the end of the maturation period, the oocyte viability, meiotic resumption, and meiotic stages were evaluated.

Assessment of oocyte viability and chromatin configuration

After IVM, oocyte (grown *in vitro* or *in vivo*) chromatin configuration and viability were assessed by fluorescence microscopy (Nikon, Eclipse 80i, Tokyo, Japan; 400x magnification). Oocytes were mechanically denuded by repeated pipetting and incubated in 100 μl droplets of PBS with 4 μM calcein-AM, 2 μM ethidium homodimer-1 (Molecular Probes Live/dead Viability/Cytotoxicity Kit for mammalian cells L3224, Invitrogen, Karlsruhe, Germany), 10 μM Hoechst 33342, and 0.5% glutaraldehyde. The emitted fluorescent signals of calcein-AM and ethidium homodimer were collected at 488 and 568 nm, respectively. Whereas the first probe detected the intracellular esterase activity of viable cells, the later labeled the nucleic acids of non-viable cells after plasma membrane disruption. Oocyte chromatin was stained by Hoescht 33342 (emission at 483 nm), and classified as germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), metaphase II (MII), or degenerated (DEG). The oocytes were considered viable when cytoplasm stained with calcein-AM (green) did not show abnormal chromatin configuration and/or did not label with ethidium homodimer (red).

Statistical analysis

All statistical procedures were carried out with Sigma Plot version 11.0 (Systat Software Inc., USA). Normality and homogeneity of variance were evaluated by Shapiro-Wilk and Levene's tests, respectively. Comparisons of means was performed by Kruskal-Wallis or Wilcoxon-Mann-Whitney tests, when appropriate. The percentage variables were analyzed among treatments and days of culture by chi-square or Fisher's exact tests. Odds ratio and confidence interval (95%) were calculated to evaluate the effect of follicular growth category on percentages of extrusion. The mRNA expression level was analyzed using the t-Tests. Data were presented as mean (± SEM) or percentage. Statistical significance was defined as $P < 0.05$ (two-sided).

Results

TLC, ^{1}H NMR and LC-MS analysis

According to the TLC procedures (Supporting information, S1 Fig.), ^{1}H NMR and LC-MS data were detected alkaloids, phenol compounds and sugars in the aqueous extract of *J. insularis*, corroborating with previous phytochemical reports on the *Justicia* species [34, 35].

Influence of *J. insularis* extract on follicular morphology during *in vitro* culture

Over the 18 days of the culture period, a significant decrease ($P < 0.05$) in the percentage of intact follicles was observed in all treatments. However, JI0.3 led to a higher ($P < 0.05$) percentage (36.8 %) of intact follicles than in control treatment (21.6 %) at the end of the culture period (Fig 1).

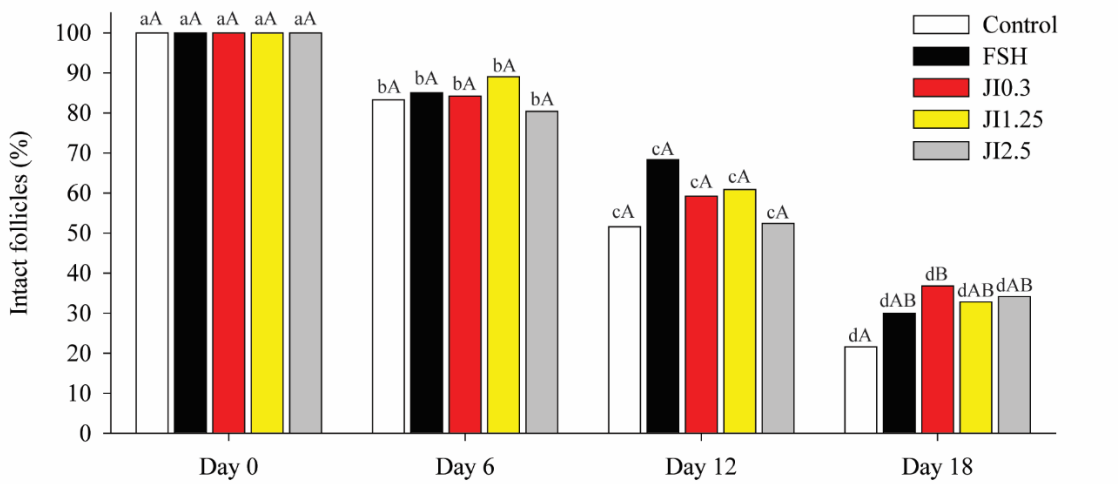


Fig 1. Percentage of morphological intact follicles at different time point (D0, D6, D12, D18) in MEM^{+} (Control), FSH or in J. insularis 0.3, 1.25 or 2.5 mg/mL (JI0.3, JI1.25 or JI2.5 respectively). D: day, MEM^{+}: Minimal essential medium supplemented, FSH: recombinant bovine follicle stimulating hormone, JI*: J. insularis*. A total of 342 secondary

follicles were distributed in the different treatments (n = 6 replicates). [a,b,c,d] Different letters denote significant differences among time for a given treatment group ($P < 0.05$). [A,B] Different letters denote significant differences among treatment groups within the same time point ($P < 0.05$).

No degenerated follicles were observed at D0 and D6. Small proportions of follicles were degenerated at D12 across all treatments. At D18, only JI1.25 treatment significantly increased the percentage of degenerated follicles compared to D0 (Fig 2).

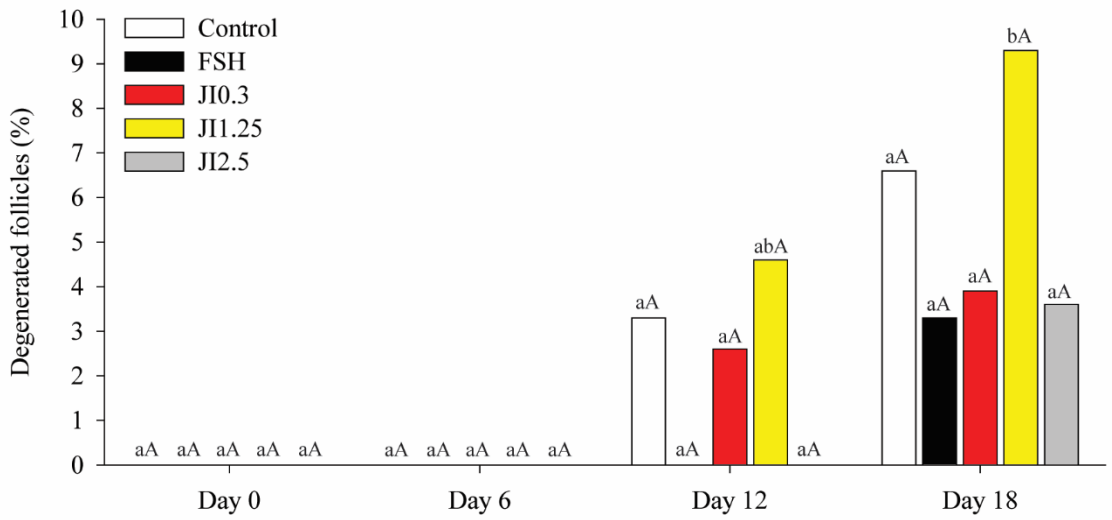


Fig 2. Percentage of degenerated follicles at different time point (D0, D6, D12, D18) in MEM$^+$ (Control), FSH or in *J. insularis* 0.3, 1.25 or 2.5 mg/mL (JI0.3, JI1.25 or JI2.5 respectively). D: day, MEM$^+$: Minimal essential medium supplemented, FSH: recombinant bovine follicle stimulating hormone, JI: *J. insularis*. A total of 342 secondary follicles were distributed in the different treatments (n = 6 replicates). [a,b] Different letters denote significant differences among time for a given treatment group ($P < 0.05$). [A] Denote no significant difference among treatment groups within the same time point ($P > 0.05$).

Influence of *J. insularis* extract on follicular growth and antral formation during *in vitro* culture

Overall, follicular diameters progressively increased ($P < 0.05$) in all treatments until D12, except for FSH and J0.3 treatments in which diameter remained unchanged from D6 onwards. At D18, JI1.25 treatment led to a larger ($P < 0.05$) follicular diameter compared to control, JI0.3 and JI2.5 (Fig 3).

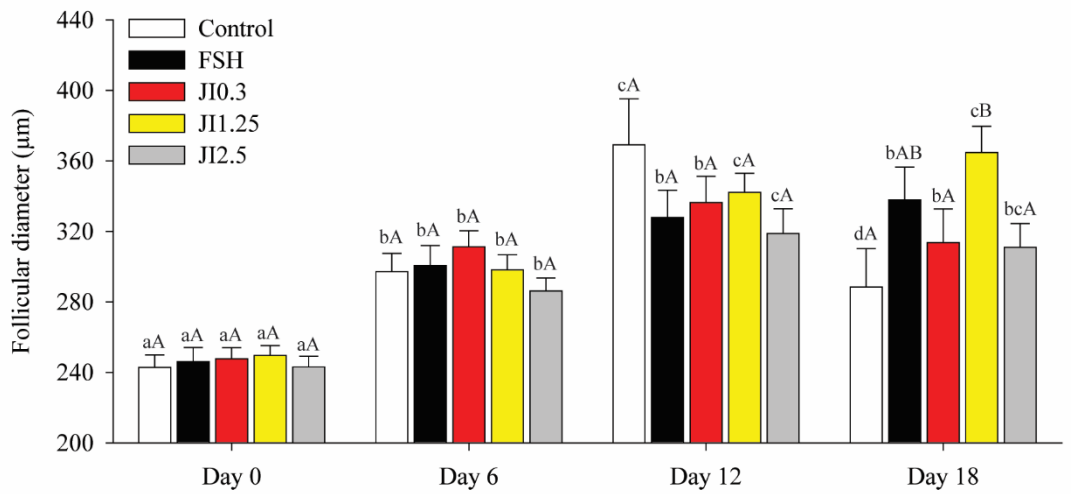


Fig 3. Follicles diameter at different time point (D0, D6, D12, D18) in MEM$^+$ (Control), FSH or in *J. insularis* 0.3, 1.25 or 2.5 mg/mL (JI0.3, JI1.25 or JI2.5 respectively). D: day, MEM$^+$: Minimal essential medium supplemented, FSH: recombinant bovine follicle stimulating hormone, JI: *J. insularis*. A total of 342 secondary follicles were distributed in the different treatments (n = 6 replicates). ^{a,b,c,d} Different letters denote significant differences among time for a given treatment group ($P < 0.05$). ^{A,B} Different letters denote significant differences among treatment groups within the same time point ($P < 0.05$).

During the three intervals (D0 - D6; D6 - D12; D12 - D18), growth rates reflected what we observed in Table 2 about the lack of differences between treatments until D12. The control, JI0.3 and JI1.25 treatments maintained growth rate until the second third of culture, although it dropped ($P < 0.05$) from the second (D6 - D12) to the last third of culture (D12 - D18). Comparing among treatments at D18, except JI0.3, all of them showed higher ($P < 0.05$) growth rate than control. Regarding the overall growth rate, none treatment differed from control. However, JI0.3 treatment showed a lower ($P < 0.05$) daily growth rate than all treatments, except the JI2.5 treatment.

Looking at the different follicular growth categories: non-growing, slow-growing and fast-growing follicles (Table 3), both treatments JI1.25 and JI2.5 presented, respectively, higher ($P < 0.05$) percentage of slow-growing follicles and lower ($P < 0.05$) percentage of fast-growing follicles than control.

Table 2. Mean (± SEM) daily growth of morphologically intact secondary follicles at different time point (D0, D6, D12, D18) in MEM$^+$ (Control), FSH or in *J. insularis* 0.3, 1.25 or 2.5 mg/mL (JI0.3, JI1.25 or JI2.5 respectively).

Mean follicular growth (µm/day ± SEM) in different culture intervals

Treatments	D0 - D6	D6 - D12	D12 - D18	Overall
Control	10.3 ± 1.2^{aA}	13.2 ± 2.4^{aA}	2.5 ± 2.0^{bA}	4.7 ± 0.6^{AB}
FSH	9.7 ± 1.2^{aA}	7.6 ± 1.7^{abA}	4.8 ± 1.3^{bB}	6.3 ± 0.8^{A}
JI0.3	10.3 ± 0.9^{aA}	7.1 ± 1.1^{bA}	1.6 ± 1.8^{cAB}	4.2 ± 0.6^{B}
JI1.25	8.1 ± 0.7^{aA}	7.2 ± 0.9^{bA}	2.8 ± 0.9^{cB}	5.6 ± 0.4^{A}
JI2.5	7.1 ± 0.7^{aA}	7.2 ± 1.1^{aA}	4.9 ± 1.0^{aB}	5.0 ± 0.3^{AB}

^{a,b,c} Different letters denote significant differences among time for a given treatment group ($P < 0.05$). ^{A,B} Different letters denote significant differences among treatment groups within the same time point ($P < 0.05$). JI: *J. insularis*. A total of 342 secondary follicles were distributed in the different treatments (n = 6 replicates).

Table 3. Percentage of secondary follicles within a growth category at different time point (D0, D6, D12, D18) in MEM^{+} (Control), FSH or in *J. insularis* 0.3, 1.25 or 2.5 mg/mL (JI0.3, JI1.25 or JI2.5 respectively).

	Growth category (%)*		
Treatments	**Non growing**	**Slow-growing**	**Fast growing**
Control	2.0 (1/50)^{A}	58.0 (29/50)^{A}	40.0 (20/50)^{A}
FSH	3.9 (2/51)^{A}	72.5 (37/51)^{AB}	23.5 (12/51)^{AB}
JI0.3	-	67.2 (4364)^{AB}	32.8 (21/54)^{A}
JI1.25	7.1 (4/56)^{A}	78.6 (44/56)^{B}	14.3 (8/56)^{B}
JI2.5	2.8 (2/72)^{A}	79.2 (57/72)^{B}	18.0 (13/72)^{B}

In vitro-cultured follicles were classified as: null, follicles that did not grow during culture; slow, follicles with a daily growth between 0.1 and 12.0 µm day $^{-}$1 and fast, follicles with a daily growth between 12.1 and 46.7 µm day $^{-}$1. ^{A,B} Different letters denote significant differences among treatment groups within the same time point ($P < 0.05$). JI: *J. insularis*. A total of 342 secondary follicles were distributed in the different treatments. We performed 6 replicates.

*Follicles were classified as: Non-growing, follicles that did not grow during the culture; Slow-growing, follicles with a daily growth rate between 0.1 and 12.0 µm/day; and fast-growing, follicles with a daily growth rate > 12.1 µm/day.

All treatments induced a progressive increase ($P < 0.05$) in the percentage of antral cavity formation compared to D0. Interestingly, at D6, JI0.3 treatment presented higher ($P < 0.05$) percentage of antral cavity formation than in the other treatments. In addition, JI0.3 was the only treatment that showed a higher ($P < 0.05$) percentage of antrum formation than control at any evaluated time point, although did not differ from the other treatments at the end of the culture or at D18 (Fig 4A).

Representative images of normal follicles, and metaphase II oocyte grown *in vitro* or *in vivo* are shown (Fig 4B).

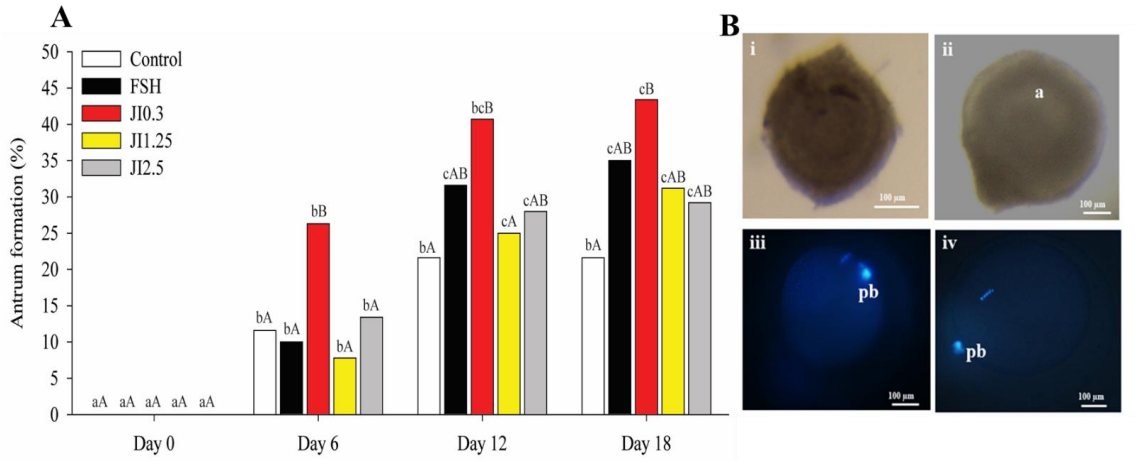


Fig 4. Percentage of antrum formation (A) at different time point (D0, D6, D12, D18) in MEM$^+$ (Control), FSH or in *J. insularis* 0.3, 1.25 or 2.5 mg/mL (JI0.3, JI1.25 or JI2.5 respectively). (B): Normal sheep secondary follicle at D0 (i) or at D18 day in J. insularis 0.3 mg/mL (JI0.3) (ii). Note formation of antrum (a) on D18. Representative images of fluorescent metaphase II oocyte grown in vitro (iii) or in vivo (iv). Note presence of the first polar body (pb). D: day, MEM$^+$: Minimal essential medium supplemented, FSH: recombinant bovine follicle stimulating hormone, JI: *J. insularis*. A total of 342 secondary follicles were distributed in the different treatments (n = 6 replicates. ^{a,b,c,d} Different letters denote significant differences among time for a given treatment group ($P < 0.05$). ^{A,B} Different letters denote significant differences among treatment groups within the same time point ($P < 0.05$).

Influence of *J. insularis* extract on follicular extrusion during *in vitro* culture

The percentage of extruded follicles increased ($P < 0.05$) in all treatments from D0 to D18. Moreover, at D12, a higher percentage ($P < 0.05$) of extruded follicles was observed in the control compared to all treatments, except JI2.5. On the other hand, at D18, the percentage of extruded follicles in the control was higher ($P < 0.05$) only than JI0.3 (Fig 5).

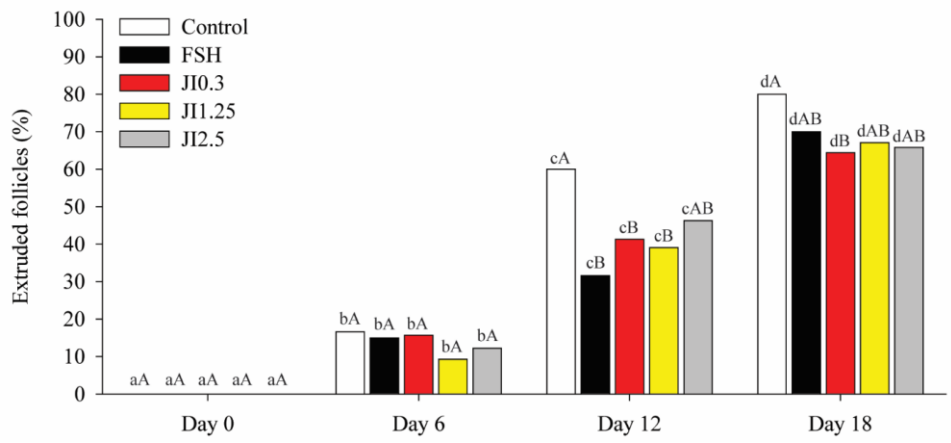


Fig 5. Percentage of extrusion at different time point (D0, D6, D12, D18) in MEM$^+$ (Control), FSH or in *J. insularis* 0.3, 1.25 or 2.5 mg/mL (JI0.3, JI1.25 or JI2.5 respectively). D: day, MEM$^+$: Minimal essential medium supplemented, FSH: recombinant bovine follicle stimulating hormone, JI*: J. insularis*. A total of 342 secondary follicles were distributed in the different treatments (n = 6 replicates). ^{a,b,c,d} Different letters denote significant differences among time for a given treatment group ($P < 0.05$). ^{A,B} Different letters denote significant differences among treatment groups within the same time point ($P < 0.05$).

Dynamic of follicular growth and extrusion of secondary follicles

Regardless the treatment, JI0.3 showed a fast-growing rate and a low percentage of extrusion compared to control. When comparing between follicle growth categories in different intervals of *in vitro* culture, the odds ratio (OR) analysis showed that this positive association was greater in fast-growing follicles compared to slow-growing follicles in the first two intervals of culture (D0 - D6 and D6 - D12) ($P = 0.0001$ and 0.0062, respectively) (Table 4).

Table 4. Association analyses between follicular growth categories and percentage of extrusion in different intervals of *in vitro* follicle culture.

Comparisons	Extrusion (%)		Odds ratio (CI 95%)		P-value	
	D0 - D6	D6 - D12	D0 - D6	D6 - D12	D0 - D6	D6 - D12
Non-growing	76.4(13/17)	45.8(11/24)	1.9 (0.6 - 6.0)	1.0 (0.4 - 2.4)	0.3915	0.8944
Slow-growing	62.9 (148/235)	46.8(66/141)				
Non-growing	76.4 (13/17)	45.8 (11/24)	1.7 (0.5 - 6.1)	2.7 (1.0 -7.2)	0.5951	0.0778
Fast-growing	85.1 (80/94)	69.6 (39/56)				
Slow-growing	62.9 (148/235)	46.8 (66/141)	3.3 (1.7 - 6.2)	2.6 (1.3 - 5.0)	0.0001	0.0062
Fast-growing	85.1 (80/94)	69.6 (39/56)				

In vitro-cultured follicles were classified as: null, follicles that did not grow during culture; slow, follicles with a daily growth between 0.1 and 12.0 µm day $^{-}$1 and fast, follicles with a daily growth between 12.1 and 46.7 µm day $^{-}$1.

Influence of J. insularis extract on levels of ROS during the in vitro culture of follicles

Differences among treatments were not found at D6. On the other hand, at D18 all treatments presented higher ($P < 0.05$) ROS concentration than the control. In addition, JI2.5 presented a higher ($P < 0.05$) levels of ROS than FSH, although did not differ from JI0.3 or JI1.25 (Table 5).

Table 5. Level of ROS measured in the culture medium from secondary follicles before (D0) and during 18 days of *in vitro* culture (D6, D12, D18) in MEM^{+} (Control), FSH or in JI0.3, JI1.25 or JI2.5.

Treatments	Day 6	Day 18
Control	14.2 ± 2.5^{A}	11.1 ± 0.2^{A}
FSH	14.6 ± 2.5^{A}	13.5 ± 0.6^{B}
JI0.3	14.9 ± 0.8^{A}	14.5 ± 0.7^{BC}
JI1.25	14.5 ± 0.9^{A}	15.1 ± 1.7^{BC}
JI2.5	16.1 ± 2.0^{A}	17.3 ± 1.9^{C}

^{A,B,C} Within a column, values without a common superscript differed ($P < 0.05$).

