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FRANCISCO LÉO NASCIMENTO DE AGUIAR

CULTIVO *IN VITRO* DE FOLÍCULOS PRÉ-ANTRAIS EQUINOS INCLUSOS EM TECIDO OVARIANO: BENEFÍCIOS DA SUPLEMENTAÇÃO CONCENTRAÇÃO-DEPENDENTE DA INSULINA, FSH E FATOR DE CRESCIMENTO EPIDERMAL

FORTALEZA
2016

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Tese apresentada ao Programa de Pós-Graduação em Ciências Veterinárias da Faculdade de Veterinária da Universidade Estadual do Ceará, como requisito parcial para a obtenção do grau de Doutor em Ciências Veterinárias.

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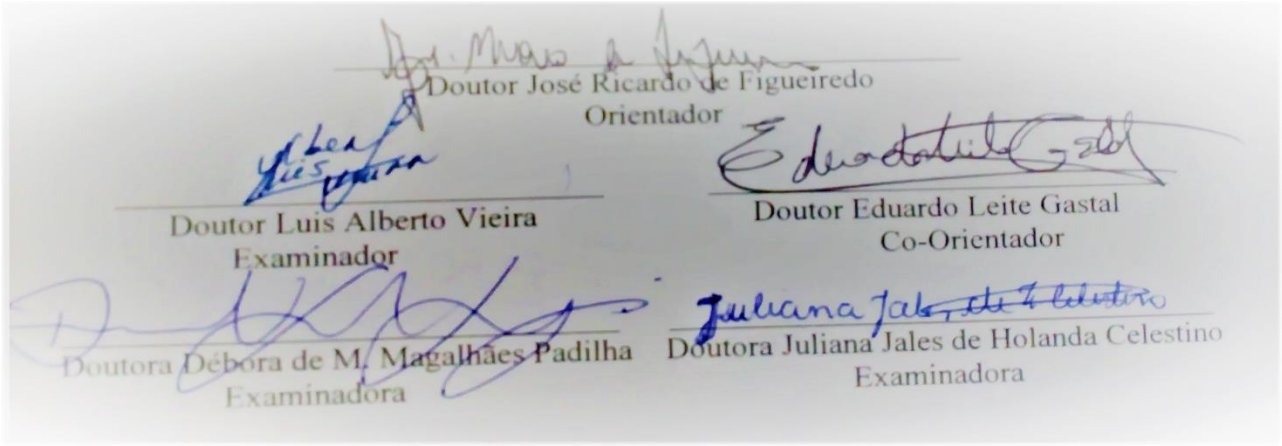
BANCA EXAMINADORA



Dedico,

A Deus, que faz tudo ganhar sentido.

Ao meu filho Estevão Carvalho de Aguiar,
Que fala comigo seu amor sem palavras.

À minha esposa Luzelena dos Santos Carvalho Aguiar,
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"...dura coisa te é recalcitrar contra os aguilhões. Atos 28:14 "