Influence of *J. insularis* extract on gene expression in follicular walls during in vitro culture

The relative expression of GPX, KL, CCNB1, and HAS2 was measured in follicular walls. No differences were observed between control and all treatments in mRNA expression for GPX, KL, CCNB1 and HAS2 (Figs 6A, B, C and D respectively).

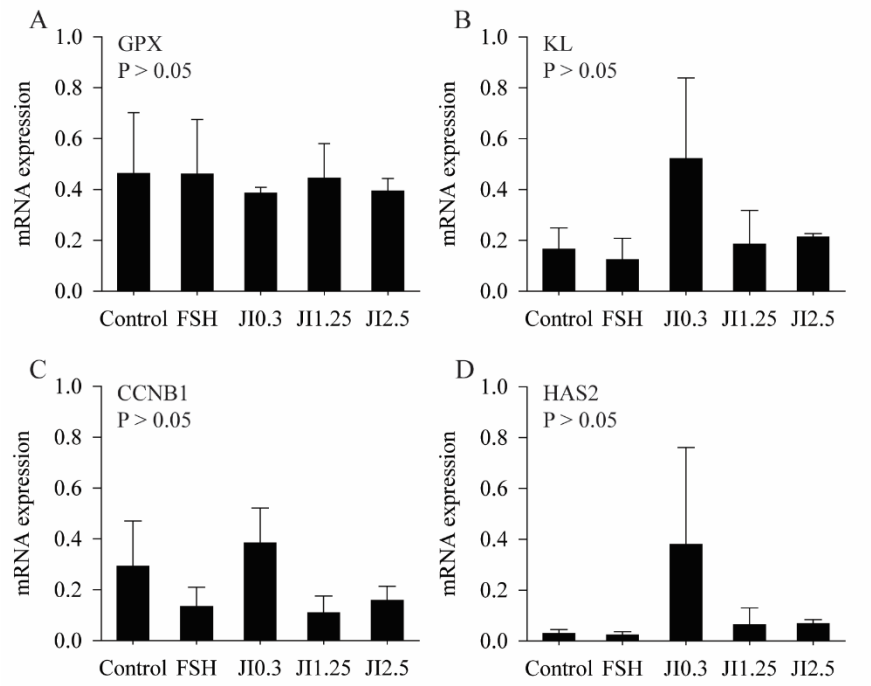


Fig 6. Relative mean (standard error of the mean) expression of mRNA of GPX (A), KL (B), CCNB1 (C), and HAS2 (D) before (D0) and during 18 days of *in vitro* culture (D6, D12, D18) in MEM$^+$ (Control), FSH or in JI0.3, JI1.25 or JI2.5.

Influence of *J. insularis* extract on structure and function of oocytes resulting from follicle cultured *in vitro*

In the first experiment, the percentage of fully grown oocytes (≥110 μm), oocyte diameter, viability rate and meiotic stages after IVM are shown in Table 6. None treatment differed from control in any evaluated end point. However, the viability rate was significantly higher in JI0.3 compared to FSH treatment.

After IVM of oocytes originate from follicles grown in vivo (Control experiment), the percentage of oocyte viability and meiotic resumption (n/total) was significantly higher ($P < 0.05$) in JI1.25 compared to JI2.5. Regarding to the percentage of metaphase II oocytes, FSH showed a similar percentage than JI0.3. Moreover, only JI0.3 showed a significant higher ($P < 0.05$) percentage than control (Table 7).

Table 6. Percentage of fully grown oocytes, oocyte diameter, viability rate, percentage of meiotic resumption, and meiotic stages of oocytes matured in TCM 199 (Control), FSH or in *J. insularis* 0.3, 1.25 or 2.5 mg/mL (JI0.3, JI1.25 or JI2.5 respectively).

Treatments	(%)Fully grown oocytes (≥110 µm/n)	Oocyte diameter (Mean±SEM)	(%)Viability (viable/n)	(%) Meiotic resumption (n/viable)	(%)GV (n/viable)	(%) GVBD (n/viable)	(%)MI (n/viable)	(%) MII (n/viable)
Control (n=43)	30.2 (13/43)^{A}	106.8 ± 2.9^{A}	76.7 (33/43)^{AB}	42.2 (14/33)^{A}	54.6 (18/33)^{A}	18.2 (6/33)^{A}	15.2 (5/33)^{A}	9.1 (3/33)^{A}
FSH (n=45)	37.8 (17/45)^{A}	106.8 ± 2.5^{A}	64.4 (29/45)^{A}	41.4 (12/29)^{A}	58.6 (17/29)^{A}	17.2 (5/29)^{A}	13.8 (4/29)^{A}	10.3 (3/29)^{A}
JI0.3 (n=45)	35.5 (16/45)^{A}	105.0 ± 3.5^{A}	84.4 (38/45)^{B}	36.8 (14/38)^{A}	63.2 (24/38)^{A}	15.8 (6/38)^{A}	10.5 (4/38)^{A}	10.5 (4/38)^{A}
JI1.25 (n=44)	36.3 (16/44)^{A}	108.1 ± 1.9^{A}	68.2 (30/44)^{AB}	40.0 (12/30)^{A}	63.3 (19/30)^{A}	20.0 (6/30)^{A}	10.0 (3/30)^{A}	10.0 (3/30)^{A}
JI2.5 (n=40)	30.0 (12/40)^{A}	105.1 ± 3.8^{A}	75.0 (30/40)^{AB}	46.7 (14/30)^{A}	56.7 (17/30)^{A}	26.7 (8/30)^{A}	6.7 (2/30)^{A}	13.3 (4/30)^{A}

^{A,B} Different letters denote significant differences among treatment groups within the same time point ($P < 0.05$). TCM: Tissue culture media 199, FSH: recombinant bovine follicle stimulating hormone, JI: *J. insularis*. A total of 217 complexes oocyte cumulus cells from secondary follicles grown *in vitro* were matured (n = 6 replicates).

Table 7. Percentage of oocyte viability, meiotic resumption, and meiotic stages of oocytes recovery from antral follicles matured in TCM 199 (Control), FSH or in *J. insularis* 0.3, 1.25 or 2.5 mg/mL (JI0.3, JI1.25 or JI2.5 respectively).

Treatments	(%) viability (viable/n)	(%) Meiotic resumption (n/total)	(%) Meiotic resumption (n/viable)	(%) VG (n/viable)	(%) GVBD (n/viable)	(%) MI (n/viable)	(%) MII (n/viable)
Control (n= 65)	58.5 (38/65)^{AB}	52.3 (34/65)^{AB}	89.5 (34/38)^{A}	10.5 (4/38)^{A}	23.7 (9/38)^{A}	44.7 (17/38)^{A}	21.1 (8/38)^{A}
FSH (n= 59)	59.3 (35/59)^{AB}	54.2 (32/59)^{AB}	91.4 (32/35)^{A}	8.6 (3/35)^{A}	22.9 (8/35)^{A}	40.0 (14/35)^{A}	28.6 (10/35)^{AB}
JI0.3 (67)	58.2 (39/67)^{AB}	56.7 (38/67)^{AB}	97.4 (38/39)^{A}	2.6 (1/39)^{A}	25.6 (10/39)^{A}	28.2 (11/39)^{A}	43.6 (17/39)^{B}
JI1.25 (66)	51.5 (34/66)^{A}	47.0 (31/66)^{A}	91.2 (31/34)^{A}	8.8 (3/34)^{A}	29.4 (10/34)^{A}	26.5(9/34)^{A}	35.3 (12/34)^{AB}
JI2.5 (71)	67.6 (48/71)^{B}	63.4 (45/71)^{B}	93.8 (45/48)^{A}	6.2 (3/48)^{A}	25.0 (12/48)^{A}	43.8 (21/48)^{A}	25.0 (12/48)^{AB}

^{A,B} Different letters denote significant differences among treatment groups within the same time point ($P < 0.05$). TCM: Tissue culture media 199, FSH: recombinant bovine follicle stimulating hormone, JI: *J. insularis*. A total of 328 complexes oocyte cumulus cells from antral follicles grown *in vivo* were mature (n = 3 replicates)

Discussion

To the best of our knowledge, this study constitutes the first report demonstrating the beneficial effects of the *J. insularis* extract on the *in vitro* culture and maturation of ovine isolated follicles. While FSH has been widely used to maintain follicle survival [41], promote the antrum formation [42] and stimulate follicular growth and oocyte maturation [11, 36], similar effects were obtained with 0.3 mg/mL of *J. insularis.* Addition of this natural substance in the culture or maturation medium represents an efficient alternative to FSH.

The phytochemical screening by TLC of the aqueous extract of *J. insularis* revealed alkaloids and flavonoids. In addition, a sample (1.0 g) of the aqueous extract was fractionated on sephadex LH-20 eluted with MeOH 8:2 to furnish four fractions which were analyzed by ^{1}H NMR and LC-MS. Inspection of these techniques allowed to identify the alkaloid trigonelline [43] and phenol compounds as eucomic acid [44] and kaempferol glycosides derivatives [45] in agreement with TLC. As expected, sugars as sucrose, and the α- and β-glucose stereoisomers [46] were also identified. Although there are no studies on the isolation of the chemical constituents of *J. insularis*, a literature survey on *Justicia* species revealed the presence of those classes of compounds [29], corroborating with our findings (Supporting information, S2 PDF file). In addition, according to the literature, plants of the genus *Justicia* are known as producers of bioactive compounds as alkaloids and flavonoids [47].

We should take into account that the seasonality of climatic elements such as temperature, relative humidity and solar radiation can alter the physiological behavior of plants and, consequently, their growth and development, as well as the chemical and biological composition of the soil [48]. Thus, for all the secondary metabolites identified from *J. insularis*, the variation of climatic elements can affect their concentrations in the plant [49]. Therefore, the environments in which the plant develops exert a direct influence on the chemical composition of the extracts.

In our study, percentages of morphologically intact follicles decreased at the end of the culture period regardless of the treatment. This expected result is common during *in vitro* culture of secondary follicles [12, 19]. Such effect could be due to the action of toxic metabolites and ROS. In excess, these metabolites can oxidize important molecules that induce the release of nucleases, proteases, and lipases from mitochondria [12], resulting in the disruption of the basal membrane [50]. This was observed in previous published articles using natural substances with the same culture period in ovine [22, 24] and goat species [19, 23]. Interestingly, *J. insularis* at 0.3 mg/mL showed a higher percentage of intact follicles than the

control. This could be due to the action of the flavonoids 3'-Metoxy-kaempferol-arabinosyl-rhamnoside, and Kaempferol-arabinosyl-rhamnoside which are phenolic compounds identified in the extract and have the capacity to neutralize damage caused by oxidation. Choi, [51] has demonstrated that pretreatment with kaempferol prior to antimycin A exposure significantly reduced cell damage by preventing mitochondrial membrane potential dissipation and ROS production. Other authors showed that cellular reactive oxygen species levels distinctly diminished by trigonelline treatment of HT-29/Caco-2 cells [52].

Percentages of extrusion increased significantly during culture period in all treatments. Moreover, the JI0.3 showed lower percentage of extruded follicles compared to control. This may be due to the increased in the follicular growth. Indeed, analyses of the chances of extrusion in non, slow or fast-growing follicles within the first (D0 - D6) and second (D6 - D12) third of the culture *in vitro* showed that in the first third (D0 - D6), fast-growing follicles were 3.3 times more likely to extrude than those with slow growth. Furthermore, in the second third (D6 - D12), the same behavior occurred (fast-growing follicles were 2.6 times more likely to extrude than slow-growing follicles).

During the culture period, all treatments increased follicle diameter. Interestingly, FSH and JI0.3 treatments showed similar follicle diameter. FSH has a well-established role in modulating granulosa cell proliferation and antrum formation which contributed to an increase in follicular diameter [53]. Erickson et al. [54] reported that FSH interacts with several growth factors such as KL to induce follicular growth. Luz et al. [21] showed that culture medium of ovine secondary follicles supplemented with KL resulted in an increased rate of follicular diameter and antrum formation. The JI 0.3 response similar to FSH could be due to the action of the trigonelline which is a phytohormone that induced the proliferation of neuronal cells [55]. How trigonelline acts on the receptor of granulosa cells awaits further investigations.

All the treatments increased the antrum formation. Such effect could be due to the secondary metabolites of the plant. The TLC, ^{1}H NMR and LC-MS analysis revealed the presence of alkaloid, flavonoids and glycosylated terpenoids. These metabolites are responsible for enhancing ovarian follicle growth and increasing in the number of corpus luteum in rats [33], and promoted the *in vitro* activation and survival of ovine preantral follicles enclosed in ovarian tissue [32]. Interestingly, at D6, JI0.3 showed a higher percentage of antrum formation. This finding is important because the ability to form an antrum is considered as a good marker of follicular functionality [56], as the mechanisms by which small cavities of fluid develop inside the follicle to form the antral cavity are related to the secretion of osmotically active

molecules into small spaces between the granulosa cells [57]. Recently, a study using *Amburana cearensis* showed that its metabolite protocatechuic acid improves the antrum formation after 18 days of ovine preantral follicle culture *in vitro* [22].

In the current study, JI0.3 caused a fast-growing of secondary follicles, however, this treatment resulted in low percentage of extrusion compared to control. This means that there was an adequate response of the oocytes without membrane damages. Similar results were reported in sheep secondary follicles, grown for 6 days with the same culture medium [58]. This may be due to the interaction between flavonoids present in the plant extract and components of the culture medium. It is known that the flavonoids protect human vascular endothelial cells against oxidative damages [59] and have antimicrobial and antioxidant properties [60]. These flavonoids interact with membrane proteins, making them stable. Therefore, we believe that this increasing hardness can give follicular membrane firmness and prevent its rupture, consequently avoiding oocyte extrusion. On the other hand, other studies using androstenedione and FSH in the culture medium of isolated caprine secondary follicles found that higher percentage of fast-growing follicles was detrimental for efficiency of caprine secondary follicles cultured *in vitro* [38].

Recent studies have shown that *J. insularis* maintains the ROS level [32], however, in the current study at D18, all treatments presented higher ROS concentration than control. We believe that these results may be attributed to an excess of antioxidants in the culture medium. It is known that the balance between ROS and antioxidants within the oocytes is critical to cell functions, such as chromosome segregation [61], mitochondria activity [62], ATP level maintenance and DNA methylation [63]. Therefore, ROS may affect follicles and oocyte growth during the *in vitro* culture. Indeed, our basic culture medium is rich in supplements among which selenium (5.5 μg/mL) and transferrin (50 ng/ mL). These supplements in addition to *J. insularis* increases the ROS level. In this study, the increase of the ROS level was not detrimental for the follicles because no significant difference was observed on the GPx mRNA expression.

Several genes have been identified in follicular walls as biomarkers to understand the dynamics of follicular development. Among them GPx related to oxidative stress [64], KL indicative of follicular growth [65]; cyclin B1 and HAS2 indicative of progression of oocyte maturation [66]. We observed that after the IVM of COCs from secondary follicles, the follicular walls (granulosa and theca cells) expressed similar transcript levels. The gene

expression similarity between J0.3 and FSH may support the use of this natural substance for the *in vitro* culture of ovine isolated secondary follicles.

Despite no effect was observed on the meiotic resumption of oocytes from *in vitro* growth secondary follicles, addition of JI0.3 on the maturation medium of oocyte from antral follicles showed similar percentage than FSH. In addition, this percentage was higher than the control. We believe that the presence of *J. Insularis* in the maturation medium could have improve the synthesis of the mitosis-promoting factor (MPF). In fact, during the meiosis resumption, the MPF, a protein complex composed of subunits cyclin B1 and p34cdc2 [67] is activated and regulate the germinal vesicle breakdown (GVBD) [68]. MPF activity is regulated by the CDK1 produced by the cells. The activity of MPF was described in many mammalian oocytes: it appears just before GVBD and increases until metaphase I stage, then its activity decreases in anaphase-telophase and increases again, reaching its maximum level in metaphase II in goat [69], sheep [70] and cow [71]. The oocyte (recovery from antral follicles) viability rate of was higher in JI1.25 compared to JI2.5. This could be due to an excess of toxic metabolites and reactive oxygen species present of the maturation medium. In fact, phytochemical analysis of the extract showed that it is constitute of secondary metabolites (flavonoids 3'-Metoxy-kaempferol-arabinosyl-rhamnoside, and Kaempferol-arabinosyl-rhamnoside, trigonelline among others) which have antioxidant activity as revealed by previous study (Bakuradze et al., 2010; Choi et al., 2011). In high concentration, those metabolite could acts as pro-oxidant and therefore increase the level of reactive oxygen species (free radical, hydrogen peroxide, hydroxyl ion…). As reported by Costa et al., 2011, although a critical amount of reactive oxygen species is essential for the physiological activities of follicles, excessive amount of them generates a contrary effect.

In summary, the present study showed for the first time that 0.3 mg/mL of *J. insularis* showed similar effect than FSH when added in the culture or maturation media of ovine isolated secondary follicles. It seems that when FSH is not available, *Justicia iusularis* could be used with the same benefits. Further studies are needed to isolate the metabolites present in *J. insularis* and assess the developmental competence of the oocytes after IVF.

Acknowledgments

The authors thank Dr. Phelix Bruno Telefo and Mr. Celestin Tchouanguep for providing the medicinal plants.

Author Contributions

Conceived and designed the experiments: GTM APRR. Performed the experiments: GTM JC NARS DDG NJD LAV FGCS. Performed the phytochemical analysis of the plant: GTM ODLP FCLP. Realized the ROS analysis: FWS. Performed the PCR analysis: GTM CHL. Performed the data analysis: BGA. Writing, review and editing of the manuscript: GTM, APRR, PC, ODLP, JS, JRF. All the authors have read and approved the final manuscript prior to submission.

References

1. Araújo VR, Gastal MO, Figueiredo JR, Gastal EL. *In vitro* culture of bovine preantral follicles: a review. Reprod Biol Endocrinol 2014; 13:12-78. doi: 10.1186/1477-7827-12-78 PMID: 25117631

2. Dela Peña EC, Takahashi Y, Katagiri S, Atabay EC, Nagano M. Birth of pups after transfer of mouse embryos derived from vitrified preantral follicles. Reproduction 2002, 123:593-600. PMID: 11914121

3. O'brien MJ, Pendola JK, Eppig JJ. A revised protocol for development of mouse oocyte from primordial follicles dramatically improves their development competence. Biol Reprod 2003; 68:1682-1686. doi: 10.1095/biolreprod.102.013029. PMID: 12606400

4. Higuchi CM, Maeda Y, Horiuchi T, Yamazaki Y. A simplified method for three-dimensional (3-D) ovarian tissue culture yielding oocytes competent to produce full-term offspring in mice. PloS one 2015; 16:e0143114. doi: 10.1371/journal.pone.0143114. PMID: 26571501

5. Agarwal A, Durairajanayagam D, Du Plessis SS. Utility of antioxidants during assisted reproductive techniques: an evidence based review. Reprod Biol Endocrinol 2014; 12:112. doi: 10.1186/1477-7827-12-112. PMID: 25421286

6. Arunakumari G, Shanmugasundaram N, Rao VH. Development of morulae from the oocytes of cultured sheep preantral follicles. Theriogenology 2010; 74:884-894. doi: 10.1016/j.theriogenology.2010.04.013. PMID: 20615540

7. Saraiva MVA., Rossetto R, Brito IR, Celestino JJH, Silva CMG, Faustino LR et al. Dynamic medium produces caprine embryo from preantral follicles grown *in vitro*. Reprod Sci 2010; 17:35-43. doi: 10.1177/1933719110379269. PMID: 20926838

8. Magalhães DM, Fernandes DD, Mororó MB, Silva CM, Rodrigues GQ, Bruno JB, et al. Effect of the medium replacement interval on the viability, growth and *in vitro* maturation of isolated caprine and ovine pre-antral follicles. Reprod Domest Anim 2011; 46:134-140. doi: 10.1111/j.1439-0531.2010.01607.x. PMID: 20403127

9. McNatty KP, Reader K, Smith P, Heath DA, Juengel JL. Control of ovarian follicular development to the gonadotrophin-dependent phase: a 2006 perspective. Soc Reprod Fertil Suppl. 2007; 64:55-68. PMID: 17491140

10. Santos J, Menezes VM, Barberino RS, Macedo TJS, Lins TLB, Gouveia BB, et al. Immunohistochemically localization of fibroblast growth factor-2 in the sheep ovary and its effects on pre-antral follicle apoptosis and development *in vitro*. Reprod Domest Anim 2014; 49:222-228. doi: 10.1111/rda.12322. PMID: 24750547

11. Ferreira ACA, Maside C, Sá NAR, Guerreiro DD, Correia HHV, Leiva-Revilla J, et al. Balance of insulin and FSH concentrations improves the *in vitro* development of isolated goat preantral follicles in medium containing GH. Anim Reprod Sci 2016; 165:1–10. doi:10.1016/j.anireprosci.2015.10.010. PMID: 26723481

12. Pessoa AFC, Rocha RMP, Brito IR, Silva GM, Chaves RN, Magalhães-Padilha DM, et al. Effect of morphological integrity, period, and type of culture system on the *in vitro* development of isolated caprine preantral follicles. Theriogenology 2014; 82:312-317. doi: 10.1016/j.theriogenology.2014.04.008. PMID: 24839921

13. Joyce IM, Pendola FL, Wigglesworth K, Eppig JJ. Oocyte regulation of kit ligand expression in mouse ovarian follicles. Dev Biol 1999; 214:342-353. doi: 10.1006/dbio.1999.9437. PMID: 10525339

14. Magalhães-Padilha DM, Geisler-Lee J, Wischral A, Gastal MO, Fonseca GR, Eloy YRG, et al. Gene Expression During Early Folliculogenesis in Goats Using Microarray Analysis. Biol Reprod 2013; 89:1-12. doi: 10.1095/biolreprod.112.106096. PMID: 23759311

15. Leiva-Revilla J, Cadenas J, Vieira AL, Campello CC, Celestino JJH, Pessoa ODL, et al. Toxicity effect of Auxemma oncocalyx fraction and its active principle oncocalyxone A on *in vitro* culture of caprine secondary follicles and *in vitro* oocyte maturation. Semin Cienc Agrar 2017; 38:1361-1374. doi: http://dx.doi.org/10.5433/1679-0359.2017v38n3p1361

16. Turpaev KT. Reactive Oxygen Species and Regulation of Gene Expression. Biochemistry (Moscow), Vol. 67, No. 3, 2002, pp. 281-292. doi: https://doi.org/10.1023/A:1014819832003. PMID: 11970728

17. Dalton TP, Shertzer HG, Puga A. Regulation of gene expression by reactive oxygen. Annu Rev Pharmacol Toxicol 1999; 39:67-101. doi: 10.1146/annurev.pharmtox.39.1.67. PMID: 10331077