Jesus

RESUMO

O principal objetivo desta tese foi estudar o efeito concentração-dependente dos hormônios insulina, hormônio folículo estimulante (FSH), bem como do fator de crescimento epidermal (EGF) no cultivo *in vitro* (CIV) de folículos pré-antrais equinos inclusos em tecido ovariano, avaliando os parâmetros morfologia e desenvolvimento folicular, produção hormonal (todas as fases), espécies reativas de oxigênio (ROS) (Fases I, II, III), perfil metabolômico (Fase III), níveis de apoptose, expressão proteíca para Ki-67 e para o receptor de EGF, bem como níveis de mRNA para GDF-9, BMP-15 e Cyclin-D2 (Fase IV). Para isso, fragmentos ovarianos equinos foram cultivados em 4 diferentes condições: CIV utilizando meio de base (α-MEM$^+$) na ausência ou presença de suplementação com insulina em concentração fisiológica (10 ng/mL) ou suprafisiológica (10 µg/mL) **(Fase I)**; CIV em meio de base suplementado com diferentes concentrações (0, 10, 50 e 100 ng/mL) de FSH (**Fase II)** ou EGF **(Fase III)**; e CIV usando um meio de base (α-MEM$^+$) enriquecido com insulina (10 ng/mL) e EGF (50 ng/mL), na ausência ou presença de FSH (50 ng/mL) **(Fase IV)**. A duração do cultivo foi de até 7 dias (Fases I, II e III) e de 15 dias (Fase IV). Na **Fase I**, observou-se que a concentração fisiológica de insulina (10 ng/mL) apresentou maiores ($P < 0,05$) percentagens de folículos morfologicamente normais e em desenvolvimento quando comparado aos demais tratamentos, após 7 dias de cultivo. Independente do período de cultivo, a produção de ROS foi menor ($P < 0,05$) no tratamento 10 ng/mL de insulina. O hormônio folículo estimulante na concentração de 50 ng/mL apresentou maior ($P < 0,05$) percentagem de folículos morfologicamente normais e em desenvolvimento, bem como maior diâmetro folicular do que os demais tratamentos após 7 dias de cultivo (**Fase II**). Adicionalmente, esta concentração de FSH manteve ($P > 0,05$) a produção de estradiol e de ROS ao longo do cultivo. De maneira geral, o uso de EGF na concentração de 50 ng/mL resultou em maior ($P < 0,05$) percentagem de folículos morfologicamente normais e em desenvolvimento, bem como maiores ($P < 0,05$) diâmetros folicular e oocitário após 7 dias de cultivo (**Fase III**). Além disso, a referida concentração de EGF manteve a produção de ROS ao longo do cultivo. A análise do perfil metabolômico do meio de cultivo após 7 dias revelou a presença de três substâncias (Dinex, Leonuriside A e Avobenzene) com potencial efeito negativo na sobrevivência, bem como sobre o diâmetro folicular e oocitário. Finalmente, independente da suplementação com FSH, o uso de um meio enriquecido contendo concentrações apropriadas de insulina e EGF manteve os níveis de apoptose do tecido ovariano cultivado similares ao controle fresco não cultivado após 15 dias de cultivo (**Fase IV**). A expressão proteíca para EGFR, Ki-67 e os níveis de RNAm para GDF-9 e Cyclin-D2 não diferiram entre os grupos tratados após 15 dias. Concluiu-se assim que: as concentrações de insulina (10 ng/mL), FSH (50 ng/mL) e EGF (50 ng/mL) são benéficas ao CIV FOPAs equinos inclusos em tecido ovariano por até 7 dias; e que um meio enriquecido contendo

insulina e EGF manteve a morfologia e funcionalidade do tecido ovariano equino após 15 dias de cultivo, independente da adição de FSH.

Palavras - chave: Folículo pré-antral. Tecido Ovariano Equino, Insulina, FSH, EGF, Cultivo *in vitro*.

ABSTRACT

The main goal of this dissertation was to study the concentration-dependent effect of the hormones insulin and follicle stimulating hormone (FSH), as well as of the epidermal growth factor (EGF) on the *in vitro* culture (IVC) of equine preantral follicles (PAFs) enclosed in ovarian tissue. The following endpoints were evaluated: morphology, follicle development, hormonal (all phases), reactive oxygen species (ROS) production (Phase I, II, III), metabolomics profile (Phase III), and apoptotic levels, expression (protein) of EGF receptors and Ki-67, as well as mRNA levels of GDF-9, BMP-15 and Cyclin-D2 (Phase IV). Hence, equine ovarian fragments were cultured *in vitro* in four different conditions: IVC using base medium (α-MEM$^+$) in the absence or presence of insulin supplementation in a physiological concentration (10 ng/mL) or supraphysiological (10 µg/mL) (**Phase I)**; IVC in base medium supplemented with different concentrations (0, 10, 50 and 100 ng/mL) of FSH **(Phase II)** or EGF **(Phase III)**; and IVC using a base medium (α-MEM$^+$) enriched with insulin (10 ng/mL) and EGF (50 ng/mL), in the absence or presence of FSH (50 ng/mL) **(Phase IV)**. The culture time lasted 7 days (Phases I, II, and III), and 15 days (Phase IV). In the Phase I, we observed that physiological concentration of insulin (10 ng/mL) had higher rates ($P < 0.05$) percentage of both morphologically normal and developing follicles when compared to the other treatments after 7 days of culture. Regardless culture time, ROS production was lower ($P < 0.05$) in the 10 ng/mL insulin treatment. Follicle stimulating hormone at 50 ng/mL had higher ($P < 0.05$) percentage of both morphologically normal and development follicles, as well as greater ($P < 0.05$) follicular diameter than the other treatments after 7 days of culture (**Phase II**). In addition, this FSH concentration maintained ($P > 0.05$) estradiol and ROS during culture. Overall, the use of EGF at 50 ng/mL resulted in higher ($P < 0.05$) percentage of morphologically normal and developing follicles, greater ($P < 0.05$) follicular and oocyte diameters after 7 days of culture (**Phase III**). In addition, the aforementioned concentration of EGF maintained ROS production during culture. The metabolomics profile of culture medium from Day 7 of culture demonstrated the presence of three substances (Dinex, Leonuriside A, and Avobenzene) with a potential negative effect on follicle survival, as well as follicular and oocyte diameters. Finally, regardless the FSH supplementation, the use of an enriched medium containing appropriate concentration of insulin and EGF maintained the apoptotic levels in the ovarian cultured tissue similar to fresh noncultured control after 15 days of culture **(Phase IV).** The protein expression for EGFR, Ki-67 and the mRNA levels of GDF-9 and Cyclin-D2 did not differ between the treated groups after 15 days of culture. In conclusion, the concentrations of insulin (10 ng/mL), FSH (50 ng/mL), and EGF (50 ng/mL) were beneficial for IVC of equine PAF enclosed in ovarian tissue at least for seven days, and an enriched medium containing insulin and EGF maintained the morphology and functionality of the ovarian tissue after 15 days of culture regardless the FSH addition.