18. Rossetto R, Saraiva MV, Dos Santos RR, Da Silva CM, Faustino LR, Chaves RN et al. Effect of medium composition on the *in vitro* culture of bovine pre-antral follicles:

morphology and viability do not guarantee functionality. Zygote. 2013; 21:125-128. doi: 10.1017/S0967199412000044. PMID: 22717039

19. Sá NAR, Araújo VR, Correia HHV, Ferreira ACA, Guerreiro DD, Sampaio AM, et al. Anethole Improves the *in vitro* Development of Isolated Caprine Secondary Follicles. Theriogenology 2016; 89:226-234. doi: 10.1016/j.theriogenology.2015.12.014. PMID: 28043356

20. Gouveia BB, Macedo TJS, Santos JMS, Barberino RS, Menezes VG, Müller MC, Almeida JRGS, et al. Supplemented base medium containing *Amburana cearensis* associated with FSH improves *in vitro* development of isolated goat preantral follicles. Theriogenology. 2016; 86:1275-1284. doi: 10.1016/j.theriogenology.2016.04.068. PMID: 27287468

21. Luz VB, Araújo VR, Duarte ABG, Silva GM, Chaves RN, Brito IR, et al. Kit ligand and insulin-like growth factor I affect the *in vitro* development of ovine preantral follicles. Small Rumin Res 2013; 115:99-102. doi: https://doi.org/10.1016/j.smallrumres.2013.09.003

22. Barberino RS, Barros VRP, Menezes VG, Santos LP, Araújo VR, Queiroz MAA, et al. *Amburana cearensis* Leaf Extract Maintains Survival and Promotes *in vitro* Development of Ovine Secondary Follicles. Zygote 2015:1-9. doi: 10.1017/S0967199415000179. PMID: 26083197

23. Gouveia BB, Macedo TJ, Santos JM, Barberino RS, Menezes VG, Müller MC, et al. Supplemented base medium containing *Amburana cearensis* associated with FSH improves *in vitro* development of isolated goat preantral follicles. Theriogenology 2016; 86:1275-1284. doi: 10.1016/j.theriogenology.2016.04.068. PMID: 27287468

24. Lins TLBG, Cavalcante AYP, Santos JMS, Menezes VG, Barros VRP, Barberino RS, et al. Rutin can replace the use of three other antioxidants in the culture medium, maintaining the viability of sheep isolated secondary follicles. Theriogenology 2017; 89:263–270. doi: 10.1016/j.theriogenology.2016.11.019. PMID: 28043362

25. Mishra A, Reddy IJ, Gupta PS, Mondal S. Expression of Apoptotic and Antioxidant Enzyme Genes in Sheep Oocytes and *In vitro* Produced Embryos. Anim Biotechnol 2017; 28:18-25. doi: 10.1080/10495398.2016.1193743. PMID: 27379566

26. Kuroda T, Naito K, Sugiura K, Yamashita M, Takakura I, Tojo H. Analysis of the roles of cyclin B1 and cyclin B2 in porcine oocyte maturation by inhibiting synthesis with antisense RNA injection. Biol Reprod 2004; 70:154-159. doi: 10.1095/biolreprod.103.021519. PMID: 12954723

27. Cadenas J, Leiva-Revilla J, Vieira LA, Apolloni LB, Aguiar FLN, Alves BG, et al. Caprine ovarian follicle requirements differ between preantral and early antral stages after IVC in medium supplemented with GH and VEGF alone or in combination. Theriogenology 2017; 87:321-332. doi: 10.1016/j.theriogenology.2016.09.008. PMID: 27729112

28. Goka MSC, Telefo PB, Mbemya GT, Awouafack MD, Lienou LL, Yemele DM, et al. Potentialisation of Pregnant Mare Serum Gonadotropin Inducing Effect on Ovarian Follicles Growth by the Aqueous Extract of *Aloe buettneri*, *Dicliptera verticillata*, *Hibiscus macranthus* and *Justicia insularis* Leaves in Immature Rats. Pharmacolology 2016; 7:328-336. doi: 10..5567/pharmacologia.2016.328.336

29. Telefo PB, Moundipa PF, Tchana AN, Tchouanguep D, Mbiapo FT. Effects of an Aqueous Extract of *Aloe buettneri, Justicia insularis, Hibiscus macranthus, Dicliptera verticillata* on Some Physiological and Biochemical Parameters of Reproduction in Immature Female Rats. J Ethnopharmacol 1998; 63:193-200. PMID: 10030723

30. Telefo PB, Moundipa PF, Tchouanguep FM. Oestrogenicity and effect on hepatic metabolism of the aqueous extract of the leaf mixture of *Aloe buettneri, Dicliptera verticillata, Hibiscus macranthus* and *Justicia insularis.* Fitoterapia. 2002; 73:472-478. PMID: 12385869

31. Telefo PB, Moundipa PF, Tchouanguep FM. Inductive Effects of the Leaf Mixture Extract of *Aloe buettneri, Justicia insularis, Dicliptera verticillata* and *Hibicus marcranthus* on *in vitro* production of oestradiol. J Ethnopharmacol 2004; 91:225-230. doi: 10.1016/j.jep.2003.12.024. PMID: 15120443

32. Mbemya GT, Guerreiro DD, Donfack NJ, Silva LM, Vieira LA, Sousa GFC, et al. Justicia insularis Improves the in vitro Survival and Development of Ovine Preantral Follicles Enclosed in Ovarian Tissue. J Pharm Pharmacol 2017;5:668-680. doi: 10.17265/2328-2150/2017.09.009

33. Telefo PB, Tagne, SR, Koona OES, Yemele DM, Tchouanguep FM, Effect of the Aqueous Extract of *Justicia Insularis* T. Anders (Acanthaceae) on Ovarian Folliculogenesis and Fertility of Female Rats. Afr J Tradit Complement Altern Med 2012; 9:197-203. PMID: 23983335

34. Da Silva NLA, Miranda FAA, Da Conceição GM. Phytochemical Screening of Cerrado Plants, from the Municipal Environmental Protection Area of Inhamum, Caxias, Maranhão, Scientia Plena 2010; 6:1-17.

35. Sherman J, Fried B. Handbook of Thin-Layer Chromatography. 3ª, 1991. Lafayette College. Easton, Pennsylvania, U.S.A. ISBN: 0-8247-0895-4

36. Lima LF, Rocha RM, Duarte AB, Brito IR, Silva GM, Rodrigues GQ, et al. Unexpected effect of the vehicle (grain ethanol) of homeopathic FSH on the *in vitro* survival and development of isolated ovine preantral follicles. Microsc Res Tech 2017; 4:406-418. doi: 10.1002/jemt.22810. PMID: 27921341.

37. Luz VB, Araujo VR, Duarte AB, Celestino JJ, Silva TF, Magalhaes-Padilha DM, et al. Eight-cell parthenotes originated from *in vitro* grown sheep preantral follicles. Reprod Sci 2012; 19:1219-1225. doi: 10.1177/1933719112446072. PMID: 22562971

38. Apolloni LB, Bruno JB, Alves BG, Ferreira ACA, Paes VM, Moreno JLRC, et al. Accelerated follicle growth during the culture of isolated caprine preantral follicles is detrimental to follicular survival and oocyte meiotic resumption. Theriogenology

39. Loetchutinat S, Kothan S, Dechsupa J, Meesungnoen J, Jay-Gerin SM. Spectrofluorometric Determination of Intracellular Levels of Reactive Oxygen Species in Drug-Sensitive and Drug-Resistant Cancer Cells Using the 2',7'-Dichlorofluorescein Diacetate Assay. Radiat Phys Chem 2005; 72:323-331. doi 10.1016/j.radphyschem.2004.06.011

40. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2 (Delta Delta C (T)) method. Methods 2001; 25:402-408. doi: 10.1006/meth.2001.1262. PMID: 11846609

41. Costa SH, Santos RR, Rondina D, Andrade ER, Ohashi OM, Rodrigues, AP, et al. Effects of IAA in combination with FSH on *in vitro* culture of ovine preantral follicles. Zygote 2010; 18:89-92. doi: 10.1017/S0967199409990104. PMID: 19586559

42. Barros VR, Cavalcante AY, Macedo TJ, Barberino RS, Lins TLB, Gouveia BB, et al. Immunolocalization of Melatonin and Follicle-Stimulating Hormone Receptors in Caprine Ovaries and their Effects During *in vitro* Development of Isolated Pre-Antral Follicles. Reprod Domest Anim 2014; 48:1025-1033. doi: 10.1111/rda.12209. PMID: 23981138

43. Farag MA, Porzel A, Wessjohann LA. Unraveling the active hypoglycemic agent trigonelline in Balanites aegyptiaca date fruit using metabolite fingerprinting by NMR. J Pharm Biomed Anal. 2015; 115:383-387. doi: 10.1016/j.jpba.2015.08.003. PMID: 26275727

44. Chahdoura H, Barreira JC, Barros L, Santos-Buelga C, Ferreira IC, Achour L. Phytochemical characterization and antioxidant activity of the cladodes of Opuntia

macrorhiza (Engelm.) and Opuntia microdasys (Lehm.). Food Funct. 2014; 9:2129-2136. doi: 10.1039/c4fo00456f . PMID: 25007233

45. Abu-Reidah IM, Arráez-Román D, Warad I, Fernández-Gutiérrez A, Segura-Carretero A. UHPLC/MS2-based approach for the comprehensive metabolite profiling of bean (Vicia faba L.) by-products: A promising source of bioactive constituents. Food Res Int. 2017; 93:87-96. doi: 10.1016/j.foodres.2017.01.014. PMID: 28290284

46. Palama TL, Menard P, Fock I, Choi YH, Bourdon E, Govinden-Soulange J, et al. Shoot differentiation from protocorm callus cultures of Vanilla planifolia (Orchidaceae): proteomic and metabolic responses at early stage. BMC Plant Biol. 2010; 10:82. doi: 10.1186/1471-2229-10-82. PMID: 20444255

47. Corrêa GM, Alcântara AFDE. Chemical constituents and biological activities of species of Justicia - a review. Braz J Pharmacog 2017; 22:220-238. doi: 10.1590/S0102-695X2011005000196

48. Inderjit, Dakshini KMM. Hesperitin 3 rutinoside (hesperidin) and taxifolin 3-arabinoside as germination and growth inhibitors in soils associated with the weed Pluchealanceolata (DC.) C.B. Clarke (Asteraceae). J Chem Ecol. 1992; 17:1585-1591. doi: 10.1007/BF00984690. PMID: 24257882

49. Suarez S, Gil A, Lorenzo E. Aloysia citriodora: morphology and density of glandular trichomes, and its relationships with essential oil content. In: Simpósio Brasileiro de Óleos Essenciais, 2, 2003, Campinas, SP. Anais… Campinas: Instituto Agronômico. pp. 78.

50. Nijveldt RJ, Van Nood E, Van Hoorn DE, Boelens PG, Van Norren K, Van Leeuwen PA. Flavonoids: a review of probable mechanisms of action and potential applications. Am J Clin Nutr. 2001;74:418-425. doi: 10.1093/ajcn/74.4.418. PMID: 11566638

51. Choi EM. Kaempferol protects MC3T3-E1 cells through antioxidant effect and regulation of mitochondrial function. Food Chem Toxicol. 2011; 49:1800-1805. doi: 10.1016/j.fct.2011.04.031. PMID: 21565246

52. Bakuradze T, Lang R, Hofmann T, Stiebitz H, Bytof G, Lantz I, et al. Baum M, Eisenbrand G, Janzowski C Antioxidant effectiveness of coffee extracts and selected constituents in cell-free systems and human colon cell lines. Mol Nutr Food Res. 2010;54:1734-1743. doi: 10.1002/mnfr.201000147. PMID: 20589861

53. Richards JS, Russell DL, Ochsner S, Hsieh M, Doyle KH, Falender AE, et al. Novel signalling pathways that control ovarian follicular development, ovulation and luteinization. Rec Prog Horm Res 2002; 57:195-220. PMID: 12017544

54. Erickson GF, Shimazak S, The spatiotemporal expression pattern of the bone morphogenetic protein family in rat ovary cell types during estrous cycle. Reprod Biol Endocrinol 2003; 5:1-9. doi: 10.1186/1477-7827-1-9. PMID: 12741959

55. Zhou J, Chan L, Zhou S. Trigonelline: a plant alkaloid with therapeutic potential for diabetes and central nervous system disease. Curr Med Chem. 2012;19:3523-3531. PMID: 22680628

56. Rossetto R, Santos RR, Silva GM, Duarte ABG, Silva CMG, Campello CC, et al. Comparative study on the *in vitro* development of caprine and bovine preantral follicles. Small Rumin Res 2012; 113:167-170. doi: https://doi.org/10.1016/j.smallrumres.2013.03.004

57. Rodgers RJ, Irving-Rodgers HF, Formation of the ovarian follicular antrum and follicular fluid. Biol Reprod 2010; 82:1021-1029. doi: 10.1095/biolreprod.109.082941 .PMID: 20164441

58. Lunardi FO, Chaves RN, Lima LF, Araújo VR, Brito IR, Souza CE, et al. Vitrified sheep isolated secondary follicles are able to grow and form antrum after a short period of *in vitro* culture. Cell Tissue Res 2015; 362:241-251. doi: 10.1007/s00441-015-2181-0. PMID: 25948481

59. Ruijters EJB, Weseler AR, Kicken C, Haenen GRMM, Bast A, The flavanol (–)-epicatechin and its metabolites protect against oxidative stress in primary endothelial cells via a direct antioxidant effect. Eur J Pharmacol 2013; 715:147-153. doi: 10.1016/j.ejphar.2013.05.029. PMID: 23747595.

60. Tatsimo SJN, Tamokou JD, Havyarimana L, Csupor D, Forgo P, Hohmann J, et al. Antimicrobial and antioxidant activity of kaempferol rhamnoside derivatives from Bryophyllum pinnatum. BMC Res 2012; 5:158-163. doi: 10.1186/1756-0500-5-158. PMID: 22433844

61. Taylor CT. Antioxidants and reactive oxygen species in human fertility. Environ Toxicol Pharmacol 2001; 10:189-98. PMID: 21782576

62. Costa RAP, Romagna CD, Pereira JL, Souza-pinto NC. The Role of Mitochondrial DNA Damage in the Citotoxicity of Reactive Oxygen Species. J Bioenerg Biomembr 2011; 43:25-29. doi: 10.1007/s10863-011-9329-8. PMID: 21286795

63. Heinzmann J, Mattern F, Aldag P, Bernal-Ulloa SM, Schneider T, Haaf T, et al. Extended *in vitro* maturation affects gene expression and DNA methylation in bovine oocytes. Mol Hum Reprod 2015; 10:770-782. doi: 10.1093/molehr/gav040. PMID: 26155800

64. Sadeghipour M, Terreux R, Phipps J. Flavonoids and Tyrosine Nitration: Structure Activity Relationship Correlation with Enthalpy of Formation. Toxicology 2005; 19:155-65. doi: 10.1016/j.tiv.2004.06.009. PMID: 15649628

65. Ribeiro RP, Portela AMLR, Silva AWB, Costa JJN, Passos JRS, Cunha EV, et al. Effects of jacalin and follicle-stimulating hormone on in vitro goat primordial follicle activation, survival and gene expression. Zygote 2014;23:537-549. doi: 10.1017/S0967199414000173. PMID: 24869637

66. Zhao G, Chen Y, Carey L, Futcher B. Cyclin-Dependent Kinase Co-Ordinates Carbohydrate Metabolism and Cell Cycle in *S. cerevisiae* Molecular Cell 2016; 62:546-557. doi: 10.1016/j.molcel.2016.04.026. PMID: 27203179

67. Robert C, Hue I, McGraw S, Gagne D, Sirard MA. Quantification of Cyclin B1 and p34cdc2 in Bovine Cumulus-Oocyte Complexes and Expression Mapping of Genes Involved in the Cell Cycle by Complementary DNA Macroarrays. Biol Reprod 2002; 67:1456-464. PMID: 12390876

68. Taieb R, Thibier C, Jessus C. On cyclins, oocytes, and eggs. Mol Reprod Dev 1997; 48:397-411. doi: 10.1002/(SICI)1098-2795(199711)48:3<397::AID-MRD14>3.0.CO;2-T. PMID: 9322253

69. Anguita B, Paramio MT, Ana R, Macedo J, Morat R, Mogasb T, Izquierdo D. Total RNA and protein content, Cyclin B1 expression and developmental competence of prepubertal goat oocytes. Anim Reprod Sci 2008; 103:290303.

doi: 10.1016/j.anireprosci.2006.12.018. PMID: 17250980

70. Ledda S, Bogliolo L, Leoni G, Naitana S. Cell coupling and maturation-promoting factor activity in *in vitro* matured prepubertal and adult sheep oocytes. Biol Reprod 2001; 65:247-52. PMID: 11420246

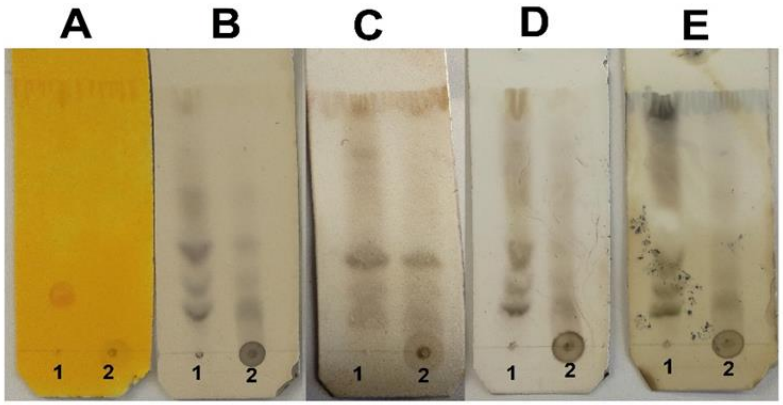
71. Mihm M, Baker PJ, Fleming LM, Monteiro AM, O'Shaughnessy AJ. Differentiation of the bovine dominant follicle from the cohort upregulates mRNA expression for new tissue development genes. Reproduction 2008; 135:253-265. doi: 10.1530/REP-06-0193. PMID: 18239053

Supporting information

S1 Fig.

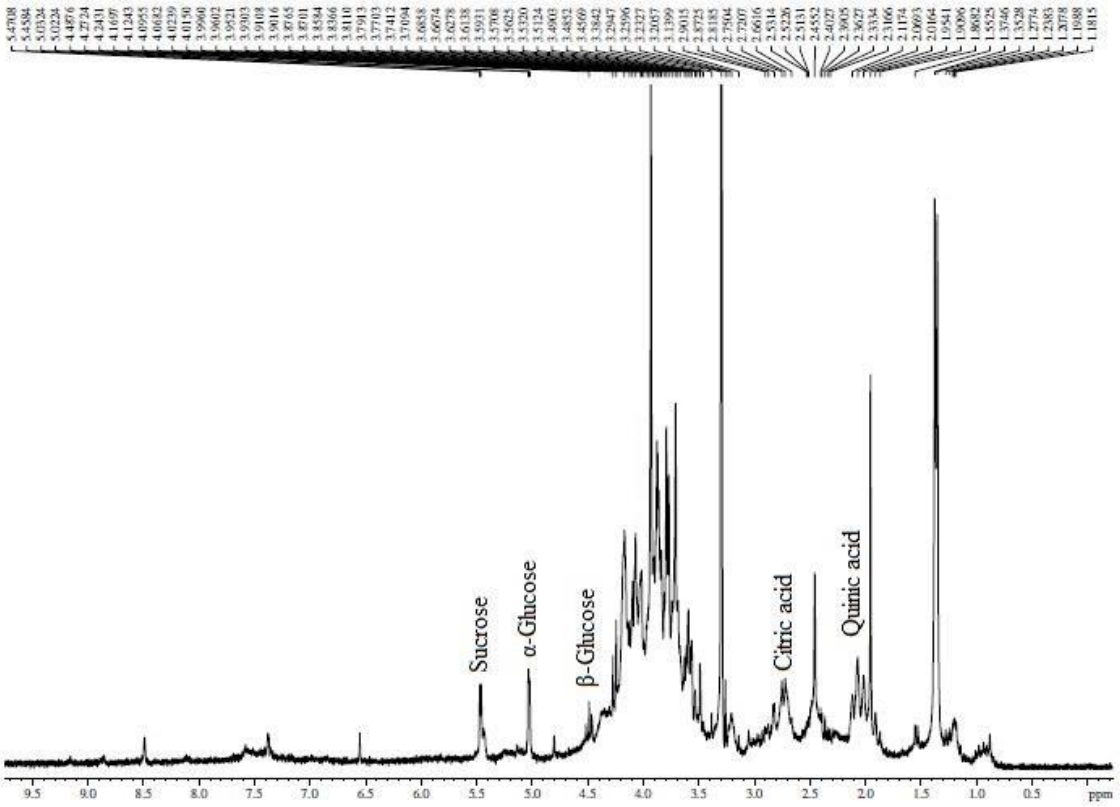
S1 Fig. Identification of secondary metabolites by thin layer chromatography (TLC). The developers used are specific to each class of metabolite as described: (A) specific Drangendorffi reagent to identify alkaloids; (B) α-Naphthol acid solution for glycosylated compounds; (C) acid solution of vanillin; (D) solution of ethanol / sulfuric acid used as universal developers and (E) acid solution of cerium sulfate for flavonoids and terpenoids.

S1 Fig. Identification of secondary metabolites by thin layer chromatography (TLC).

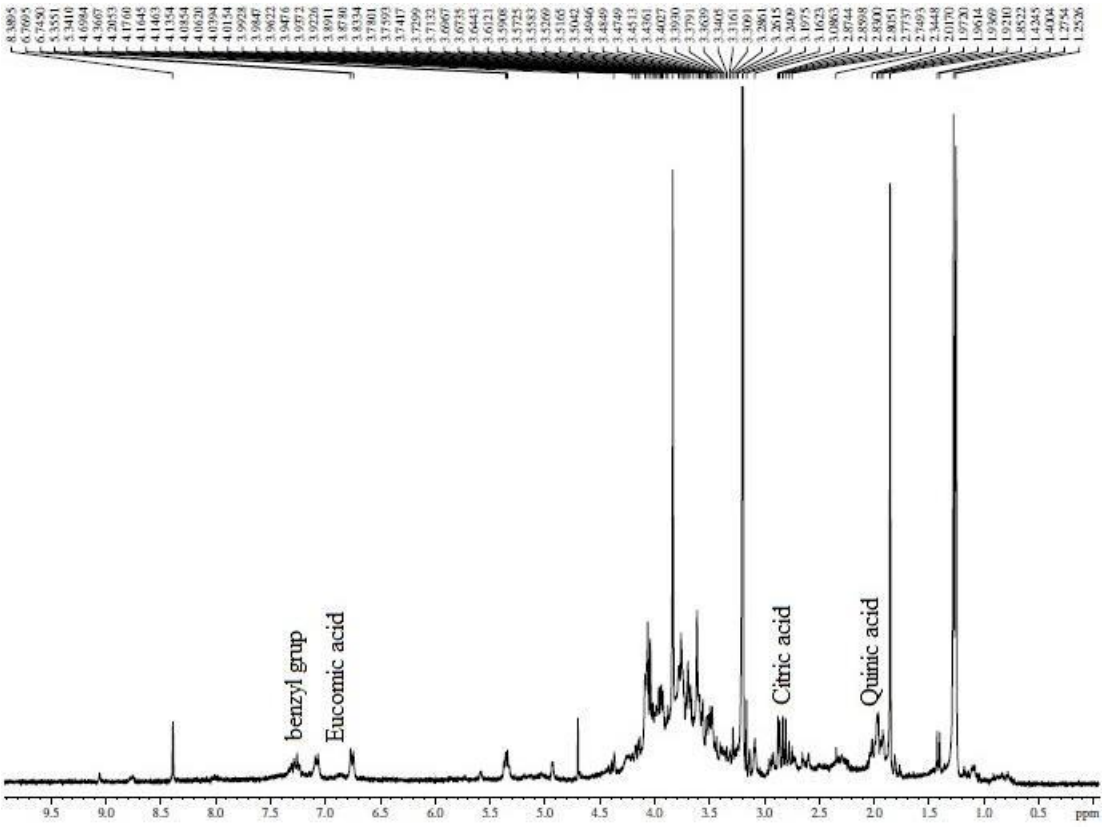


S 2. Fig. Metabolites of *J. insularis* identified by proton nuclear magnetic resonance (^{1}H NMR) and liquid chromatography-mass spectrometry (LC-MS).