Keywords: Preantral follicle. Equine Ovarian Tissue, Insulin, FSH, EGF, *In vitro* culture.

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LISTA DE ABREVIATURAS E SIGLAS

Akt	Protein Kinase B (proteina quinase B)
ANOVA	Análise de variância
AP-1	Activator protein 1
BAD	Bcl-2-associated death promoter
Bax	BCL2 Associated X Protein
Bcl-2	B-cell lymphoma 2
Bim	BH3-containing protein
BMP-15	Bone morphogenetic protein 15
BPU	Biopsy Pick-Up method
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
Ccnd-d2	G1/S-specific cyclin-D2
CEUA	Comitê de Ética no Uso de Animais
CGP	Células Germinativas Primordiais
CIV	Cultivo *In vitro*
CO_2	Dióxido de Carbono
CREB	cAMP response element-binding protein
Cyclin D1	G1/S-specific cyclin-D1
Cyclin-D2	G1/S-specific cyclin-D2
DCHF-DA	2',7'-dihidrodiclorofluorescein diacetate
DNA	deoxyribonucleic acid (ácido desoxirribonucleico)
EGF	Epidermal growth factor (fator de crescimento epidermal)
EGFR	Epidermal growth factor Receptor (receptor do fator de crescimento epidermal)
EIA	Equine Infectious Anemia
ERK	extracellular signal–regulated kinases
EROs	Espécies Reativas de Oxigênio
FasL	Fas ligand
FOXO	Forkhead box
FSH	Follicle-Stimulating Hormone
GDF-9	Growth/differentiation factor 9
h	hours
HC	Histologia Clássica

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IGF-I	Insulin-Like Growth Factor 1 (IGF-1)
ITS	Insulin-Transferrin-Selenium
IVC	*In Vitro* Culture
JAK	Janus Kinase
KL	Kit Ligand
LH	Luteinizing Hormone
MAPK	Mitogen Activated Protein Kinases
MEM	Minimum Essential Medium
METLIN	Metabolomics Database
min	minutos
mL	mililitros
mm	milímetros
MOIFOPA	Manipulação de Oócitos Inclusos em Folículos Ovarianos Pré-Antrais
mRNA	Messenger RNA
NFkB	Nuclear factor kappa *B*
ng	nanogramas
P450arom	Cytochrome P450 aromatase
P450scc	Cholesterol side-chain cleavage enzyme P450
PAF	Preantral Follicles
PAS	Periodic Acid-Schiff (ácido periódico-Schiff)
PC	Principal Component
PCA	Principal Component Analysis
PI3-K	phosphatidylinositol 3-kinase
PLS-DA	partial least square discrimination analysis
qPCR	real-time PCR; quantitative PCR
R^2	Linear regression
rbFSH	Recombinant bovine Follicle Stimulating Hormone
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SEM	Standard Error of the Mean
SNRPD3	Small Nuclear Ribonucleoprotein D3 Polypeptide
STAT	Signal Transducer and Activator of Transcription
TCM-199	Tissue Culture Media -199
TGF-β	Transforming Growth Factor beta

TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
VIP	Variable Importance in Projection
α-MEM	Alpha Minimum Essential Medium
α-MEM^{+}	Alpha Minimum Essential Medium with supplementation
μm	micrômetros
°C	Celsius degree (Graus Celsius)
17β-HSD	17 – beta - hydroxylsteroid dehydrogenase

SUMÁRIO

1. INTRODUÇÃO

A importância do estudo dos folículos pré-antrais (FOPAs) tem se tornado mais perceptível nas últimas duas décadas. Em condições fisiológicas, os folículos ovarianos pré-antrais têm três possíveis destinos: permanecer em estado de dormência sobre a influência de fatores inibitórios, iniciar o desenvolvimento após ativação folicular, ou morrer pelo processo de atresia. Somente uma minoria (0,01%) alcançará o estágio pré-ovulatório, e consequentemente, irá liberar um oócito fertilizável. Portanto, a manipulação *in vitro* de FOPAs, os quais representam 90% da população folicular, visando evitar a atresia e promover o desenvolvimento desses folículos até estádios mais avançados, é um atrativo desafio no campo da pesquisa reprodutiva (XU et al., 2013; GRENN; SHIKANOV, 2016).

Apesar dos excelentes resultados alcançados até agora em camundongos com à produção de oócitos maturos a partir de FOPAs cultivados *in vitro* (O'BRIEN e EPPIG 2003), a repetibilidade destes resultados em animais de produção é baixa (ARUNAKUMARI et al., 2010; MAGALHÃES et al., 2011).

Grupos de pesquisa têm avaliado modelos animais para estudo comparativo com a foliculogênese humana, sendo portanto uma alternativa devido a barreiras éticas para o estudo de material biológico humano. Neste cenário, a égua surge como um modelo atrativo para o estudo da foliculogênese comparativa com a mulher, por compartilhar similaridades, tais como a dinâmica folicular (GINTHER, 2012), eventos endócrinos (GINTHER et al., 2004a, MIHM e EVANS; 2008), efeito da idade (CARNEVALE, 2008), e mais recentemente, a dinâmica de folículos pré-antrais (ALVES et al., 2016b). Contudo, estudos da foliculogênese ovariana em éguas têm alguns obstáculos para se transpor, uma vez que em alguns países, tais como os Estados Unidos, o abate de equinos é proibido desde 2007. Para superar este problema, uma alternativa é o uso do método de biópsia ovariana por pick-up (BPU; HAAG et al., 2013d; ALVES et al., 2015; 2016a,b), que representa uma ferramenta valiosa para recuperar o tecido ovariano para diversas abordagens. Quando aplicável, ovários de abatedouro (GOMES et al., 2015) ou oriundos de animais eutanasiados, também podem ser utilizados, provendo material biológico para diversos estudos.

Tal material biológico poderá então fornecer folículos ovarianos pré-antrais, e os mesmos serem cultivados *in vitro*. A eficiência do cultivo *in vitro* vai depender de diferentes fatores,

podendo-se destacar dentre eles a composição de meio. Neste sentido, a adição de substâncias importantes para o sucesso do cultivo *in vitro*, capazes de permitir o desenvolvimento de um grande número de folículos pré-antrais, melhorando assim a taxa de produção de embriões no futuro, são de grande importância. Neste contexto pelo seu papel como importantes reguladores da foliulogênese ovariana, destacam-se a insulina, o FSH e o EGF.

Para uma melhor compreensão do tema investigado nesta tese, a revisão de literatura a seguir fará uma breve abordagem sobre ovário equino, o cultivo *in vitro* de folículos pré-antrais na espécie equina, com destaque para a utilização da insulina, FSH e EGF no meio de cultivo e sua ação, bem como as principais técnicas de avaliação da qualidade folicular.

2 REVISÃO DE LITERATURA

2.1 O ovário dos mamíferos com destaque para o ovário da égua: Sítio promotor da oogênese e foliculogênese

O ovário constitui-se um órgão do trato reprodutivo da fêmea capaz de produzir oócitos e liberar diferente fatores, incluindo hormônios. Assim, o ovário atua, fornecendo os gametas femininos através da ovulação, para a produção de descendentes, além de produzir moléculas bioativas, tais como esteroides (principalmente estradiol e progesterona) e fatores de crescimento capazes de regular diferentes aspectos da fisiologia reprodutiva da fêmea (EDSON et al., 2009).