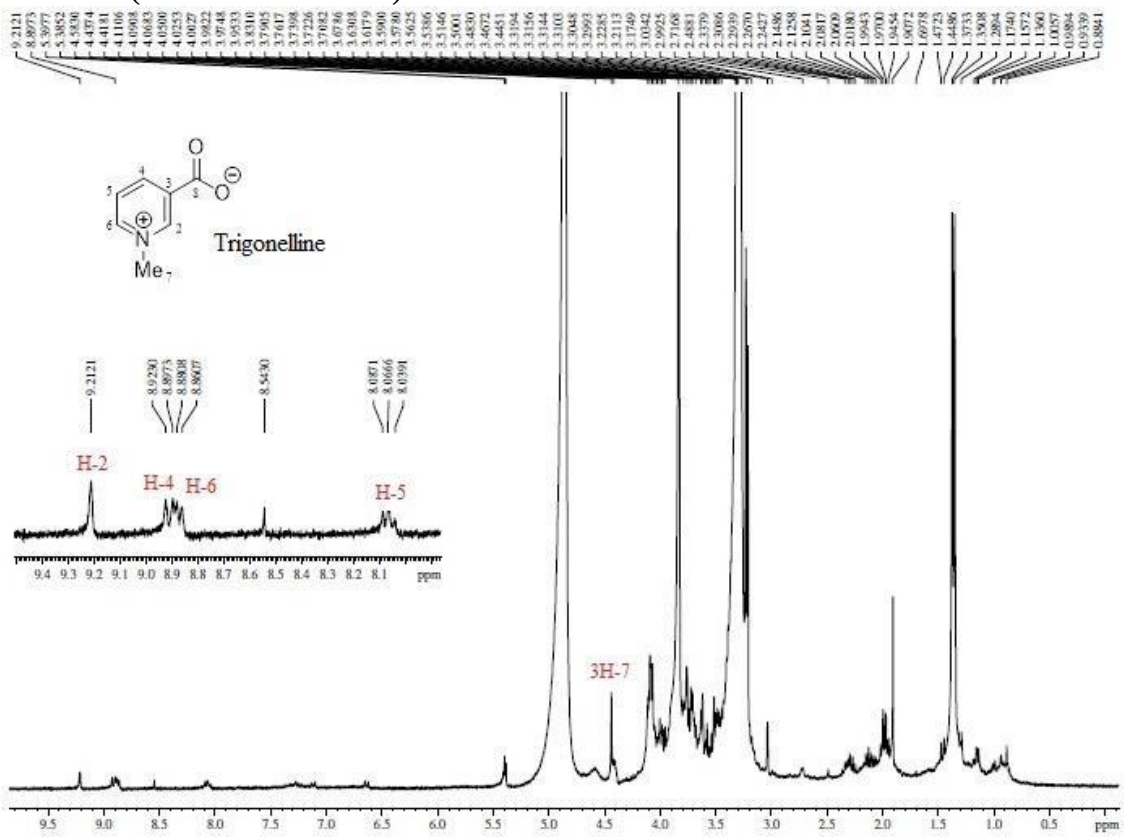
^{1}H NMR (300 MHz in D_2O) fraction **JI-1**


Sucrose
α-Glucose
β-Glucose
Citric acid
Quinic acid
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
ppm

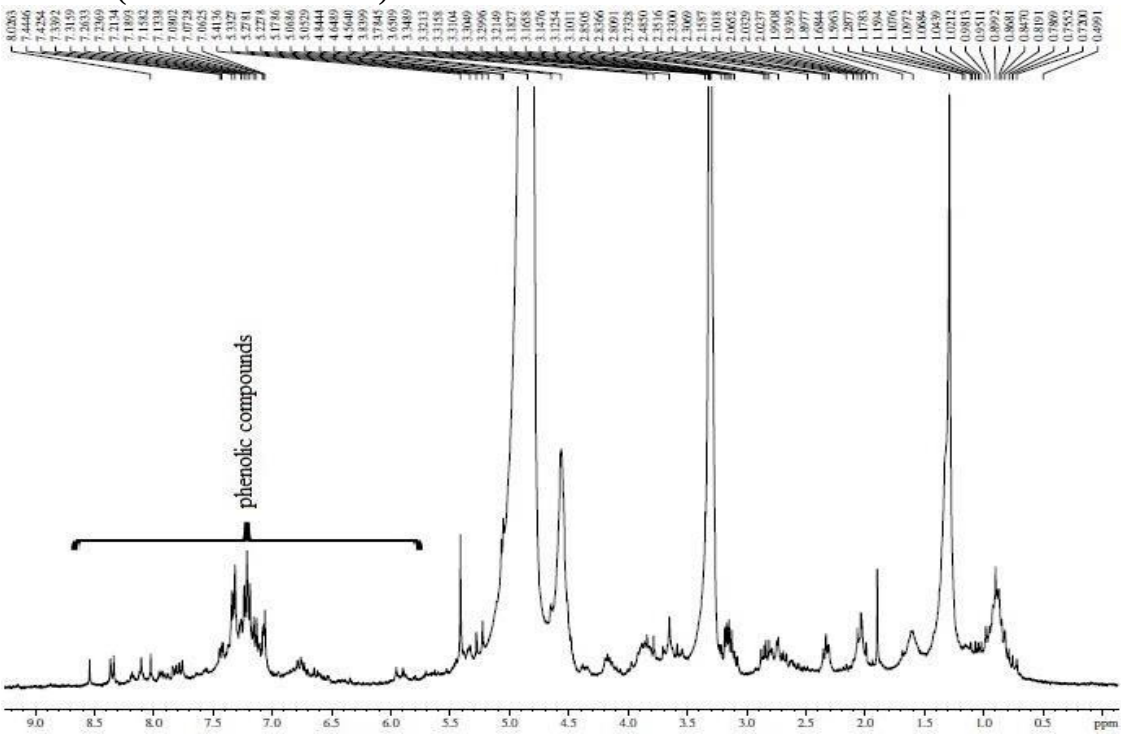
^{1}H NMR (300 MHz in D_2O) fraction **JI-2**



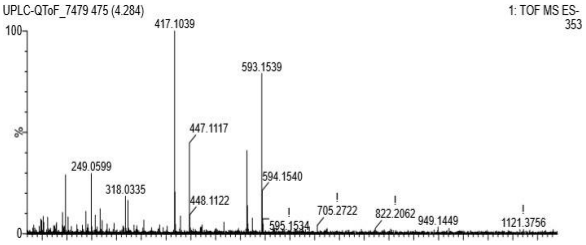
^{1}H NMR (300 MHz in MeOD) fraction **JI-3**

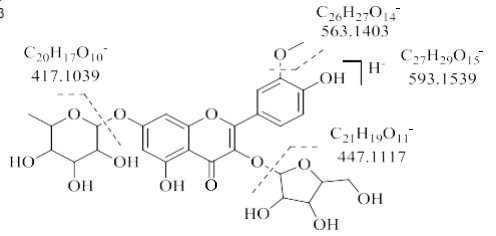


^{1}H NMR (300 MHz in MeOD) fraction **JI-4**

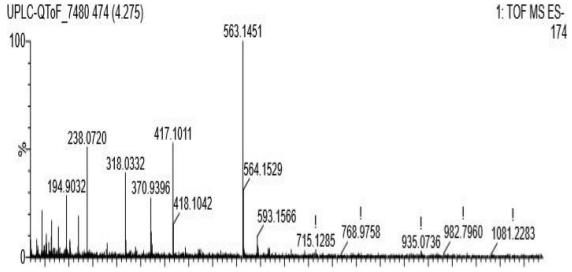


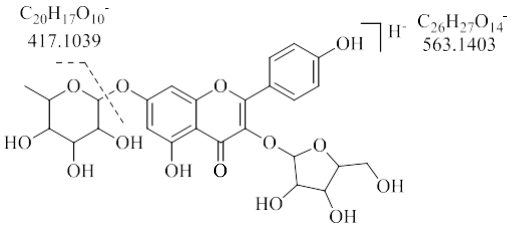
3’-Metoxy-kaempferol-arabinosyl-rhamnoside





Kaempferol-arabinosyl-rhamnoside





9 CAPÍTULO 4

Os folículos pré-antrais iniciais têm potencial para crescer até o estágio antral no sistema de cultivo em duas etapas na presença do extrato aquoso de Justicia insularis.

Early preantral follicles have a potential to grow until antral stage in two-step culture system in the presence of aqueous extract Justicia insularis.

Periódico: submetido na revista Aninal Reproduction Science.

Qualis A2

RESUMO

O objetivo deste estudo foi determinar se é possível isolar e cultivar folículos secundários intactos após o cultivo *in vitro* do tecido ovariano por 7 dias até a fase antral durante um período adicional de 6 dias de cultivo *in vitro* na presença de extrato aquoso de *Justicia insularis.* Fragmentos ovarianos frescos de 16 ovelhas adultas foram fixados para análise histológica (Controle 1) ou cultivados *in vitro* em α-MEM^{+} suplementado com 0,3 mg/mL de *J. insularis* (Etapa 1) por 7 dias. Parte dos fragmentos foram então fixados para análise histológica (grupo cultivado *in vitro*). Os fragmentos remanescentes foram expostos em concentrações crescentes de trealose antes do isolamento imediato de folículos secundários iniciais (FS) e a viabilidade foi avaliada antes (Controle 2) e após 6 dias de cultivo (Etapa 2). No Passo 1, a porcentagem de ativação folicular foi de 80%. No Passo 2, foi observado um aumento significativo ($P < 0,05$) no diâmetro folicular e na formação de antro em 6 dias de cultivo *in vitro* de folículos isolados. A capacidade antioxidante total em ambas as etapas aumentou significativamente ($P < 0,05$) do dia 2 para o dia 6. A análise confocal dos oócitos mostrou 57,14% de oócitos com distribuição homogênea e 42,86% com distribuição pericortical. Em conclusão, os folículos secundários podem ser isolados com sucesso do córtex ovariano de ovelhas após 7 dias de cultivo e são capazes de sobreviver e formar uma cavidade antral se cultivados *in vitro* por mais 6 dias na presença de 0,3 mg/mL de *J. insularis*.

Palavras-chave: Córtex ovariano; Folículos secundários precoces; Folículos antrais, Justicia insularis, Cultura in vitro.

Early preantral follicles have a potential to grow until antral stage in two-step culture system in the presence of aqueous extract *Justicia insularis*.

G.T. Mbemya[a], N.a.R. de Sá[a], D.D. Guerreiro[a], F.G.C. de Sousa[a], S.N. Nguedia[a], B.G. Alves[b], F.W. Santos[c], O.D.L. Pessoa[d], P. Comizzoli[e], J.R. Figueiredo[a], A.P.R. Rodrigues[a,*]

[a] Laboratory of Manipulation of Oocyte and Ovarian Preantral Follicles (LAMOFOPA), Faculty of Veterinary (FAVET), State University of Ceará, Fortaleza, Brazil

[b] Laboratory of Biology of Reproduction, Federal University of Uberlândia, Minas Gerais, Brazil

[c] Laboratory of Reproduction Biotechnology (Biotech), State of University of Pampa, Uruguaiana, Brazil

[d] Laboratory of Phytochemical Analysis of Medicinal Plants (LAFIPLAN), Federal University of Ceará, Fortaleza, Brazil

[e] Center for Species Survival, Smithsonian Conservation Biology Institute, Front Royal, VA, United States of America.

[*] Corresponding author:

E-mail address: aprrodriguespapers@gmail.com (A.P.R. Rodrigues)

Prof. Dr. Ana Paula Ribeiro Rodrigues, Laboratory of Manipulation of Oocyte and Ovarian Preantral Follicles, Faculty of Veterinary, State University of Ceará, Av. Dr. Silas Munguba, 1700-Fortaleza-CE- 60.714-903. Phone: + 55 8531019852 Fax: + 55 8531019840.

ABSTRACT

The objective of this study was to determine whether preantral follicles cultured *in vitro* for 7 days within ovine ovarian cortical strips could be isolated at the secondary follicles and grown until antral stage during an additional 6 days period of *in vitro* culture in the presence of aqueous extract of *Justicia insularis*. Fresh ovarian fragments from 16 adults sheep were fixed for histological analysis (Control 1) or *in vitro* cultured in α-MEM^{+} supplemented with 0.3 mg/mL *J. insularis* (Step 1) for 7 days. Part of the fragments then were fixed for histological analysis (*in vitro* culture group). Remaining fragments were exposed step-wise to increasing trehalose concentrations before immediate isolation of early secondary follicles (SF) and viability assessment (Control 2) or after 6 days of culture (Step 2). In Step 1, percentage of follicular activation was 80%. In Step 2, a significant increase ($P < 0.05$) in follicular diameter and antrum formation within 6 days *in vitro* culture of isolated follicles was achieved. The total antioxidant capacity from both steps significantly increase ($P < 0.05$) from day 2 to day 6. Confocal analysis of oocytes showed 57.14 % oocytes with homogeneous distribution and 42.86 % with pericortical distribution. In conclusion, secondary follicles can be successfully isolated from sheep ovarian cortex after 7 days of culture and are capable of surviving and forming an antral cavity if cultured *in vitro* for an additional 6 days in the presence of 0.3 mg / mL *J. insularis*.

Keywords: Ovarian cortex; Early secondary follicles; Antral follicles, *Justicia insularis*, *In vitro* culture.

1. Introduction

In vitro early preantral follicle growth has great potential to provide additional female fertility preservation option for young patients with cancer (Shea et al., 2014) and others mammalian species (livestock and wild species) (Cortvrindt and Smitz, 2001; Fortune et al., 2011). Although, several *in vitro* follicle culture systems have successfully supported the growth and maturation of ovarian follicles in mice (O'Brien et al., 2003), translation of this technique to large animals has been challenging. Therefore, multi-step systems have been designed to support *in vitro* follicle development in human (Xu et al., 2009; McLaughlin et al., 2018) and mice (Eppig and O'Brien, 1996; O'Brien et al., 2003; Telfer et al., 2008). In other species, scarce results have only been reported in cattle (McLaughlin et al., 2010).

In sheep, some results are promising, for instance, the growth and antrum formation have been successfully achieved (Kamalamma et al., 2015; Nascimento et al., 2018). Moreover, our team has also reported that it is possible to produce parthenotes embryos from large secondary follicles (> 200 µm), grown *in vitro* (Luz et al., 2012). This result was obtained from the secondary follicles isolated from the fresh tissue. Generally, the number of follicles isolated from this material is less than 10. Thus, we hypothesized that culture of ovarian tissue prior to isolation and *in vitro* culture of secondary follicles may improve the technique. However, after *in vitro* culture, ovarian tissue shows a swollen appearance due to perfusion of the culture medium, which may hinder the isolation of secondary follicles. Therefore, the use of an osmotic buffer, such as sugars, commonly used for the washing and removal of cryoprotectants, in cryopreservation protocols of ovarian tissue (Marsella et al., 2008; Sanfilippo et al., 2015; Tian et al., 2015) seems to be an alternative to reduce tissue swelling. Among those sugar, trehalose should be a viable chosen, which has been classified as a kosmotrope or water-structure maker that is the interaction between trehalose/water is much stronger than water/water interaction and may be involved in its bioprotective action (Branca et al., 2005). The trehalose helps biomolecules to maintain a glassy spatial structure (Paolantoni et al., 2009), binds water molecules around the protein surface instead of interacting directly with the protein (Lupi et al., 2012). Furthermore, trehalose is able to counteract osmotic stress, hypoxia, and oxidative stress (Zhang et al., 2015).

As *in vitro* follicle culture is maintained at higher oxygen atmosphere than the *in vivo* environment, this lead to an increased level of reactive oxygen species (ROS). According to Costa et al. (2011), *in vitro* follicular development and maturation are dramatically affected by excess amount of ROS generation. Therefore, the addition of antioxidants to the culture medium

is critical to maintain the balance of ROS production, preventing the damage induced by *in vitro* oxidative stress. Several teams have dedicated huge efforts to evaluate the antioxidant potential of natural compounds (Abedi et al., 2014; Barberino et al., 2015; Ibrahim et al., 2015). Recently, our group reported that the addition of *J. insularis* as antioxidant improves the *in vitro* survival, activation and growth of preantral follicles enclosed in ovarian cortex cultured *in vitro* for 7 days (Mbemya et al., 2017). Furthermore, *J. insularis* demonstrates similar effect as FSH on ovine secondary follicles *in vitro* cultured for 18 days (Mbemya et al., 2018). In this context, the presence of FSH is crucial to promote the development of follicles beyond the early antral stage (Oktay et al., 1997), and previous report shown that FSH added to culture medium maintains follicle viability and promotes the development of isolated preantral follicles and antrum formation (Rodrigues et al., 2010). Although some information on the *in vitro* growth of ovine secondary follicles is already available, a two-step culture system, not yet employed, may offers more promising results.

The objective of this study was to verify if secondary follicles could be harvested from ovarian tissue cultured *in vitro* for 7 days (first step) and culture them for follow 6 days (second step) in the presence of *J. insularis*.

2. Materials and methods

This study was approved and performed according to the recommendations of the Committee of Animal Handling and Ethical Regulation from the State University of Ceará (N° 6004720/2015).

2.1. Source of ovaries and experimental design

Ovaries (n = 32) were collected at a local slaughterhouse from 16 adults (1-3 years old) mixed-breed sheep (*Ovis aries*). 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 (Lima et al., 2013) and intended for a two-step *in vitro* culture, i.e. first step (*in vitro* culture of ovarian tissue) and second step (*in vitro* culture of secondary isolated follicles), as we can see on figure 1.

Ovarian cortex from each ovarian pair were cut using a surgical blade into 15 fragments and placed in MEM-HEPES. For each replicate, five fragments were took randomly and

immediately fixed for histological analysis and identified as *Control 1* (CTR1), the remaining fragments were *in vitro* cultured for 7 days. After that, five fragments for each replicate were took randomly and also fixed for histological analysis and called *in vitro culture ovarian tissue* (IVC-OT). The remaining fragments (n = 5) were submitted to four-step bath solutions (MEM-HEPES) supplemented with increased concentrations of trehalose for 10 minutes each at room temperature to reduce swelling after *in vitro* culture. After that, secondary follicles (SF) were isolated and immediately submitted to viability test (*Control 2* - CRT2) or *in vitro cultured* (IVC-SF) for 6 days.

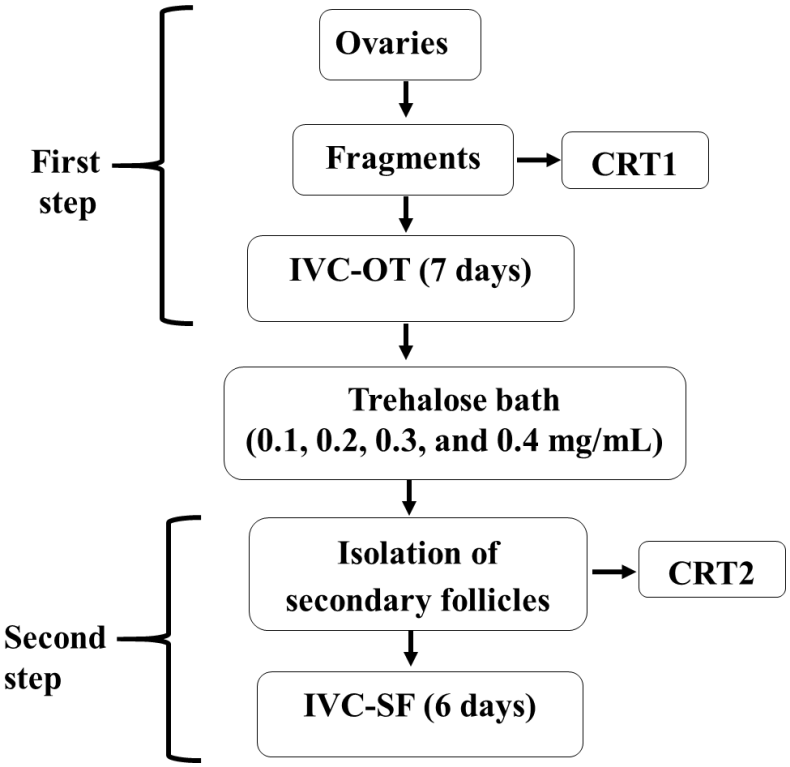


Fig. 1. Experimental design of two-steps *in vitro* culture of preantral follicles. **CRT:** Control**, IVC-OT:** *in vitro* cultured of ovarian tissue**, IVC-SF:** *in vitro* cultured of secondary follicles.

2.2. Chemicals, in vitro culture media and trehalose solution

Unless mentioned otherwise, the culture media, trehalose and other chemicals used in the present study were purchased from Sigma Chemical Co. (St. Louis, Mo, USA).

For *in vitro* culture of ovarian fragments, the culture medium used was α-MEM (M5650, pH 7.2-7.4) supplemented with 1.25 mg/mL bovine serum albumin, ITS (10 µg/mL insulin, 5.5 mg/mL transferrin, 5 ng/mL selenium), 2 mM glutamine, 2 mM hypoxanthine and antibiotics (100 µg/mL penicillin-streptomycin) which was referred to as α-MEM^{+} (Bandeira et al., 2015). Whereas for culture of isolated secondary follicles, the medium was (α-MEM - M5650, pH 7.2-7.4), supplemented with 3 mg/mL of bovine serum albumin (BSA), ITS (10 µg/mL insulin, 5.5 µg/mL transferrin, 5 ng/mL selenium), 2 mM of glutamine, 2 mM of hypoxanthine, 50 ng/mL of Leukemia Inhibitory Factor (LIF) and 50 ng/mL of Kit Ligant (KL) as mentioned by Luz et al. (2012) was referred to as α-MEM^{++}.