O ovário possui como unidade funcional o folículo ovariano, composto por um oócito circundado por células somáticas, incluindo células da granulosa e da teca, cuja função é proporcionar um ambiente ideal para a sobrevivência, o crescimento e maturação do oócito, bem como produzir hormônios (MCGEE et al., 2000) e outros peptídeos (PENG et al., 2010). O folículo ovariano é uma estrutura resultante dos processos de oogênese e foliculogênese que ocorrem no ovário ao longo da vida reprodutiva da fêmea. O ovário é constituído por duas regiões: o córtex e a medula. O córtex consiste na região funcional do órgão, e é formado por tecido conjuntivo (fibroblastos, colágeno e fibras reticulares), folículos ovarianos e corpos lúteos em diversos estádios de crescimento ou regressão. A medula é composta primariamente de vasos sanguíneos e linfáticos, nervos e tecido conjuntivo, responsáveis pela nutrição e estruturação do ovário (SAMUELSON, 2007).

A égua tem um ovário em formato anatômico semelhante ao rim, que mede aproximadamente 6-8 cm de comprimento e 3-4 cm de largura durante a estação de cobertura. Éguas maduras tendem a ter ovários maiores, algumas vezes alcançando 10 cm de comprimento. Durante o anestro, o ovário reduz de tamanho, apresentando de 2-4 cm de comprimento por 2-3 cm de largura. O córtex ovariano da égua, ao contrário da maioria das espécies mamíferas, é localizado na área central do ovário, circundado pela área medular (MOSSMAN e DUKE, 1973). Esta zona parenquimatosa contém folículos e, com o crescimento dos folículos dentro do ovário, uma ampla cavidade repleta de fluído pode ser percebida via palpação retal. Inicialmente este

folículo tem consistência firme à palpação, mas tende a diminuir a sua consistência com a aproximação da ovulação.

Precedendo a ovulação, o folículo pré-ovulatório torna-se triangular no formato, posicionando seu ápice em direção à fossa ovulatória. A fossa ovulatória forma uma área côncava no ovário onde a ovulação ocorre. Essa região é mais parenquimatosa do que as áreas circundadas e o epitélio germinal é exposto para o exterior do ovário equino (GINTHER, 1992), garantindo que a ovulação ocorra somente neste local (WITHERSPOON e TALBOT, 1970; STABENFELDT et al., 1975). O verdadeiro propósito pelo qual a fossa ovulatória existe na égua é ainda desconhecido, mas uma especulação é a de que a fossa reduz a possibilidade de ocorrência de múltiplas ovulações que colocariam a égua em risco de desenvolver gestação gemelar. Durante a estação de anestro, os ovários tornam-se mais firmes à palpação, devido a um decréscimo no desenvolvimento dos folículos ovarianos e da perfusão vascular ovariana (GINTHER, 1992).

A oogênese consiste na etapa pela qual as células germinativas primordiais (CGP) transformam-se até oócitos maduros, ou aptos a fertilização, tendo seu início ainda na vida intra-uterina, e concluindo-se após o fim da maturidade sexual (OLIVEIRA, 2009). Na vida fetal, as CGPs migram a partir do endoderma do saco vitelínico para a gônada primitiva, onde iniciam um processo de multiplicação, através de sucessivas mitoses, originando as oogônias meioticamente ativas (SUH et al., 2002). Quando estas oogônias entram em prófase I da meiose, estas são agora denominadas oócitos primários. Estes oócitos são encontrados em "ninhos", sendo envoltos por células somáticas planas conhecidas como células da pré-granulosa. Em seguida, estes oócitos circundados pelas células da pré-granulosasaem dos ninhos, formando os folículos primordiais (HARTSHORE et al., 2009). Oócitos inclusos em folículos primordiais interrompem seu desenvolvimento e entram em um período de quiescência. A retomada da divisão meiótica e a completa maturação oocitária, tanto nuclear quanto citoplasmática, ocorrerão somente a partir da puberdade. Em equinos, não há pico pré-ovulatório de LH, e sim um aumento progressivo do FSH durante os dias de estro (HINRICHS et al., 1993a, BERGFELT e GINTHER, 1993). Se este oócito for ovulado e posteriormente fecundado pelo espermatozoide, ocorrerá a formação do oócito haploide fecundado, finalizando assim a oogênese (FIGUEIREDO et al., 2008).