2.3. First step: In vitro culture of ovarian tissue (in situ)

In the laboratory, ovarian cortex from each ovarian pair were cut using a surgical blade into 15 fragments (approximately 3 x 3 x 1 mm) and placed in MEM-HEPES. Ovarian fragments were *in vitro* cultured in 1 mL of α-MEM^{+} supplemented with lyophilized plant extract 0.3 mg/mL *J. insularis* whose concentration was defined based on our previous study (Mbemya et al., 2017). The *in vitro* culture was performed for 7 days at 38.5 °C in 5% CO_2 in air. The culture medium was equilibrated at least 3 h prior to use. Every two days, whole culture medium was replaced. At the end of the culture, the fragments were subjected to the trehalose bath and then follicular isolation for the accomplishment of the second step.

2.4. Ovarian tissue bath with trehalose and isolation of secondary follicles

After *in vitro* culture of ovarian tissue, the fragments were submitted to four-step bath solutions (MEM-HEPES) supplemented with increased concentrations of trehalose (0.1, 0.2, 0.3 and 0.4 mg/mL respectively) for 10 minutes each at room temperature. At the end of bath, the fragments were transferred to MEM-HEPES supplemented with 0.1 mg/mL of trehalose and taken for mechanical isolation of SF.

2.5. Second step: In vitro culture of secondary follicles

Secondary follicles (approximately 132-200 μm in diameter) were visualized under a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan) and manually dissected from the slices of ovarian tissue cultured *in vitro* previously (first step), using 26-gauge needles. After isolation, follicles were transferred to 100 μL drops of medium under mineral oil to further evaluate follicular quality. Secondary follicles with a visible oocyte, surrounded by granulosa cells, an intact basement membrane were selected for *in vitro* culture according to Lima et al. (2017).

The *in vitro* culture of SF (second step) was performed as described previously by Telfer et al. (2008). Briefly, immediately after isolation, follicles were individually cultured in 100 μL drops of culture medium on Petri dishes (60 ×15 mm; Corning, USA) under mineral oil for 6 days. The medium used was α-MEM^{++} supplemented with lyophilized plant extract 0.3 mg/mL *J. insularis* whose concentration was defined based on our previous study (Mbemya et al., 2018). Incubation was carried out at 38.5 °C in 5% CO_2 in air. Fresh medium was prepared immediately before use and incubated for 2 h prior to use with 60 μL medium being replaced in each drop every 2 days (Lins et al. 2017). The experimental conditions were replicated five times and at least 15 secondary follicles were used per replicate.

2.6. Morphological evaluation of preantral (primordial, intermediary, primary and secondary) follicles in situ

Before and after 7 days of culture, the ovarian fragments were fixed individually in Carnoy for 4 h at room temperature. Subsequently, fragments were dehydrated in a graded concentrations of ethanol. After paraffin embedding (Synth, São Paulo, Brazil), the ovarian fragments were cut into 7 μm sections and mounted on glass slides and stained by periodic acid schiff-hematoxylin. Follicle stage and morphology were assessed microscopically on serial sections, according to Lima et al. (2016). The developmental stages of follicles were *primordial* (one layer of flattened pregranulosa 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). Morphologically, these follicles were still classified individually as *normal* or *atretic*. Normal follicles contained an intact oocyte was present, surrounded by granulosa cells which are well organized in one or more layers and that have 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. One hundred and fifty follicles were evaluated for each treatment (30 follicles per each five repetitions).

To evaluate follicular activation, the percentages of healthy primordial and growing follicles were calculated before and after *in vitro* culture. 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.

2.7. Normal appearance and viability assessment of secondary follicles

The appearance of follicles was considered normal if they presented bright and homogeneous granulosa and theca cells, and homogeneous oocyte cytoplasm**.**

The follicular viability before and after 6 days of *in vitro* culture was assessed using the trypan blue dye exclusion test. Briefly, 5 mL of 0.4% trypan blue (Sigma Chemical Co., St. Louis, MO, USA) was added to 100 mL of isolated and suspended SF, which were incubated for 1 minute at room temperature. Subsequently, follicles were examined with an inverted microscope (Nikon, Tokyo, Japan) and classified as nonviable or viable if they were not stained or stained by trypan blue, respectively (Castro et al., 2014).

2.8. Growth and antrum formation of secondary follicles

Considering the visual analysis, the isolated SF were classified as *intact* (no rupture of basement membrane), *extruded* (follicles were those that underwent rupture of their basement membrane) or *degenerated* (follicles showed darkened oocyte and/or misshapen granulosa cells). The percentage of intact follicles and follicular diameter were calculated only in non-extruded and non-degenerated follicles. Follicular diameter was calculated as the mean of two perpendicular measures of each follicle every 2 days with the aid of an ocular micrometer attached to a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan; 100 x magnification). The average follicular daily growth was calculated as follows: the diameter of intact follicles on the last day minus their diameter on day zero divided by the number of days (Luz et al., 2012).

2.9. Ferric reducing antioxidant potential (FRAP) analysis in culture medium of both steps

To determinate the antioxidant total capacity, the FRAP assay with slight modifications (Benzie and Strain 1996) was used. For this, the antioxidants present in the medium collected

(from both *in situ* and isolated follicles culture) were evaluated as reducers of Fe^{+3} to Fe^{+2}, which is chelated by 2,4,6-Tri-(2-pyridyl)-s-triazine (TPTZ) to form the complex Fe^{+2}-TPTZ, evaluated using the maximal absorption at 593 nm. An ascorbic acid standard curve was performed as a control, and the results are expressed as the equivalent to micrograms of ascorbic acid.

2.10. Distribution and activity of oocyte mitochondria

For the evaluation of the mitochondrial distribution and activity, oocytes recovery after 6 days of *in vitro* culture were fixed with glutaraldehyde in 1% phosphate buffered saline solution (PBS). Thereafter, all oocytes were incubated for 30 minutes in 1999 μL of PBS with 1 μl of MitoTracker (0.5 μM) Orange CMTMRos (M7510, 38.5 °C and 5% CO_2 probes to detect and locate active mitochondria). Then the oocytes were washed (3x) in PBS medium. The stained oocytes were then mounted on glass slides and observed using a confocal laser scanning microscope (LSM 710, Zeiss, Oberkochen, Germany). The distribution of the active mitochondria in each oocyte was classified as (i) *homogeneous* if the organelle was observed along the cytoplasm of the oocyte or (ii) *pericortical* if the organelle was aggregated along the outer edge of the gamete (cortex region) and the fluorescence intensity, software Zen lite 2.3 SP1 was used (Leoni et al., 2015).

2.11. Statistical analyses

Statistical analyses were performed by Sigma Plot version 11.0 (Systat Software Inc., USA). The variables morphology, category, antrum formation, intact, extruded, and degenerated follicles were evaluated by chi-square or Fisher exact tests, when appropriate. Data for daily growth, follicular growth, and FRAP were not normally distributed (Shapiro- Wilk test); therefore were analyzed using Friedman ANOVA on ranks repeated measure followed by Student-Newman-Keuls post hoc test (daily growth and follicular growth) or Mann-Whitney test (FRAP). Statistical significance was defined as $P < 0.05$ (two sided) and values are presented as mean ± (standard error of the mean) or percentage.

3. Results

3.1. In vitro culture of preantral follicles within ovarian cortex

The swelling of ovarian cortex observed during the first step (7 days of *in vitro* culture - Fig. 2A) was reduced after trehalose bath (Fig. 2B). Moreover, the ovarian fragments become more suitable for visualization and isolation of SF. Growing follicles were found before (Fig. 2C) and after 7 days of culture (Fig. 2D). As shown in Fig. 2E, SF isolated from the ovarian cortex after the first step had a normal appearance (bright and homogeneous granulosa and theca cells and homogeneous oocyte cytoplasm as well). In addition, those SF further cultured *in vitro* individually for 6 days were able to form an antral cavity (Fig. 2F).

The percentage of morphologically normal follicles was reduced ($P < 0.05$) after 7 days of culture when compared to CTR1 group (Fig. 2G). Despite this, the rate of developing follicles increased ($P < 0.05$) during the same period of culture, compared to CTR1.

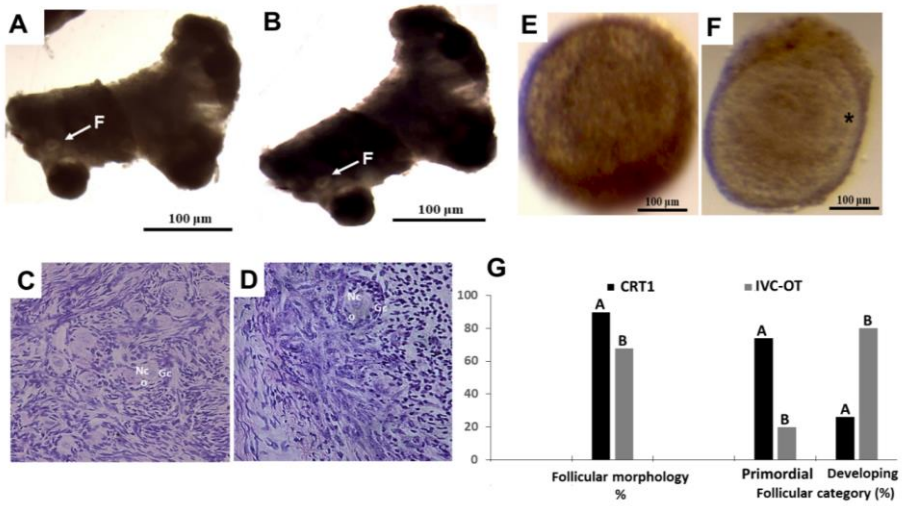


Fig. 2. Representative photographs of *in vitro* culture ovarian**.** Note that in figures **A** and **B**, F indicates an included follicle in tissue. Histological section of ovarian tissue stained with periodic acid Schiff (PAS) - hematoxylin in CTR1 **(C)** or after 7 days of culture **(D)**. Normal primordial follicles are shown in CTR1, while normal SF follicles in *J. insularis* 0.3 mg/mL is represented. Photomicrographs of normal sheep secondary follicle after trehalose bath **(E)** or at

day 6 in *J. insularis* 0.3 mg/mL **(F).** Note formation of antrum (∗). (**G**): Percentage of normal and developing follicles before and after *in vitro* culture. F: follicle; O: oocyte; Nc: oocyte nucleus; Gc: granulosa cells. [A,B] Values without a common superscript differed ($P < 0.05$).

3.2. In vitro growth of secondary follicles

When dividing the culture period into three intervals (D0-D2; D2-D4; D4-D6), we observed a reduction ($P < 0.05$) in both parameters from the first (D0-D2) to the second one (D2-D4) (Table 1). However, the daily and follicular growth rates remained unchanged from the second interval onwards.

Table 1

Mean (± SEM) daily and follicular growth of morphologically normal secondary follicles at different time point (D0, D2, D4, D6).

Mean follicular growth (µm/day ± SEM) in different culture intervals				
	D0 – D2	D2 – D4	D4 – D6	Overall
Daily growth	13.1 ± 1.6^{A}	7.2 ± 1.6^{B}	6.8 ± 2.1^{B}	9.0 ± 1.0
Follicle growth	26.2 ± 3.2^{A}	14.4 ± 3.2^{B}	13.6 ± 4.3^{B}	59.9 ± 6.4

[A,B] Different letters denote significant differences among culture intervals ($P < 0.05$).

Overall, follicular diameter and antrum formation progressively increased ($P < 0.05$) during the culture period (Fig. 3A).

Over the 6 days of the culture period, a significant decrease ($P < 0.05$) in the percentage of intact follicles was observed, although it has remained unchanged from D2 to D4 and from D4 onwards (Fig. 3B). Similar results were observed regarding the percentage of extruded follicles. Looking at the degeneration, no difference was observed between days of *in vitro* culture.

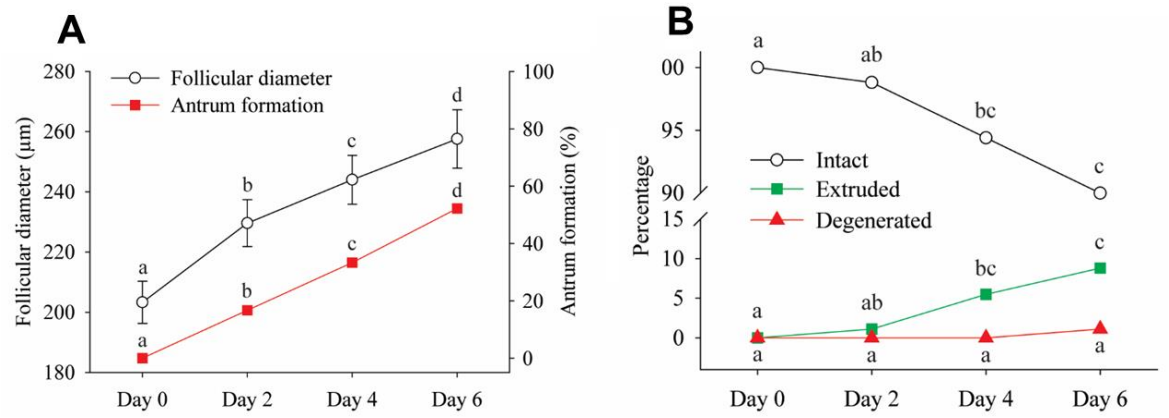


Fig. 3. Follicular diameter (mean ± SEM) and antrum formation rate (**A**), and percentage of intact, extruded, and degenerated follicles (**B**) at different time points of *in vitro* culture. [a,b,c,d] Different letters denote significant differences among periods of time ($P < 0.05$).

3.3. Total antioxidant capacity, oocyte mitochondrial activity and distribution

The total antioxidant capacity (Fig. 4A) of *J. insularis* was evaluated after the first (*in vitro* culture of OT) and second step (*in vitro* culture of SF). As we can see, there was a significant increase ($P < 0.05$) in the ferric reduction antioxidant potential in both steps. In relation to the distribution and activity of oocyte mitochondria, as we can see in Fig. 4B, 57.1 % of oocyte showed homogeneous distribution whereas 42.9 % presented pericortical distribution.

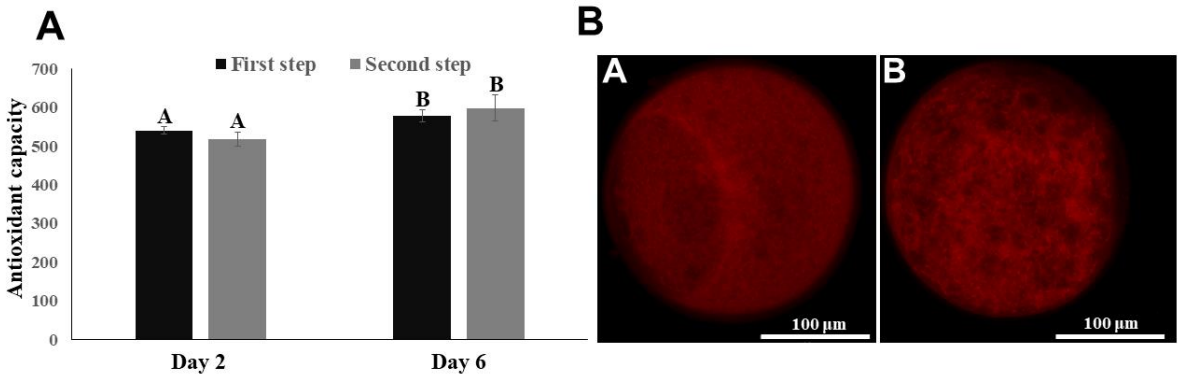


Fig. 4. Total antioxidant capacity (Mean ± SEM) from both *in situ* and isolated follicles culture on days 2 and 4 (**A**) and representative image of oocytes (**B**) showing homogeneously (A) and pericortical distribution of active mitochondria (B) obtained by confocal microscopy. Note the redistribution of mitochondria organelles after six days in vitro culture. [A,B] Within the same step culture system and between days ($P < 0.05$)

4. Discussion

Here in we reported for the first time that ovine preantral follicles have developed in a two-step culture system after a total of 13 days in culture (7 days within cortical strips – *first step* and additional 6 days as isolated follicles – *second step*). Using this strategy we demonstrated that early preantral follicles can grow until early antral (mean diameter 258 µm) stage in a medium containing 0.3 mg/mL of an aqueous plant extract (*J. insularis*).

During the first step, we found a significant reduction of morphologically normal follicles rate in ovarian cortex treated with 0.3 mg/mL *J. insularis*, compared to control after 7 days of culture, a very common outcome observed in *in vitro* culture protocols (Andrade et al., 2005; Mery et al., 2007; Costa et al., 2010; Esmaielzadeh et al., 2013; Lima et al., 2016). We believe that this effect may be due to when cultured *in vitro*, even when they are inside the cortex, the follicles can suffer the effects caused by blood supply deprivation and several components, such as growth factors, hormones, nutrients, etc.

The rate of growing follicles increased concomitant to reduction of primordial follicles rate at day 7 of culture, indicating that follicular activation had occurred in the presence of 0.3 mg/mL of *J. insularis* added to culture medium, similar to observed in our previous study (Mbemya et al., 2017). This result was not also different from several others carried out in ovine (Mery et al., 2007; Esmaielzadeh et al., 2013; Lima et al., 2016) or in many other species (caprine: Silva et al., 2004; Martins et al., 2010, bovine: Andrade et al., 2012; McLaughlin et al., 2010, human: Wright et al., 1999; McLaughlin et al., 2018).This phenomenon is common and could be due to the fact that when the ovarian cortex is outside of the body it may be deprived of inhibitory factors that can reach it through the bloodstream and stimulate the primordial follicles to pass into the pool of growing follicles (Wandji et al., 1996; Hovatta et al., 1997, 1999). Furthermore, the absence of inhibitory substances, in *in vitro* conditions, the follicles are permanently exposed to a huge number of factors (Rosairo et al., 2008; Fujihara et al., 2014) and hormones that promote the follicular activation, such as FSH (Andrade et al., 2005; Esmaielzadeh et al., 2013). In the case of this study, we had the effect of *J. insularis* which has previously been shown to have a similar effect as FSH (Telefo et al., 2012; Mbemya et al., 2017; 2018).

After the first step, secondary follicles were observed microscopically in several cultured pieces within 7 days; however, it was difficult to isolate all of them because of the clustering of follicles within tightly packed stromal cells, as also reported by Telfer et al.,

(2008). Therefore, we designed a protocol using trehalose bath to reduce swelling after *in vitro* culture of ovarian cortex; which enable follicles recuperation without any damage on follicles. Previous works of our own team showed that the average obtained does not reach, respectively, 5/10 SF per ovary/animal neither in caprine (Sá et al., 2016: 222 SF per 50ov/25an; Apolloni et al., 2016: 169 SF per 60ov/30an) nor ovine (Luz et al., 2012: 179 SF per 40ov/20an; Paz et al., 2018: 340 SF per 114ov/57an). Interestingly, in this study using a two-step culture system, we recovered 159 SF (4.96 per 32 ovaries or 9.93 per 16 animals). These data showed that the mean of SF obtained per ovary/animal in our study was better than in previous studies, which might be possible to fact that the follicles may have grown to the stage of secondary follicles, providing a greater number of follicles obtained.

We observed a reduction in both daily and follicular growth rates from the first (D0 – D2) to the second interval (D2 – D4), however, remained unchanged from the second interval onwards. This means that there was an adequate response of the follicles without damages since no difference was observed on the percentage of degenerated follicles. Furthermore, all follicles evaluated were negatively stained by the trypan blue.

Into two-steps culture system, we were able to achieve significant follicular diameter and antrum formation within a total of 13 days *in vitro* (7 days within cortical strips and 6 days as isolated follicles) when 0.3 mg/mL *J. insularis* was present in the culture media. These results could be related to a rich culture medium used, supplemented with LIF and KL, among other additives. Luz et al. (2012) also reported the positive effect of these substances on ovine follicles development *in vitro*.

In our present system culture, we achieved significant follicular growth (mean follicular diameter 252 µm after 13 days of *in vitro* culture; whereas in bovine, using the two step culture system, after the same culture period, McLaughlin and Telfer (2010) reported a mean diameter of 200 µm. In human, after 10 days of *in vitro* culture, Telfer et al. (2008) obtained a mean diameter 150 µm to isolated secondary follicles. We believe that the presence of *J. insularis* in our culture medium improved the growth of secondary follicles. This action could be due to the secondary metabolite trigonelline. Phytochemical analysis of *J. insularis* revealed the presence of trigomelline (Mbemya et al., 2018), which is a phytohormone that induced the proliferation of neuronal cells (Zhou et al., 2012). The molecular mechanism of trigonelline on the receptor of granulosa cells awaits further investigations.

Over the 6 days of the culture period, a significant decrease in the percentage of intact follicles was observed, interestingly, it has remained unchanged from D2 to D4 and from D4

onwards. This could be due to the action of the metabolites of the plant extract (flavonoids: 3'-Metoxy-kaempferol-arabinosyl-rhamnoside, Kaempferol-arabinosyl-rhamnoside and alkaloid: trigomelline) which are antioxidant compounds that have the capacity to neutralize damage caused by oxidation (Bakuradze et al., 2010; Choi et al., 2011).

In the present study, a significant increase in the percentage of extruded follicles was observed, although it has remained unchanged from D2 to D4 and from D4 onwards. We believe that the extrusion could be due to the presence of the LIF on the culture medium. It have been reported that LIF can increase secretion of tissue inhibitors of metalloproteinases (TIMPs), resulting in increased membrane damage and, consequently, extrusion (Luz et al., 2011). Tapia et al. (2008) also found that the addition of LIF to the culture medium of human placental cells promoted secretion increase in TIMPs, which are enzymes involved in the remodeling of the basement membrane, suggesting a possible explanation for the extrusion observed in this study.

This study also focuses on the ferric reducing antioxidant potential analysis which represent the capacity of an antioxidant to reduce Fe^{+3} into Fe^{+2} (Benzie and Strain 1996). Our data show that the total antioxidant capacity from both *in situ* and isolated follicles culture increased from day 2 to day 6. This is the first time that a natural compound reduced the free radical within a two-steps system. Such effect could be due to the phenolic compound of *J. insularis* which is constituted of several secondary metabolites among which the phenols (Goka et al. 2016), we believed that phenolic compounds acts as donor of hydrogen involved in the reduction of free radical produced during *in vitro* culture. Similar mechanism has been found during *in vitro* culture of ovine preantral follicles with *Amburana cearensis* (Barberino et al., 2015).