A foliculogênese é um processo que vai desde a formação dos folículos primordiais até o estádio de folículos pré-ovulatórios. A população folicular na espécie equina é de em média 36,000 folículos, apresentando grande variação individual entre os animais (DRIANCOURT et al., 1982).

Durante a vida reprodutiva da fêmea, um pequeno grupo de folículos é gradualmente estimulado a crescer, iniciando o processo de ativação folicular. A ativação é um processo que ocorre através da passagem dos folículos primordiais quiescentes para os diferentes estádios de desenvolvimento folicular (transição, primário, secundário, terciário e pré-ovulatório), sendo este o maior evento biológico que controla o potencial reprodutivo das fêmeas.

2.2 Atresia folicular

A atresia folicular consiste na morte dos folículos após a sua formação, através da ativação de alguma via de morte celular. A atresia folicular pode ocorrer em qualquer estádio de desenvolvimento folicular, ocorrendo por via degenerativa ou apoptótica. A morte celular por degeneração, conhecida como uma morte celular passiva, ocorre geralmente como consequência de estresse físico-químico extremo associado ao calor, choque osmótico, estresse mecânico, congelação-descongelação e altas concentrações de peróxido de hidrogênio (KRYSKO et al., 2008), ou ainda por exemplo, por isquemia em que o ovário sofre restrição dos suprimentos adequados de oxigênio ou nutrientes (MIKKELSEN et al., 2001). Esse tipo de morte celular é caracterizado morfologicamente pelo aumento do volume celular, desorganização do citoplasma, disfunção mitocondrial, colapso de organelas e perda da integridade da membrana plasmática. Consequentemente, ocorre a ruptura da célula com liberação de seu conteúdo para o meio extracelular, causando dano às células vizinhas e uma reação inflamatória no local (ZONG e THOMPSON, 2006).

Já a apoptose, também conhecida como morte celular programada, é um processo determinado geneticamente, e como tal, dependente da expressão de genes pró e anti-apoptóticos. Este processo é morfologicamente caracterizado pela condensação da cromatina (picnose nuclear), fragmentação específica do DNA, perda de volume celular e formação de protuberâncias na membrana plasmática e de corpos celulares condensados, conhecidos como corpos apoptóticos (HUSSEIN, 2005).

Apesar de ser um fenômeno natural, a atresia reduz significativamente o número de oócitos que seriam ovulados, diminuindo assim o potencial reprodutivo do animal. Entretanto, o cultivo *in vitro* pode se caracterizar como uma excelente estratégia para reverter ou reduzir o impacto da perda folicular. Esses folículos podem então ser cultivados *in vitro* para a obtenção de oócitos

maturos, aptos à fecundação, garantindo assim a manutenção da função reprodutiva de um determinado animal, ou até mesmo a multiplicação de animais de alto valor genético ou em vias de extinção.

2.3 A biotécnica de Manipulação de Oócitos Inclusos em Folículos Ovarianos Pré-Antrais (MOIFOPA)

Conforme visto anteriormente, existe uma grande perda folicular que ocorre naturalmente *in vivo*. Assim, a disponibilidade de oócitos é um fator limitante para o desenvolvimento de novas técnicas reprodutivas (SMITZ e CORTVRINDT, 2002). Os métodos atuais para a produção *in vitro* de embriões dependem de uma oferta de oócitos competentes provenientes de grandes folículos antrais ou pré-ovulatórios, os quais estão presentes no ovário em número reduzido (TELFER, 1998). Dessa forma, a possibilidade de desenvolver sistemas *in vitro* que explorem o grande número de oócitos provenientes de folículos pré-antrais deve ser considerada. Neste contexto, a biotécnica de Manipulação de Oócitos Inclusos em Folículos Ovarianos Pré-Antrais (MOIFOPA) visa previnir a atresia observada *in vivo*, maximizando a recuperação de oócitos potencialmente fertilizáveis. Tal biotécnica consiste no isolamento, conservação (resfriamento e criopreservação) e/ou cultivo *in vitro* de folículos ovarianos pré-antrais, visando a estocagem, ativação, crescimento e maturação *in vitro* do folículo primordial até o folículo pré-ovulatório (FIGUEIREDO et al., 2008). Dentre as possíveis aplicações da MOIFOPA, podem-se exemplificar: a pequisa fundamental, com o aumento dos conhecimentos acerca da foliculogênese; a criopreservação de material biológico para a produção de biobancos de células germinativas; fonte para testes toxicológicos para a indústria farmacêutica; incremento na produtividade de animais de alto valor genético, bem como preservação de espécies ameaçadas de extinção (HAAG et al., 2013d).