In the present study, 57.14 % of oocyte showed homogeneous distribution whereas 42.86 % presented pericortical distribution. Although more analysis need to be performed, in our opinion, the redistribution of oocyte mitochondria is an indication of cytoplasmic maturation of oocyte. According to Ferreira et al. (2009) and Leoni et al. (2015), cytoplasmic maturation of oocyte is associated with considerable changes among which redistribution of mitochondria organelles which are indicator of ATP contents and oocytes meiotic competence. Moreover, cytoplasmic ATP content promotes all the energy requiring processes which determine the timing of the cell cycle and the acquisition of developmental competence (Leoni et al., 2015).

5. Conclusion

In conclusion, in this study we used for the first time a two-step system to culture *in vitro* ovine preantral follicles. We demonstrated that secondary follicles can be successfully isolated from ovarian cortex after 7 days in culture and are able to survive and form an antral cavity if cultured *in vitro* for additional 6 days in the presence of 0.3 mg/mL *J. insularis*. However, we know that huge attempts will be needed to achieve more consistent results, for example, the improvement of ovarian cortex *in vitro* culture conditions to increase the number of secondary follicles obtained. Thus, certain that by using this strategy, isolated secondary follicles can resist up to 6 days in culture, we can try to extend this period for an additional 6 or 12 days to obtain a larger number of viable oocytes for *in vitro* maturation. This might be an additional option to obtain embryos produced *in vitro* from genetically valuable animals, which cannot reproduce naturally or after death. In addition, the data of this study may also reinforce some researchers' ideas (Xu et al., 2009; Xiao et al., 2015; McLaughlin et al., 2018) who stated that the multi-step strategy may be, in the future, an alternative to ovary transplantation for preserving the fertility of young women undergoing cancer treatment.

Conflicts of interest

None.

Acknowledgments

This research was supported by grants from the National Council for Scientific and Technological Development (CNPq Brazil) for financial support. Gildas Tetaping Mbemya is the recipient of a doctoral scholarship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (PEC-PG/CAPES, Brazil). Ana Paula Ribeiro Rodrigues is recipient of a grant from CNPq Brazil through the process 308071/2016-6.

References

Abedi, A., Rouhi, L., Pirbalouti, A.G., 2014. Effect of *Gundelia tournefortii* leaves extract on in immature mouse oocytes. J. Herb. Drugs. 5, 84–89.

Andrade, E.R., Marcondes, S.M., Alfieri, A.A., De Oliveira, J.A., Frederico, R.L.B., Figueiredo, J.R., Toniolli, R., 2005. Interactions of indole acetic acid with EGF and FSH in the culture of ovine preantral follicles. Theriogenology 5, 1104–1113. https://doi:10.1016/j.theriogenology.2005.03.001.

Andrade, ER., Van den Hurk, R., Lisboa, L.A., Hertel, M.F., Melo-Sterza, F.A., Moreno, K., Bracarense, A.P., Landim-Alvarenga, F.C., Seneda, M.M., Alfieri, A.A., 2012. Effects of ascorbic acid on *in vitro* culture of bovine preantral follicles. Zygote 20, 379–388. https://doi:10.1017/S0967199412000056.

Apolloni, L.B., Bruno, J.B., Alves, B.G., Ferreira, A.C., Paes, V.M., Moreno, J.L. Aguiar, F.L., Brandão, F.Z., Smitz, J., Apgar, G., Figueiredo, J.R., 2016. Accelerated follicle growth during the culture of isolated caprine preantral follicles is detrimental to follicular survival and oocyte meiotic resumption. Theriogenology 6, 1530–1540. https://doi: 10.1016/j.theriogenology.2016.05.012.

Bakuradze, T., Lang, R., Hofmann, T., Stiebitz, H., Bytof, G., Lantz, I., Baum, M., Eisenbrand, G., Janzowski, C., 2010. Antioxidant effectiveness of coffee extracts and selected constituents in cell-free systems and human colon cell lines. Mol. Nutr. Food Res. 54, 1734–1743. https://doi:10.1002/mnfr.201000147.

Bandeira, F.T., Carvalho, A.A., Castro, S.V., Lima, L.F., Viana, D.A., Evangelista J., Pereira, M., Campello, C.C., Figueiredo, J.R., Rodrigues, A., 2015. Two Methods of Vitrification Followed by *in vitro* Culture of the Ovine Ovary: Evaluation of the Follicular Development and Ovarian Extracellular Matrix. Reprod. Domest. Anim. 50, 177–185. https://doi:10.1111/rda.12463.

Barberino, R.S, Barros, V.R., Menezes, V.G., Santos, L.P., Araújo, V.R., Queiroz, M.A., Almeida, J.R., Palheta, R.C., Matos, M.H., 2015. *Amburana cearensis* Leaf Extract Maintains Survival and Promotes *in vitro* Development of Ovine Secondary Follicles. Zygote 2, 277–285. https://doi:10.1017/S0967199415000179.

Benzie, I.F., Strain, J.J., 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal. Biochem. 1, 70–76. https://doi:10.1006/abio.1996.0292.

Branca, C., Maccarrone, S., Magazù, S., Maisano, G., Bennington, S.M., Taylor, J., 2005. Tetrahedral order in homologous disaccharide-water mixtures. J. Chem. Phys. 17, 174513-6. https://doi:10.1063/1.1887167.

Castro, S.V., Carvalho, A.A., Silva, C.M., Santos, F.W., Campello, C.C., Figueiredo, J.R., Rodrigues, A.P., 2014. Fresh and vitrified bovine preantral follicles have different nutritional requirements during *in vitro* culture. Cell. Tissue Bank 24, 591–601. https://doi:10.1007/s10561-014-9432-2.

Choi, E.M., 2011. Kaempferol protects MC3T3-E1 cells through antioxidant effect and regulation of mitochondrial function. Food Chem. Toxicol. 49, 1800–1805. https://doi:10.1016/j.fct.2011.04.031.

Costa, R.A., Romagna, C.D., Pereira, J.L., Souza-pinto, N.C., 2011. The Role of Mitochondrial DNA Damage in the Citotoxicity of Reactive Oxygen Species. *J.* Bioenerg. Biomembr. 43, 25–29. https://doi:10.1007/s10863-011-9329-8.

Costa, S.H., Santos, R.R., Rondina, D., Andrade, E.R., Ohashi, O.M., Rodrigues, A.P, Figueiredo, J.R. 2010. Effects of IAA in combination with FSH on *in vitro* culture of ovine preantral follicles. Zygote 1, 89–92. https://doi: 10.1017/S0967199409990104.

Cortvrindt R., Smitz, J., 2001. *In vitro* Follicle Growth: Achievements in Mammalian Species. Reprod. Domes. Anim. 36, 3–9.

Eppig, J.J., O'Brien, M.J. 1996. Development *in vitro* of mouse oocytes from primordial follicles. Biol. Reprod. 54, 197–207.

Esmaielzadeh, F., Hosseini, S.M., Hajian, M., Nasiri, Z., Chamani, M., Afshar, A.M., Nasr-Esfahani, M.H. 2013. Role of Follicle Stimulating Hormone in the Survival, Activation and Further Growth of *in vitro* Cultured Sheep Primordial Follicles. Iran. J. Appl. Anim. *Sci.* 3, 785–90.

Ferreira, E.M., Vireque, A.A., Adona, P.R., Meirelles, F.V., Ferriani, R.A., Navarro, P.A., 2009. Cytoplasmic maturation of bovine oocytes: Structural and biochemical modifications and acquisition of developmental competence. Theriogenology 5, 836-848. https://doi:10.1016/j.theriogenology.2008.10.023.

Fortune, J.E., Yang, M.Y., Muruvi, W., 2011. *In vitro* and *in vivo* regulation of follicular formation and activation in cattle. Reprod. Fertil. Dev. 23, 15–22. https://doi: 10.1071/RD10250.

Fujihara, M., Comizzoli, P., Keefer, C.L., Wildt, D.E., Songsasen, N., 2014. Epidermal growth factor (EGF) sustains *in vitro* primordial follicle viability by enhancing stromal cell proliferation via MAPK and PI3K pathways in the prepubertal, but not adult, cat ovary. Biol. Reprod. 4, 86. https://doi:10.1095/biolreprod.113.115089.

Goka, M.S., Telefo, P.B., Mbemya, G.T., Awouafack, M.D., Lienou, L.L., Yemele, D.M., Njina, S.N., Donfack, N.J., Tagne, R.S., Fekam, F.B., 2016. Potentialisation of Pregnant Mare Serum Gonadotropin Inducing Effect on Ovarian Follicles Growth by the Aqueous Extract of *Aloe buettneri, Dicliptera verticillata*, *Hibiscus macranthus* and *Justicia insularis* Leaves in Immature Rats. *Pharmacolologia* 7; 328–336. https://doi:10.5567/pharmacologia.2016.328.336.

Hovatta, O., Silye, R., Abir, R., Krausz, T., Winston, R.M., 1997. Extracellular matrix improves survival of both stored and fresh human primordial and primary ovarian follicles in long-term culture. Hum. Reprod. 5, 1032–1036.

Hovatta, O., Wright, C., Krausz, T., Hardy, K., Winston, R.M. 1999. Human primordial, primary and secondary ovarian follicles in long-term culture: effect of partial isolation. Hum. Reprod. 14, 2519–2524.

Ibrahim, A.H., Barakata, B., Khalilb, W.K.B., Al-Himaidi, A,R. 2015. *Moringa oleifera* extract modulates the expression of fertility related genes and elevation of calcium ions in sheep oocytes. Small. Rumin. Res. 2015;130, 67–75. https://doi.org/10.1016/j.smallrumres.2015.06.011.

Kamalamma, P., Kona, S.S, Praveen, Chakravarthi, V., Siva, Kumar., A.V, Punyakumari, B., and Rao, V.H. 2016. Effect of leptin on *in vitro* development of ovine preantral ovarian follicles. Theriogenology 85, 224–229. https://doi:10.1016/j.theriogenology.2015.09.045.

Leoni, G.G., Palmerini, M.G., Satta, V., Succu, S., Pasciu, V., Zinellu, A., Carru, C., Macchiarelli, G., Nottola, S.A., Naitana, S., Berlinguer, F., 2015. Differences in the Kinetic of the First Meiotic Division and in Active Mitochondrial Distribution between Prepubertal and Adult Oocytes Mirror Differences in their Developmental Competence in a Sheep Model. PLoS One 4. https://doi:10.1371/journal.pone.012491.

Lima, L.F., Rocha, R.M. P., Alves, A.M.C.V., Saraiva, M.V.A., Araújo, V.R., Lima, I.M., Lopes, C. A, Báo, S.N., Campello, C.C., Rodrigues, A.P., Figueiredo, J.R., 2013. Dynamized Follicle-Stimulating Hormone Affects the Development of Ovine Preantral Follicles Cultured *in vitro*. Homeopathy 102, 41–48. https://doi:10.1016/j.homp.2012.11.002.

Lima, L.F., Rocha, R.M., Alves, A.M., Carvalho, A.A., Chaves, R.N., Lopes, C.A., Báo, S.N., Campello, C.C., Rodrigues, A.P., Figueiredo, J.R., 2016. Comparison between the additive effects of diluted (rFSH) and diluted/dynamized (FSH 6 cH) recombinant follicle-stimulating hormone on the *in vitro* culture of ovine preantral follicles enclosed in ovarian tissue. Complement Ther. Med. 25, 39–44.

Lima, L.F., Rocha, R.M., Duarte A.B., Brito, I.R., Silva, G.M., Rodrigues, G.Q., Nunes-Pinheiro, D.C., Sales, A. D., Moura, A.A., Wheeler, M.B., Rodrigues, A.P., Campello, C.C., Figueiredo, J.R., 2017. Unexpected effect of the vehicle (grain ethanol) of homeopathic FSH on the *in vitro* survival and development of isolated ovine preantral follicles. Microsc. Res. Tech. 4, 406–418. https://doi: 10.1002/jemt.22810.

Lins, T.L., Cavalcante, A.Y., Santos, J.M., Menezes, V.G., Barros, V.R., Barberino, R.S., Bezerra, M.É., Macedo, T.J., Matos, M.H., 2017. Rutin can replace the use of three other antioxidants in the culture medium, maintaining the viability of sheep isolated secondary follicles. Theriogenology 89, 263–270. https://doi:10.1016/j.theriogenology.2016.11.019.

Lupi, L., Comez, L., Paolantoni, M., Perticaroli, S., Sassi, P., Morresi, A., Ladanyi, B.M., Fioretto, D., 2012. Hydration and aggregation in mono- and disaccharide aqueous solutions by gigahertz-to-terahertz light scattering and molecular dynamics simulations. J. Phys. Chem. B. 51, 14760–4767. https://doi:10.1021/jp3079869.

Luz, V.B., Araujo, V.R., Duarte, A.B, Celestino, J.J, Silva, T.F., Magalhaes-Padilha, D.M., Chaves, R.N, Brito, I.R, Almeida, A.P, Campello, C.C, Feltrin, C., Bertolini, M., Santos, R.R, Figueiredo, J.R., 2012. Eight-cell parthenotes originated from *in vitro* grown sheep preantral follicles. Reprod. Sci. 19, 1219–1225. https://doi:10.1177/1933719112446072.

Marsella, T., Sena, P., Xella, S., La Marca, A., Giulini, S., De Pol, A., Volpe, A., Marzona, L., 2008. Human ovarian tissue cryopreservation: effect of sucrose concentration on morphological features after thawing. Reprod. Biomed. Online. 16, 257–267.

Martins, F.S., Celestino, J.J., Saraiva, M.V., Chaves, R.N., Rossetto, R., Silva, C.M., Lima-Verde, I.B., Lopes, C.A., Campello, C.C., Figueiredo, J.R., 2010. Interaction between growth differentiation factor 9, insulin-like growth factor I and growth hormone on the *in vitro* development and survival of goat preantral follicles. Braz. J. Med. Biol. Res. 8, 728–736.

Mbemya, G.T., Guerreiro, D.D., Donfack, N.J., Silva, L.M., Vieira, L.A., Sousa, G.F., Alves, B.J., Izaguirry, A.P., Santos, F.W., Telefo, P.B., Pessoa, O.D., Johan, S., Figueiredo, J.R., and Rodrigues, A.P., 2017. *Justicia insularis* Improves the *in vitro* Survival and

Development of Ovine Preantral Follicles Enclosed in Ovarian Tissue. J. Pharm. Pharmacol. 5, 668–680. https://doi:10.17265/2328-2150/2017.09.009.

Mbemya, G.T., Cadenas, J., Sá, R.N.A., Guerreiro, D.D., Jiatsa, N.D., Vieira A.L., Sousa, F.G., Alves, G.B., Lobo, C.H., Santos, F.W., Lima, P.F.D.C., Otília, L.P., Smitz, J., Comizzoli, P., Figueiredo, J.R., Rodrigues, A.P.R., 2018. Supplementation of *in vitro* culture medium with FSH to grow follicles and mature oocytes can be replaced by extracts of *Justicia insularis*. PLoS One. 7, 1–21. https://doi: 10.1371/journal.pone.0208760.

McLaughlin, M., Albertini, D.F., Wallace, W.H., Anderson, R.A., Telfer, E.E., 2018. Metaphase II oocytes from human unilaminar follicles grown in a multistep culture system. Mol. Hum. Reprod. 3, 135–142. https://doi:10.1093/molehr/gay002.Molecular

McLaughlin, M., Telfer, E. E., 2010. Oocyte development in bovine primordial follicles is promoted by activin and FSH within a two-step serum-free culture system. Reproduction 139, 971–978. https://doi: 10.1530/REP-10-0025.

McLaughlin, M., Bromfield, J.J., Albertini, D.F., Telfer, E.E., 2010. Activin promotes follicular integrity and oogenesis in cultured pre-antral bovine follicles. Mol. Hum. Reprod. 16, 644–653. https://doi:10.1093/molehr/gaq021.

Mery, L., Lefevre, A., Benchaib, M., Demirci, B., Salle, B., Guerin, J.F., and Lornage, J., 2007. Follicular Growth *in vitro*: Detection of Growth Differentiation Factor 9 (GDF9) and Bone Morphogenetic Protein 15 (BMP15) during *in vitro* Culture of Ovine Cortical Slices. Mol. Reprod. Dev. 74, 767–74. https://doi:10.1002/mrd.20661.

Nascimento, T.S., Silva, I.S., Alves, M.C., Gouveia, B.B., Barbosa, L.M., Macedo, T.J., Santos, J.M., Monte, A.P., Matos, M.H., Padilha, F.F., Lima-Verde, I.B., 2018. Effect of red propolis extract isolated or encapsulated in nanoparticles on the *in vitro* culture of sheep preantral follicle: impacts on antrum formation, mitochondrial activity and glutathione levels. Reprod. Domest. Anim. https://doi:10.1111/rda.13347.

O'Brien, M.J., Pendola, J.K., Eppig J.J., 2003. A revised protocol for *in vitro* development of mouse oocytes from primordial follicles dramatically improves their developmental competence. Biol. Reprod. 68, 1682–1686. https://doi:10.1095/biolreprod.102.013029.

Oktay, K., Briggs, D., and Gosden, R.G. 1997. Ontogeny of follicle-stimulating hormone receptor gene expression in isolated human ovarian follicles. J. Clin. Endocrinol. Metab. 82, 3748–3751. https://doi:10.1210/jcem.82.11.4346.

Paolantoni, M., Comez, L., Gallina, M.E, Sassi, P., Scarponi, F., Fioretto, D., Morresi, A., 2009. Light scattering spectra of water in trehalose aqueous solutions: evidence for two different

solvent relaxation processes. J. Phys. Chem. B. 22, 7874–7878. https://doi:10.1021/jp9004983.

Paz, M.P., Sousa, F.G., Alves, B.G., Lobo, C.H., Sales, A.D, Tavares, K.C., Sá, N.A., Guerreiro, D.D., Maside, C., Rocha, R.M., Bertolini, L. R., Bordignon, V., Figueiredo, J.R., Rodrigues, A.P., 2018. Effect of aquaporin 3 knockdown by RNA interference on antrum formation in sheep secondary follicles cultured *in vitro*. Zygote 5, 1–9. https://doi: 10.1017/S096719941800031X.

Rodrigues, G.Q., Silva, C.M., Faustino, L.R., Bruno, J.B., Magalhães, D.M., Campello, C. C., Figueiredo, J.R., 2010. Bovine serum albumin improves *in vitro* development of caprine preantral follicles. Anim. Reprod. 7, 382–388.

Rosairo, D., Kuyznierewicz, I., Findlay, J., Drummond, A., 2008. Transforming growth factor-beta: its role in ovarian follicle development. Reprod. 136, 799–809. https://doi:10.1530/REP-08-0310.

Sá, N.A., Araújo, V.R., Correia, H.H., Ferreira, A.C., Guerreiro, D.D., Sampaio, A.M., Escobar, E., Santos, F.W., Moura, A.A., Lôbo, C.H., Ceccatto, V.M., Campello, C.C., Rodrigues, A.P., Leal-Cardoso, J.H., Figueiredo, J.R., 2016. Anethole Improves the *in vitro* Development of Isolated Caprine Secondary Follicles. Theriogenology 89, 226–234. doi:10.1016/j.theriogenology.2015.12.014.

Sanfilippo, S., Canis, M., Smitz, J., Sion, B., Darcha, C., Janny, L., Brugnon, F., 2015. Vitrification of human ovarian tissue: a practical and relevant alternative to slow freezing. Reprod. Biol. Endocrinol. 25, 2–7. https://doi:10.1186/s12958-015-0065-5.

Shea, L.D., Woodruff, T.K., Shikanov, A., 2014. Bioengineering the ovarian follicle microenvironment. Annu. Rev. Biomed. Eng. 16, 29–52. https://doi:10.1146/annurev-bioeng-071813-105131.

Silva, J.R., Hurk, V.D., Matos, M.H., Santos, R.R., Pessoa, C., Moraes, M.O., Figueiredo, J.R., 2004. Influences of FSH and EGF on Primordial Follicles during *in vitro* Culture of Caprine Ovarian Cortical Tissue. Theriogenology 61, 1691–704. https://doi:10.1016/j.theriogenology.2003.09.014.

Telefo, P.B., Tagne, S.R., Koona, O.E., Yemele, D.M., Tchouanguep, F.M., 2012). Effect of the Aqueous Extract of *Justicia Insularis* T. Anders (Acanthaceae) on Ovarian Folliculogenesis and Fertility of Female Rats. Afr. J. Tradit. Complement. Altern. Med. 9, 197–203.

Telfer, E.E., McLaughlin, M., Ding, C., and Thong, K.J., 2008. A two-step serum-free culture system supports development of human oocytes from primordial follicles in the presence of activin. Hum. Reprod. 23, 1151–1158. https://doi:10.1093/humrep/den070.

Tian, T., Zhao, G., Han, D., Zhu, K., Chen, D., Zhang, Z., Wei, Z., Cao, Y., Zhou, P., 2015. Effects of vitrification cryopreservation on follicular morphology and stress relaxation behaviors of human ovarian tissues: sucrose versus trehalose as the non-permeable protective agent. Hum. Reprod. 30, 877–883. https://doi:10.1093/humrep/dev012.

Wandji, S.A, Srsen, V., Voss, A. K., Eppig, J.J., Fortune, J.E. 1996. Initiation *in vitro* of growth of bovine primordial follicles. Biol. Reprod. 5, 942–948.

Wright, C.S., Hovatta, O., Margara, R., Trew, G., Winston, R.M., Franks, S., Hardy, K.,1999. Effects of Follicle-Stimulating Hormone and Serum Substitution on the *in vitro* Growth of Human Ovarian Follicles. Hum. Reprod. 14, 1555–1562.

Xiao, S., Zhang, J., Romero, M.M., Smith, K.N., Shea, L.D., and Woodruff, T.K. 2015. *In vitro* follicle growth supports human oocyte meiotic maturation. Sci. Rep. 5, 17323. https://doi:10.1038/srep17323.

Xu, M., Barrett, S.L., West-Farrell, E., Kondapalli, L.A., Kiesewetter, S.E., Shea, L.D., Woodruff, T.K., 2009. *In vitro* grown human ovarian follicles from cancer patients support oocyte growth. Hum. Reprod. 24, 2531–2540. https://doi:10.1093/humrep/dep228.

Zhang, X.G, Wang, Y.H, Han, C., Hu, S., Wang, L.Q., and Hu, J.H., 2015. Effects of trehalose supplementation on cell viability and oxidative stress variables in frozen-thawed bovine calf testicular tissue. Cryobiology 3, 246–252. https://doi:10.1016/j.cryobiol.2015.03.004.

10 CONCLUSÕES

Resumidamente, de acordo com os resultados encontrados nesse estudo nós vimos que;

- A *Justicia insularis* exerceu um efeito positivo sobre a sobrevivência e a ativação folicular, bem como manteve os níveis de ROS no tecido ovariano cultivado *in vitro*;
- A concentração de 0,3 mg/mL do extrato aquoso de *Justicia insularis* adicionado ao meio de cultivo *in vitro* de folículos secundários ou ao meio de maturação dos oócitos obtidos desses folículos, mostrou um efeito similar ao FSH;
- Nós demonstramos pela primeira vez que folículos secundários podem ser isolados com sucesso do córtex ovariano após 7 dias de cultivo e são capazes de sobreviver e formar antro se cultivados *in vitro* por 6 dias adicionais na presença de 0,3 mg/mL *J. insularis*.

Portanto, conclui-se que o extrato aquoso de JI pode ser utilizado como um suplemento alternativo ao FSH, no meio de cultivo *in vitro* de folículos pré-antrais ovinos, seja em um sistema simples ou em um sistema de dois passos.

11 PERSPECTIVAS

Plantas medicinais como a *Justicia insularis* possuem um vasto potencial farmacológico ainda a ser explorado, principalmente no que diz respeito à saúde reprodutiva feminina. Embora os mecanismos de interação não sejam claros, os resultados obtidos em nossos estudos abrem um vasto campo de pesquisa para elucidar o papel de extratos vegetais no desenvolvimento dos folículos ovarianos e gera novas possibilidades para o uso de plantas medicinais em experimentos mais complexos que visam o desenvolvimento completo de folículos pré-antrais *in vitro*.

Nossos resultados mostraram que o extrato aquoso de *Justicia insularis* parece exercer um efeito semelhante ao FSH e também reduz a produção de ROS. Entretanto, ainda não se sabe como os compostos metabólicos presentes neste extrato são responsáveis por estas propriedades químicas que culminam com resultados positivos sobre a foliculogênese *in vitro*. Portanto, novos estudos são necessários no intuito de isolar os metabólitos presentes na *Justicia insularis* capazes de exercer esses efeitos.

Além disso, são necessárias outras tentativas tão desafiadoras quanto esta para obter resultados mais consistentes, por exemplo, melhorar as condições de cultivo in vitro do córtex ovariano visando aumentar o número de folículos secundários obtidos. Assim, certos que usando esta estratégia, folículos secundários isolados podem resistir até 6 dias em cultivo *in vitro*, podemos estender este período por mais 6 ou 12 dias para obter um número maior de oócitos viáveis para maturação *in vitro*. Essa estratégia pode ser uma alterantiva para obter embriões produzidos *in vitro* a partir de animais geneticamente valiosos, que não podem se reproduzir naturalmente ou após a morte. Além disso, os dados deste estudo também podem reforçar as idéias de alguns pesquisadores que afirmaram que a estratégia de cultivo de múltiplos passos de folículos pré-antrais pode ser, no futuro, uma alternativa ao transplante de ovário para preservar a fertilidade de mulheres jovens em tratamento de câncer.

Por fim, apesar da grande expectativa gerada com os resultados obtidos no cultivo de dois passos, nesta tese, os grandes desafios agora são limitar as perdas de folículos secundários após o primeiro passo e aumentar o tempo de cultivo de folículos secundários isolados do tecido ovariano cultivado. Neste caso, acredita-se que esses folículos poderiam manter melhor a morfologia, bem como o potencial de desenvolvimento folicular e oocitário.

REFERÊNCIAS

AALTONEN, J.; LAITINEN, M. P.; VUOJOLAINEN, K.; JAATINEN, R.; HORELLI-KUITUNEN, N.; SEPPÄ, L.; LOUHIO, H.; TUURI, T.; SJÖBERG, J.; BÜTZOW, R.; HOVATA, O.; DALE, L.; RITVOS, O. Human growth differentiation factor 9 (GDF- 9) and its novel homolog GDF-9B are expressed in oocytes during early folliculogenesis. **Journal of Clinical Endocrinology and Metabolism**, v. 84, n. 8, p. 2744–2750, 1999.

ABEDI, A.; ROUHI, L.; PIRBALOUTI, A. G. Effect of *Gundelia tournefortii* leaves extract on in immature mouse oocytes. **Journal of Herbal Drugs**, v. 5, n. 1, p. 84–89, 2014.

ABIR, R.; NITKE, S.; BEN-HAROUSH, A.; FISCH, B. *In vitro* maturation of human primordial ovarian follicles: Clinical significance, progress in mammals, and methods for growth evaluation. **Histology and Histopathology**, v. 21, n. 8, p. 887–898, 2006.

ABIR, R.; ROIZMAN, P.; FISCH, B.; NITKE, S.; OKON, E.; ORVIETO, R.; BEN RAFAEL, Z. Pilot study of isolated early human follicles cultured in collagen gels for 24 hours. **Human Reproduction**, v. 14, n. 5, p. 1299–1301, 1999.

ADJANOHOUN, J. E.; ABOUBAKAR, N.; DRAMANE, K.; EBOT, M. E.; EKPERE, J. A.; ENOW-OROCK, E. G. Traditional medicine andpharmacopoeia. In: Contribution to ethnobotanical floristic studies in Cameroon. **Porto-Novo Benin**; p. 5–8, 1996.

AGARWAL, A.; DURAIRAJANAYAGAM, D.; DU PLESSIS, S. S. Utility of antioxidants during assisted reproductive techniques: an evidence based review. **Reproductive Biology and Endocrinology**, v. 12, p. 1–13, 2014.

AGUIAR, F. L.; LUNARDI, F. O.; LIMA, L. F.; ROCHA, R. M.; BRUNO, J. B.; MAGALHÃES-PADILHA, D. M.; CIBIN, F. W.; NUNES-PINHEIRO, D. C.; GASTAL, M. O.; RODRIGUES, A. P.; APGAR, G. A.; GASTAL, E. L.; FIGUEIREDO, J. R. FSH supplementation to culture medium is beneficial for activation and survival of preantral follicles enclosed in equine ovarian tissue. **Theriogenology**, v. 85, n. 6, p. 1106–12, 2016.

AJIBESIN, K. K., EKPO, B. A.; BALA, D. N.; ESSIEN, E. E.; ADESANYA, S. A. Ethnobotanical survey of Akwa Ibom State of Nigeria. **Journal of Ethnopharmacology**, v. 115, p. 387–408, 2008.

AKOKA G, AKOKA A. Medicine. **Medical Encyclopedia**, v. 3, n. 692, 1972 p.

ALVES, A. M. C. V.; CHAVES, R. N.; ROCHA, R. M. P.; LIMA, L. F., ANDRADE, P. M.; LOPES, C. A. P.; SOUZA, C. E.; MOURA, A. A.; CAMPELLO, C. C.; BÁO, S. N.; SMITZ, J.; FIGUEIREDO, J. R. Dynamic medium containing growth differentiation factor-9 and FSH maintains survival and promotes *in vitro* growth of caprine preantral follicles after long-term *in vitro* culture. **Reproductive Fertility and. Development**, n. 25, v. 6, p. 955–965, 2013.

APOLLONI, L. B.; BRUNO, J. B.; ALVES, B. G.; FERREIRA, A. C. A.; PAES, V. M.; CADENAS, J.; DE AGUIAR, F. L. N.; BRANDÃO, F. Z.; SMITZ, J.; APGAR, G.; FIGUEIREDO, J. R. Accelerated follicle growth during the culture of isolated caprine preantral follicles is detrimental to follicular survival and oocyte meiotic resumption. **Theriogenology**, v. 86, n. 6, p. 1530–1540, 2015.

ARMSTRON, D. G.; BAXTER, G.; GUTIERREZ, C. G.; HOGG, C. O.; GLAZYRIN, A. L.; CAMPBELL B. K.; BRAMLEY, T. A.; WEBB, R. Insulin-like growth factor binding protein-2 and -4 messenger ribonucleic acid expression in bovine ovarian follicles: effect of gonadotropins and developmental status. **Endocrinology**, v. 139, n. 4, p. 2146–2154, 1998.

ARUNAKUMARI, G.; SHANMUGASUNDARAM, N.; RAO, V. H. Development of morulae from the oocytes of cultured sheep preantral follicles. **Theriogenology**, v. 74, n. 5, p. 884–894, 2010.

BARBERINO, R. S.; BARROS, V. R.; MENEZES, V. G.; SANTOS, L. P.; ARAÚJO, V. R.; QUEIROZ, M. A.; ALMEIDA, J. R.; PALHETA, R. C.; MATOS, M. H. *Amburana cearensis* leaf extract maintains survival and promotes *in vitro* development of ovine secondary follicles, **Zygote,** v. 24. n. 2, p. 277–285, 2015.

BARROS, V. R.; CAVALCANTE, A. Y.; MACEDO, T. J.; BARBERINO, R. S.; LINS, T. L.; GOUVEIA, B. B.; MENEZES, V. G.; QUEIROZ, M. A.; ARAÚJO, V. R.; PALHETA, R. C. JR.; LEITE, M. C.; MATOS, M. H. Immunolocalization of melatonin and follicle-

stimulating hormone receptors in caprine ovaries and their effects during in vitro development of isolated pre-antral follicles. **Reproduction in Domestic Animals**, v. 48, n. 6, p. 1025–1033, 2013.

BAUER, A. K.; DWYER-NIELD, L. D.; KEIL, K.; KOSKI, K.; MALKINSON, A. M. Butylated hydroxytoluene (BHT) induction of pulmonary inflamation: A role in tumor promotion. **Experimental Lung Research,** v. 7, n. 3, p. 197–216, 2001.

BECKERS, J.; DRION, P.; FIGUEIREDO, J. The ovarian follicle in cow: in vivo growth and *in vitro* culture. **Reproduction in Domestic Animals**, v. 31, p. 543–548, 1996.

BERHAUT, H. J. Flore Illustrée du Sénégal: Acanthacées à Avicienniacées, **Maisonneuve, Dakar,** v. 1, p. 57–61, 1971.

BERTOLDO, M. J.; DUFFARD, N.; BERNARD, J.; FRAPSAUCE, C.; CALAIS, L.; RICO, C.; MERMILLOD, P.; LOCATELLI, Y. Effects of bone morphogenetic protein 4 (BMP4) supplementation during culture of the sheep ovarian cortex. **Animal Reproduction Science**, v. 149, n. (3-4), p. 124–134, 2018.

BODENSTEINER, K. J.; CLAY, C. M.; MOELLER, C. L.; SAWYER, H. R. Molecular cloning of the ovine growth/differentiation factor-9 gene and expression of growth/differentiation factor-9 in ovine and bovine ovaries. **Biology of Reproduction**, v. 60, n. 2, p. 381–386. 1999.

BRISTOL-GOULD, S.; WOODRUFF, T. K. Folliculogenesis in the domestic cat (*Felis catus*). **Theriogenology**, v. 66, n. 1, p. 5–13, 2006.

BRITO, I. R.; SILVA, C. M. G.; DUARTE, A. B. G.; LIMA, I. M. T.; RODRIGUES, G. Q.; ROSSETTO, R.; SALES, A. D.; LOBO, C. H.; BERNUCI, M. P.; ROSA-E-SILVA, A. C. J. S.; CAMPELLO, C. C.; XU, M.; FIGUEIREDO, J. R. Alginate hydrogel matrix stiffness influences the in vitro development of caprine preantral follicles. Molecular **Reproduction and Development**, v. 81, n. 7, p. 636–645, 2014.

CAI, Y. Z., SUN, M., CORKE, H. Antioxidant activity of betalains from plants of the Amaranthaceae. **Journal of Agricultural and Food Chemistry**, v. 51, n. 8, p. 2288–2294, 2003.

CARABATSOS, M.; SELLITTO, C.; GOODENOUGH, D.; ALBERTINI, D. Oocyte-granulosa cell heterologous gap junctions are required for the coordination of nuclear and cytoplasmic meiotic competence. **Developmental Biology**, v. 226, n. 2, p. 167–179, 2000.

CARROLL, J.; WHITTINGHAM, D.; WOOD, M. J. Effect of dibutyryl cyclic adenosine monophosphate on granulosa cell proliferation , oocyte growth and meiotic maturation in isolated mouse primary ovarian follicles cultured in collagen gels. **Journal of Reproduction and Fertility**, v. 92, n. 1, p. 197–207, 1991.

CECCONI, S.; BARBONI, COCCIA, M.; MATTIOLI, M. In vitro development of sheep preantral follicles. **Biology of Reproduction**, v.60, n. 3, p. 594–601, 1999.

CELESTINO, J. J.; BRUNO, J. B.; SARAIVA, M. V.; ROCHA, R. M.; BRITO, I. R.; DUARTE, A. B.; ARAÚJO, V. R.; SILVA, C. M.; MATOS, M. H.; CAMPELLO, CC, SILVA JR, FIGUEIREDO JR. Steady-state level of epidermal growth factor (EGF) mRNA and effect of EGF on in vitro culture of caprine preantral follicles. **Cell Tissue Research**, v. 344, n. 3, p.539–550, 2011.

CELESTINO, J. J. H.; BRUNO, J. B.; LIMA-VERDE, I. B.; MATOS, M. H. T.; SARAIVA, M. V. A.; CHAVES, R. N.; MARTINS, F. S.; LIMA, L. F.; NAME, K. P. O.; CAMPELLO, C. C.; SILVA, J. R. V.; BÁO, S. N.; FIGUEIREDO, J. R. Recombinant epidermal growth factor maintains follicular ultrastructure and promotes the transition to primary follicles in caprine ovarian tissue cultured *in vitro*. **Reproductive Science**, [S.l.], v. 16, n. 3, p. 239–246, 2009.

CLARK, E. J. A.; WANG, A. T. P.; WOLFMAN, N. M.; MATZUK, M. M. Paracrine actions of growth differentiation factor-9 in the mammalian ovary. **Molecular Endocrinology**, v. 13, n. 6, 1035–1048. 1999.

CORRÊA, G. M.; ALCÂNTARA, A. F. C. Chemical constituents and biological activities of species of *Justicia* - a review. **Brazilian Journal of Pharmacognosy**, v. 22, n. 1, p. 220–238, 2012.

CORTVRINDT, R., SMITZ, J. *In vitro* follicle growth: Achievements in mammalian species. **Reproduction Domestic Animal**, v. 36, n. 1, p. 3–9, 2001.

CORTVRINDT, R.; SMITZ, J.; VAN STEIRTEGHEM, A. C. *In vitro* maturation, fertilization and embryo development of immature oocytes from early preantral follicles from prepuberal mice in a simplified culture system. **Human Reproduction**, v. 11 n. 12 p. 2656–2666, 1996.

DEMEESTERE, I.; CENTNER, J.; GERVY, C.; ENGLERT, Y.; DELBAERE, A. Impact of various endocrine and paracrine factors on *in vitro* culture of preantral follicles in rodents. **Reproduction (Cambridge, England),** v. 130, n. 2, p. 147–156, 2005.

DONEDA, L.; KLINGER, F. G.; LARIZZA, L.; DE FELICI, M. KL/KIT co-expression in mouse fetal oocytes. International **Journal of Developmental Biology**, v. 46, n. 8, p. 1015–1021, 2002.

DRIANCOUR, T. M. A. Follicular dynamics in sheep and cattle. **Theriogenology**, v.35, p.55–68, 1991.

EDSON, M. A.; NAGARAJA, A. K.; MATZUK, M. M. The mammalian ovary from genesis to revelation. **Endocrine Reviews**, v. 30, n. 6, p. 624–712, 2009.

ELVIN, J. A.; CLARK, A. T.; WANG, P.; WOLFMAN, N. M.; MATZUK, M. M. Paracrine actions of growth differentiation factor-9 in the mammalian ovary. **Molecular Endocrinology**, v. 13, n. 6, p. 1035–1048, 1999.

EPPIG, J. J.; O'BRIEN, M. J. Development *in vitro* of mouse oocytes from primordial follicles. **Biology of Reproduction,** v. 54, n. 1, p. 197–207, 1996.

EPPIG, J. J.; SCHROEDER, A. C. Capacity of mouse oocytes from preantral follicles to undergo embryogenesis and development to live young after growth, maturation, and fertilization *in vitro*. **Biology of Reproduction**, v. 41, n. 2, p. 268–276, 1989.

EPPIG, J.; PENDOLA, F.; WIIGGLESWORTH, K.; PENDOLA, J. Mouse oocytes regulate metabolic cooperativity between granulosa cells and oocytes: amino acid transport. **Biology of Reproduction**, v. 73, n. 2, p. 351–357, 2005.

ESMAIELZADEH, F.; HOSSEINI, S. M.; HAJIAN, M.; NASIRI, Z.; CHAMANI, AMIN AFSHAR, M.; NASR-ESFAHANI, M. H. Role of follicle stimulating hormone in the survival, activation and further growth of *in vitro* cultured sheep primordial follicles. **Iranian Journal of Applied Animal Science.** v. 3, 785–90, 2013.

FARNWORTH, P. G. Gonadotrophin secretion revised. How many ways can FSH leave a gonadotroph? **Journal of Endocrinology**, v.145, n. 3, p.387–395, 1995.

FIGUEIREDO, J. R.; RODRIGUES, A. P. R.; AMORIM, C. A.; SILVA, J. R. V. Manipulação de oócitos inclusos em folículos pré-antrais – MOIFOPA. In: GONÇALVES, P. B. D.; FIGUEIREDO, J. R.; FREITAS, V. J. F. **Biotécnicas Aplicadas à Reprodução Animal**. São Paulo: Roca, p. 303–327, 2008.

FILATOV, M.; KHRAMOVA, Y.; PARSHINA, E.; BAGAEVA, T.; SEMENOVA, M. Influence of gonadotropins on ovarian follicle growth and development *in vivo* and *in vitro*. **Zygote**, v. 25, n. 3, p. 235–243, 2017.

FOREST, M. G.; BERTRAND, J. Puberté féminine. In: Mauvais-Jarvis P, Sitruck-Ware R. Médecine de la reproduction. Gynécologie endocrinienne. Paris, **Flammarion Médecine-Sciences**: p. 185–218, 1986.

FORTUNE, J. E. The early stages of follicular development, p. activation of primordial follicles and growth of preantral follicles. **Animal Reproduction Science**, v.78, n. 3–4, p.135-163, 2003.

GEORGES, A.; AUGUSTE, A.; BESSIÈRE, L.; VANET, A.; TODESCHINI, A. L.; VEITIA, R. A. FOXL2: a central transcription factor of the ovary. **Journal of Molecular Endocrinology**, v. 52, n. 1, p. 17–33, 2014.

GOKA, M. S. C.; AWOUAFACK, M. D.; LAMSHÖFT, M.; LIENOU, L. L.; MBEMYA, G. T.; FEKAM F. B.; TANE, P.; TELEFO, P. B. Comparative effect of the aqueous extracts of *Aloe buettneri, Dicliptera verticillata, Hibiscus macranthus* and *Justicia insularis* on the sexual maturation of pregnant mare serum gonadotrophin-primed immature female rats. **Journal of Basic and Clinical Physiology and Pharmacology**, v. 29, n. 5, p. 473–481, 2018.

GOKA, M. S. C.; TELEFO, P. B.; MBEMYA, G. T.; AWOUAFACK, M. D, LIENOU, L. L.; YEMELE, D. M.; NJINA, N. S.; NJIATSA, D. N.; TAGNE, R. S.; FEKAM, F. B.. Potentialisation of Pregnant Mare Serum Gonadotropin Inducing Effect on Ovarian Follicles Growth by the Aqueous Extract of Aloe buettneri, Dicliptera verticillata, Hibiscus macranthus and *Justicia insularis* Leaves in Immature Rats. **Pharmacolologia,** v. 7, n. 6–7, p.328–336, 2016.

GOUVEIA, B. B.; MACEDO, T. J.; SANTOS, J. M.; BARBERINO, R. S.; MENEZES, V. G.; MÜLLER, M. C.; ALMEIDA, J. R.; FIGUEIREDO, J. R.; MATOS, M. H. Supplemented base medium containing *Amburana cearensis* associated with FSH improves *in vitro* development of isolated goat preantral follicles. Theriogenology, v. 86, n. 5, p. 1275–84, 2016.

GOUVEIA, B. B.; BARROS, V. R. P.; GONÇALVES, R. J. S.; BARBERINO, R. S.; MENEZES, V. G.; LINS, T. L. B.; MACEDO, T. J. S.; SANTOS, J. M. S.; ROLIM, L. A.; NETO, R. P. J.; ALMEIDA, J. R. G. S.; MATOS, M. H. T. Effect of ovarian tissue transportation in *Amburana cearensis* extract on the morphology and apoptosis of goat preantral follicles. **Animal Reproduction, Belo Horizonte**, v.12, n.2, p.316–323, 2015.

GUEDES, J. S.; RODRIGUES, J. K.; CAMPOS, A. L. M.; MORAES, C. C.; CAETANO, J. P. J.; MARINHO, R. M. Follicle Viability after Vitrification of Bovine Ovarian Tissue. **Revista Brasileira de Ginecologia e Obstetrícia**, v. 39, n. 11, p. 614–621, 2017.

GUPTA, P. S.; RAMESH, H. S.; MANJUNATHA, B. M.; NANDI, S.; RAVINDRA, J. P. Production of buffalo embryos using oocytes from *in vitro* grown preantral follicles. **Zygote**, v.16, n. 1, p.57–63, 2008.

GURIB-FAKIM, A. Medicinal plants: Traditions of yesterdayand drugs of tomorrow. **Molecular Aspects of medicine**, v. 27, n. 1, p. 1–93, 2006.

GUTIERREZ, C. G.; RALPH, J. H.; TELFER, E. E.; WILMUT, I.; WEBB, R. Growth and antrum formation of bovine preantral follicles in long-term culture *in vitro*. **Biology of Reproduction**, v.62, n. 5, p. 1322–28, 2000.

HARBORNE, J. B. Classes and functions of secondary products. In: Walton NJ, Brown DE, editors. Chemicals from plants, perspectives on secondary plant products. **Imperial College Press**, p. 1–25.53, 1999.

HAUACHE, O. M. Receptores Acoplados à Proteína G: Implicações para a Fisiologia e Doenças Endócrinas. **Arquivos Brasileiros de Endocrinologia & Metabologia**, v. 45, n. 3, p. 228–239, 2001.

HIRAO, Y.; NAGAI, T.; KUBO, M.; MIYANO, T.; MIYAKE, M.; KATO, S. *In vitro* growth and maturation of pig oocytes. **Journal of Reproduction and Fertility**, v. 100, n. 2, p. 333–339, 1994.

HIRSHFIELD, A. N. Development of follicles in the mammalian ovary. **International Review of Cytology**, v. 124, p. 43–101. 1991.

HOYER, P. E.; BYSKOV, A. G.; MOLLGARD, K. Stem cell factor and c-Kit in human primordial germ cells and fetal ovaries. **Molecular and Cellular Endocrinology**, v. 234, n. (1-2), p.1–10, 2005.

HREINSSON, J. G.; SCOTT, J. E.; RASMUSSEN, C.; SWAHN, M. L.; HSUEH, A. J. W.; HOVATTA, O. Growth differentiation factor-9 promotes the growth, development, and survival of human ovarian follicles in organ culture. **Journal of Clinical Endocrinology and Metabolism**, v. 87, n. 1, p. 316–321, 2002.

HTTP://FACULTY.SOUTHWEST.TN.EDU/RBURKETT/A&P2 REPRODUCTIVE SYSTEM LAB.HTM

HUNZICKER-DUNN, M.; MAIZELS, E. T. FSH signaling pathways in immature granulosa cells that regulate target gene expression: branching out from protein kinase A. **Cell Signal**, v. 18, n. 9, p. 1351–1359, 2006.

HUSSEIN, T. S.; FROILAND, D. A.; AMATO, F.; THOMPSON, J. G.; GILCHRIST, R. B. Oocytes prevent cumulus cell apoptosis by maintaining a morphogenic paracrine gradient of bone morphogenetic proteins. **Journal of Cell Science**, v. 118, n. 22, p. 5257–5268, 2005.

HUTT, K. J.; ALBERTINI, D. F. An oocentric view of folliculogenesis and embryogenesis. **Reproductive Biomedicine Online**, v. 14, n. 6, p. 758–642, 2007.

ISMAIL, R. S.; DUBE, M.; VANDERHYDEN, B. C. Hormonally regulated expression and alternative splicing of kit ligand may regulate kit-induced inhibition of meiosis in rat oocytes. **Developmental Biology**, v.184, n. 2, p. 333–342, 1997.

ITOH, T.; KACCHI, M.; ABE, H.; SENDAI, Y.; HOSHI, H. Growth, antrum formation, and estradiol production of bovine preantral follicles cultured in a serum-free medium. **Biology of reproduction**, v. 67, n. 4, p.1099–1105, 2002.

JAATINEN, R.; LAITINEN, M. P.; VUOJOLAINEN, K.; AALTONEN, J.; LOUHIO, H.; HEIKINHEIMO, K.; LEHTONEN, E.; RITVOS, O. Localization of growth differentiation factor-9 (GDF-9) mRNA and protein in rat ovaries and cDNA cloning of rat GDF-9 and its novel homolog GDF-9B. **Molecular and Cellular Endocrinology**, 156, n. (1-2), p. 189–193, 1999.

JIMENEZ, C. R.; DE AZEVEDO, J. L.; SILVEIRA, R. G.; PENITENTE-FILHO, J.; CARRASCAL-TRIANA, E. L.; ZOLINI, A. M.; ARAÚJO, V. R.; TORRES, C. Effects of Growth Hormone on *In Situ* Culture of Bovine Preantral Follicles are Dose Dependent. **Reproduction in Domestic Animals**, v. 51, n. 4, p. 575–84, 2016.

JIN, X.; HAN, C. S.; YU, F. Q.; WEI, P.; HU, Z. Y.; LIU, Y. X. Anti-apoptotic action of stem cell factor on oocytes in primordial follicles and its signal transduction. **Molecular Reproduction and Development**, v.70, n. 1, p.82–90, 2005.

KIM, I. W.; GONG, S. P.; YOO, C. R.; CHOI, J. H.; KIM, D. Y.; LIM, J. M. Derivation of developmentally competent oocytes by the culture of preantral follicles retrieved from adult ovaries: maturation, blastocyst formation, and embryonic stem cell transformation. **Fertility and Sterility,** v. 92, n. 5, 1716–1724, 2009.

KNOBIL E, NEILL JD. **Physiology of Reproduction**. 4th ed: Academic Press, 2684 p. April 2015.

KURZER, M. S.; XU, X. Dietary phytoestrogens. **Annual Review of Nutrition**, v. 17, p. 353–381, 1997.

LEIVA-REVILLA, J.; CADENAS, J.; VIEIRA, L. A.; CAMPELLO, C. C.; CELESTINO, J. J. H. OTÍLIA D. L. P.; APGAR, G. A.; RODRIGUES, A. P. R.; FIGUEIREDO, J. R.; MASIDE, C. Toxicity effect of *Auxemma oncocalyx* fraction and its active principle *oncocalyxone A* on *in vitro* culture of caprine secondary follicles and *in vitro* oocyte maturation. **Semina: Ciências Agrárias**, v. 38, n. 3, p. 1361–1373, 2017.

LEIVA-REVILLA, J; LIMA, L. F.; CASTRO, S. V.; CAMPELLO, C. C.; ARAÚJO, V. R.; CELESTINO, J. J.; PESSOA, O. D, SILVEIRA. E. R.; RODRIGUES, A. P.; FIGUEIREDO, J. R. Fraction of *Auxemma oncocalyx* and *Oncocalyxone A* Affects the *In Vitro* Survival and Development of Caprine Preantral Follicles Enclosed in Ovarian Cortical Tissue. **Forsch Komplementmed**, v. 23, n. 5, p. 307–313, 2016.

LIU, J.; VAN DER ELST, J.; VAN DEN BROECK, R.; DHONT, M.; Live offspring by *in vitro* oocytes from cryopreserved primordial mouse follicles after sequential *in vivo* transplantation and *in vitro* maturation. **Biology of Reproduction**, [S.l.], v. 64, n.1, p.171–178, 2001.

LUZ, V. B.; ARAUJO, V. R.; DUARTE, A. B. G.; CELESTINO, J. J. H.; SILVA, T. F. P.; MAGALHAES-PADILHA, D. M.; CHAVES, R. N.; BRITO, I. R.; ALMEIDA, A. P.; CAMPELLO, C. C.; FELTRIN, C.; BERTOLINI, M.; SANTOS, R. R.; FIGUEIREDO, J. R. Eight-cell parthenotes originated from *in vitro* grown sheep preantral follicles. **Reproductive Sciences**, v. 19, n. 11, p. 1219–1225, 2012.

MAGALHÃES, D. M.; DUARTE, A. B. G.; ARAÚJO, V. R.; BRITO, I. R.; SOARES, T. G.; LIMA, I. M. T.; LOPES, C. A. P.; CAMPELLO, C. C.; RODRIGUES, A. P. R.; FIGUEIREDO, J. R. *In vitro* production of a caprine embryo from a preantral follicle cultured in media supplemented with growth hormone. **Theriogenology**, v. 75, p. 182–188, 2011.

MAO, J.; WU, G.; SMITH, M.F.; MCCAULEY, T.C.; CANTLEY, T.C.; PRATHER, R.S.; DIDION, B.A.; DAY, B.N. Effects of culture medium, serum type, and various concentrations of follicle stimulating hormone on porcine preantral follicle development and antrum formation *in vitro*. **Biology of Reproduction**, v. 67, n. 4, p. 1197–1203, 2002.

MARTINS, F. S.; MATOS, M. H. T.; CELESTINO, J. J. H.; FIGUEIREDO, J. R. Effect of cryopreservation, activation and growth of *in situ* and isolated ovine early-stage follicles. **Animal Reproduction Science**, v.99, n. (1-2), p.53–64, 2007.

MCGEE, E. A.; HSUEH, A. J.; Initial and cyclic recruitment of ovarian follicles. **Endocrine Reviews**, v. 21, n. 2, p. 200–214, 2000.

MCGEE, E. A.; PERLAS, E.; LAPOLT, P. S.; TSAFRIRI, A.; HSUEH, A. J. Follicle-stimulating hormone enhances the development of preantral follicles in juvenile rats. **Biology of Reproduction**, v. 57, n. 5, p. 990–998, 1997.

MCLAUGHLIN, M.; ALBERTINI, D. F.; WALLACE, W. H. B.; ANDERSON, R. A.; TELFER, E. E. Metaphase II oocytes from human unilaminar follicles grown in a multi-step culture system. **Molecular Human Reproduction,** v. 24, n.3, p.135–142, 2018.

MCLAUGHLIN, M.; BROMFIELD, J. J.; ALBERTINI, D. F.; TELFER, E. E. Activin promotes follicular integrity and oogenesis in cultured pre-antral bovine follicles. **Molecular Human Reproduction**, v. 16, n. 9, p. 644–653, 2010.

MCLAUGHLIN, M.; TELFER, E. E. Oocyte development in bovine primordial follicles is promoted by activin and FSH within a two-step serum-free culture system. **Reproduction**, v. 139, n. 6, 971–978, 2010.

MURRAY, A. A.; MOLINEK, M. D, BAKER, S. J.; KOJIMA, F. N.; SMITH, M. F.; HILLIER, S. G.; SPEARS, N. Role of ascorbic acid in promoting follicle integrity and survival in intact mouse ovarian follicles *in vitro*. **Reproduction**. v. 121, n. 1, p. 89–96, 2001.

NILSSON, E. E.; SKINNER, M. K. Growth and differentiation factor-9 stimulates progression of early primary but not primordial rat ovarian follicle development. **Biology of reproduction,** v. 67, n. 3, p. 1018–1024. 2002.

NUTTINCK, F.; MERMILLOD, P.; MASSIP, A.; DESSY, F.; Characterization of *in vitro* growth of bovine preantral ovarian follicles - a preliminary study. **Theriogenology**, v. 39, n. 4, p. 811–821, 1993.

O'BRIEN, M. J. A Revised protocol for *in vitro* development of mouse oocytes from primordial follicles dramatically improves their developmental competence. **Biology of Reproduction**, v. 68, n. 5, p. 1682–1686, 2003.

OKTEM O, OKTAY K. The role of extracellular matrix and activin-A in *in vitro* growth and survival of murine preantral follicles. **Reproductive Sciences**, v. 14, n. 4, p. 358–366, 2007.

OLIVEIRA, A. F. M.; XAVIER, H. S.; SILVA, N. H.; ANDRADE, L. H. C. Screening Cromatográfico de Acanthaceae Medicinais: *Justicia pectoralis Jacq* e *J. gendarussa Burm*. **Revista Brasileira de Plantas Medicinais**, v.3, n.1, p. 37–41, 2000.

OTSUKA, F.; SHIMASAKI, S. A negative feedback system between oocyte bone morphogenetic protein 15 and granulosa cell kit ligand: its role in regulating granulosa cell mitosis. **Proceedings of the National Academy of Sciences**; v. 99, n. 12, 8060 – 8065, 2002.

PANGAS. S. A. Regulation of the ovarian reserve by members of the transforming growth factor beta family. **Molecular Reproduction and Development**, v. 79. n. 10, p. 666–679, 2012.

PARROTT, J. A.; SKINNER, M. K. Direct actions of kit-ligand on theca cell growth and differentiation during follicle development. **Endocrinology**, v.138, n. 9, p.3819–3827, 1997.

POLZIN, G. M.; STANFILL, S. B.; BROWN, C. R.; ASHLEY, D. L.; WATSON, C. H. Determination of eugenol, anethole, and coumarin in the mainstream cigarette smoke of Indonesian clove cigarettes. **Food and Chemical Toxicology.** v. 45, n. 10, p. 1948–1953. 2007.

RAJABI-TOUSTANI, R.; MOTAMEDI-MOJDEHI, R.; MEHR, M.; MOTAMEDI-MOJDEHI, R. Effect of *Papaver rhoeas* L. extract on *in vitro* maturation of sheep oocytes. **Small Ruminant Research**, v. 9, n. 4, p. 146–51. 2013.

RATES, S. M. K. Promoção do uso racional de fitoterápicos: uma abordagem no ensino de Farmacognosia. **Revista Brasileira de Farmacognosia**, v. 11, n. 2, p. 57–69, 2001.

ROY, S. K. Epidermal growth factor and transforming growth factor-beta modulation of follicle-stimulating hormone-induced deoxyribonucleic acid synthesis in hamster pre-antral and early antral follicles. Biology of Reproduction, v. 48, n. 3, p. 552–557, 1993.

SÁ, N. A. R.; ARAÚJO, V. R.; CORREIA, H. H. V.; FERREIRA, A. C. A.; GUERREIRO, D. D. SAMPAIO, A. M.; ESCOBAR, E.; SANTOS, F. W.; MOURA, A. A.; LÔBO, C. H.; CECCATTO, V. M.; CAMPELLO, C. C.; RODRIGUES, A. P. R.; LEAL-CARDOSO, J. H.; FIGUEIREDO, J. R. Anethole Improves the *in vitro* Development of Isolated Caprine Secondary Follicles. **Theriogenology**, v. 89, p. 226-234, 2016.

SADEU, J. C.; CORTVRINDT, R.; RON-EL, R.; KASTERTEIN, E.; SMITZ, J. Morphological and ultrastructural evaluation of cultured froze-thawed human fetal ovarian tissue. **Fertility and sterility**, v.85, n. 1, p.1130–1141, 2006.

SÁNCHEZ, F.; SMITZ, J. Molecular control of oogenesis. **Biochimica et Biophysica Acta,** v.1822, n. 12, p. 1896–912, 2012.

SARAIVA, M. V. A.; ROSSETTO, R.; BRITO, I. R.; CELESTINO, J. J. H.; SILVA, C. M. G.; FAUSTINO, L. R.; ALMEIDA, A. P.; BRUNO, J. B.; MAGALHAES, D. M.; MATOS, M. H. T.; CAMPELLO, C. C.; FIGUEIREDO, J. R. Dynamic medium produces caprine embryo from preantral follicles grown *in vitro*. **Reproductive Sciences**, v. 17, n. 12, p. 1135–1143, 2010.

SHEA, L. D.; WOODRUFF, T. K.; SHIKANOV, A. Bioengineering the ovarian follicle microenvironment. **Annual Review of Biomedical Engineering**, v. 16, p. 29–52, 2014.

SILVA, G. M.; ROSSETTO, R.; CHAVES, R. N.; DUARTE, A. B.; ARAÚJO, V. R.; FELTRIN, C.; BERNUCI, M. P.; ANSELMO-FRANCI, J. A.; XU, M.; WOODRUFF, T. K.; CAMPELLO, C. C.; FIGUEIREDO, J. R.; *In vitro* development of secondary follicles from pre-pubertal and adult goats cultured in two-dimensional or three-dimensional systems. **Zygote**, v. 23, n. 4, p. 475–84. 2015.

SILVA, R. V.; VAN DEN HURK, R.; FIGUEIREDO, J. R. Expression of mRNA and protein localization of epidermal growth factor and its receptor in goat ovaries. **Zygote,** v. 14, n. 2, p. 107–117, 2006.

SILVA, J. R. V.; VAN DEN HURK, R.; VAN TOL, H. T. A.; ROELEN, B. A. J.; FIGUEIREDO J. R. Expression of Growth Differentiation Factor 9 (GDF9), Bone Morphogenetic Protein 15 (BMP15) and BMP Receptors in the Ovaries of Goats. **Molecular Reproduction and Development,** v.70, n. 1, p.11–19, 2004.

SILVA, L. M.; MBEMYA, G. T.; GUERREIRO, D. D.; BRITO, D. C. C.; DONFACK, N. J.; MORAIS M. L. G. S.; RODRIGUES, G. Q.; BRUNO, J. B.; ROCHA, R. M. P.; ALVES, B. G.; APGAR, G. A.; CIBIN, F. W. S.; FIGUEIREDO, J. R.; RODRIGUES, A. P. R. Effect of Catalase or Alpha Lipoic Acid Supplementation in the Vitrification Solution of Ovine Ovarian Tissue. **Biopreservation and Biobanking**, v. 16, n. 4, p. 258–269, 2018.

SOARES, M. A. A, COSTA, J. J. N.; VASCONCELOS, G. L.; RIBEIRO, R. P.; SOUZA, J. C.; SILVA, A. L. C.; VAN DEN HURK, R.; SILVA, J. R. V.; Effects of frutalin on early follicle morphology, ultrastructure and gene expression in cultured goat ovarian cortical tissue. **Histology and Histopathology**, v. 33, n. 1, p. 41–53, 2018.

SPEARS, N.; BOLAND, N.; MURRAY, A.; GOSDEN, R. Mouse oocytes derived from *in vitro* grown primary ovarian follicles are fertile. **Human Reproduction**, v. 9, n. 3, p. 527–532, 1994.

TALEBI, A.; ZAVAREH, S.; KASHANI, M. H, LASHGARBLUKI, T. KARIMI, I. The effect of alpha lipoic acid on the developmental competence of mouse isolated preantral follicles. **Journal of Assisted Reproduction and Genetics**, n. 29, v. 2, p 175–183, 2012.

TELEFO, P. B.; TAGNE, S. R.; KOONA, O. E. S.; YEMELE, D. M.; TCHOUANGUEP, F. M. Effect of the Aqueous Extract of *Justicia Insularis* T. Anders (Acanthaceae) on Ovarian Folliculogenesis and Fertility of Female Rats. **African Journal of Traditional, Complementary and Alternative Medicines**, v. 9, n. 2, p. 197-203, 2012.

TELEFO, P. B.; MOUNDIPA, P. F.; TCHOUANGUEP, F. M. Inductive effects of the leaf mixture extract of *Aloe buettneri, Justicia insularis, Dicliptera verticillata* and *Hibicus marcranthus* on *in vitro* production of oestradiol. **Journal of Ethnopharmacology**, v. 91, n. 2-3, p. 225–230, 2004.

TELEFO, P. B.; MOUNDIPA, P. F.; TCHOUANGUEP, F. M. Oestrogenicity and effect on hepatic metabolism of the aqueous extract of the leaf mixture of *Aloe buettneri, Dicliptera verticillata, Hibiscus macranthus* and *Justicia insularis*. **Fitoterapia**, v. 73, n. 6, p. 472–478, 2002.

TELEFO, P. B.; MOUNDIPA, P. F; TCHANA, A. N.; TCHOUANGUEP, DZICKOTZE, C.; MBIAPO, F. T. Effects of an aqueous extract of *Aloe buettneri, Justicia insularis, Hibiscus macranthus, Dicliptera verticillata* on some physiological and biochemical parameters of reproduction in immature female rats. **Journal of Ethnopharmacology**, v. 63, n. 3, p. 193–200, 1998.

TELFER, E. E.; ZELINSKI, M. B; Ovarian follicle culture: advances and challenges for human and nonhuman primates. **Fertility and sterility**, v. 99. n. 6, p. 1523–1533, 2013.

TELFER, E. E.; MCLAUGHLIN, M.; DING, C.; THONG, K. J. A two-step serum-free culture system supports development of human oocytes from primordial follicles in the presence of activin. **Human Reproduction**, v. 23, n. 5, p. 1151–1158, 2008.

TISDALL, D. J.; QUIRKE, L. D.; SMITH, P.; MCNATTY, K. P. Expression of the ovine stem cell factor gene during folliculogenesis in late fetal and adult ovaries. **Journal of Molecular Endocrinology**, v.18, n. 2, p.127–135, 1997.

VAN DEN HURK, R.; ZHAO, H. Formation of mammalian oocytes and their growth, differentiation and maturation within ovarian follicles. **Theriogenology**, v. 63, n. 6, p. 1717–1751, 2005.

VANACKER, J.; AMORIM, C. A. Alginate: A Versatile Biomaterial to Encapsulate Isolated Ovarian Follicles. Annals of Biomedical Engineering, v. 45, n. 7, p. 1633–1649, 2017.

VANORNY, D. A.; MAYO, K. E. The role of Notch signaling in the mammalian ovary. **Reproduction**, v. 153, n. 6, p. 187–204, 2017.

WANDJI, S.; SRSEN, V.; NATHANIELSZ, P. W.; EPPIG, J. J.; FORTUNE, J. E. Initiation of growth of baboon primordial follicles *in vitro*. **Human Reproduction**, v. 12, n. 9, p. 1993–2001, 1997.

WEST, E.; SHEA, L. D.; WOODRUFF, T. K. Engineering the Follicle Microenvironment. **Seminars in Reproductive Medicine**, v. 25, n. 4, p. 287–299, 2007.

WIGGLESWORTH, K.; LEE, K. B.; EMORI, C.; SUGIURA, K.; EPPIG, J. J. Transcriptomic diversification of developing cumulus and mural granulosa cells in mouse ovarian follicles. **Biology of Reproduction**, v. 92, n. 1, p. 1–14, 2015.

WINKLER-CREPAZ, K.; NEDEREGGER, V.; AYUANDARI, S.; ROSENFELLNER, D.; ZERVOMANOLAKIS, I.; HOFER, S.; WILDT, L.; ZIEHR, S. C. Novel dynamic culture system to support initiation of primordial follicle growth in prepubertal mouse ovaries. **Fertility and sterility**, v. 102, n. 3, p. 864–870, 2014.

WRIGHT CS, HOVATTA O, MARGARA R, TREW G, WINSTON RML, FRANKS S, HARDY K. Effects of follicle-stimulating hormone and serum substitution on the in-vitro growth of human ovarian follicles. **Human Reproduction**, v. 14, n. 6, p.1555-1562, 1999.

WU, J.; EMERY, B. R.; CARRELL, D. T. *In vitro* growth, maturation, fertilization, and embryonic development of oocytes from porcine preantral follicles. **Biology of Reproduction**, v. 64, n. 1, p. 375–381, 2001.

XU, J.; LAWSON, M. S.; MITALIPOV, S. M.; PARK, B. S.; XU, F. Stage-specific modulation of antimüllerian hormone promotes primate follicular development and oocyte maturation in the matrix-free three-dimensional culture. **Fertility and Sterility**, v. 110, n. 6, p. 1162–1172, 2018.

XU, M.; BANC, A.; WOODRUFF, T. K.; SHEA, L. D. Secondary follicle growth and oocyte maturation by culture in alginate hydrogel following cryopreservation of the ovary or individual follicles. **Biotechnology and Bioengineering**. v. 103, n. 2, p. 378–86, 2009.

WU, J.; TIAN, Q. Role of follicle stimulating hormone and epidermal growth factor in the development of porcine preantral follicle *in vitro*. **Zygote**, v. 15, n. 3, p. 233–240, 2007.

XU, Z. Z.; GARVERICK, H. A.; SMITH, G. W.; SMITH, M. F.; HAMILTON, S. A.; YOUNGQUIST, R. S. Expression of messenger-ribonucleic-acid encoding cytochrome-p450 side-chain cleavage, cytochrome-p450 17-alpha-hydroxylase, and cytochrome-p450

aromatase in bovine follicles during the first follicular wave. **Endocrinology**, v. 136, n. 3. p. 981–989. 1995.

YEO, C. X.; GILCHRIST, R. B.; THOMPSON, J. G.; LANE, M. Exogenous growth differentiation factor 9 in oocyte maturation media enhances subsequent embryo development and fetal viability in mice. **Human Reproduction**, v. 23, n. 1, p. 67–73, 2008.

ZAMA, A. M.; HUDSON, F. P.; BEDELL, M. A. Analysis of Hypomorphic KitLSL mutants suggests different requirements for KITL in proliferation and migration of mouse primordial germ cells. **Biology of Reproduction**, v.73, n. 4, p.639–47, 2005.

ZHANG, P.; LOUHIO, H.; TUURI, T.; SJÖBERG, J.; HREINSSON, J.; TELFER, E. E.; HOVATTA, O. In vitro effect of cyclic adenosine 30, 50-monophosphate (cAMP) on early human ovarian follicles. **Journal of Assisted Reproduction and Genetics**, v. 21, n. 8, p. 21:301–306, 2004.