2.3.1 Recuperação de tecido ovariano equino e estudo da população, morfologia e densidade folicular.

Em equinos, estudos recentes demonstraram que diversos fatores podem influenciar na qualidade do tecido ovariano recuperado, no que tange a integridade morfológica folicular e no

número de folículos obtidos para análise. Sabe-se que a população folicular no ovário equino apresenta uma alta variabilidade, estimada em média de 35.950 folículos primordiais e 100 folículos em crescimento, variando entre 5.600 a 75.000 folículos primordiais, com 20 a 300 folículos em crescimento (DRIANCOURT et al., 1982). Além disso, o diâmetro folicular e oocitário médio são de 31.0 ± 0.5 e 27.6 ± 0.6, respectivamente (HAAG et al., 2013d). Após a recuperação tecidual, o tempo, a temperatura, bem como o meio de manutenção, pode influenciar na qualidade dos folículos após recuperação do tecido ovariano. Em relato prévio, demonstrou-se que a temperatura de 4ºC por até 4 horas em meio PBS foi capaz de preservar maiores percentagens de folículos morfologicamente normais do que o meio MEM (GOMES et al., 2012).

Outro fator limitante para a manipulação de tecido ovariano equino consiste na obtenção do tecido ovariano, uma vez que em alguns países, como por exemplo nos Estados Unidos, o abate de equinos está proibido desde 2007. Neste contexto, uma excelente alternativa consiste no uso da técnica de biópsia ovariana por pick-up (BPU; AERTS et al., 2005; 2008). Tal procedimento foi validado na espécie equina com sucesso (HAAG et al., 2013a), com a obtenção de fragmentos de biópsia contendo folículos ovarianos em diferentes estádios de desenvolvimento, sendo aptos a manipulações subsequentes, como o isolamento mecânico por *tissue chopper* (HAAG et al., 2013b), com a manutenção da viabilidade folicular.

Após a manipulação, o fragmento de biópsia geralmente é destinado à análise por histologia clássica. A eficácia da análise morfológica vai depender do correto processamento do material obtido. Desta maneira, alguns fatores podem afetar o processamento, como por exemplo, o tipo de fixador escolhido. A fixação é uma das etapas mais importantes da técnica histológica, pois visa interromper o metabolismo celular, estabilizando os componentes bioquímicos e estruturas intra e extracelulares, além de permitir a penetração de substâncias subsequentes à fixação (O`LEARY, 2001). Em estudo prévio avaliando diferentes tipos de fixadores para o tecido ovariano equino, demonstrou-se que para fragmentos ovarianos (5 x 5 x 1 mm), o fixador Carnoy utilizado por 24 horas foi o mais benéfico para a manutenção da morfologia folicular, quando comparado ao Bouin e à formalina a 10%. Em outros estudos, o Bouin (HAAG et al., 2013a,b,c), bem como o paraformaldeído a 4 % (AGUIAR et al., 2016a,b), têm sido utilizados com sucesso para a classificação morfológica folicular.

Adicionalmente, a espessura de corte do tecido ovariano pode interferir na identificação de estruturas morfológicas após processamento histológico. Analisando diferente espessuras de corte

(3 µm, 5 µm e 7 µm; ALVES et al., 2015) verificou-se que um maior número de folículos morfologicamente normais foram encontrados na espessura de 7 µm (ALVES et al., 2015).

O ovário equino pode sofrer alterações na densidade folicular por conta da idade, bem como devido à heterogeneidade da distribuição folicular no ovário (HAAG et al., 2013a). Neste contexto, um estudo anterior demonstrou que a densidade folicular difere entre animais e dentro de cada fragmento ovariano. Adicionalmente, a morfologia folicular é afetada negativamente pelo aumento da idade (11-17 anos), sendo necessários 3 a 4 fragmentos ovarianos, combinados com 65 secções histológicas, para detectar a densidade folicular do tecido ovariano equino, independentemente da heterogeneidade (ALVES et al., 2016a).

Mais recentemente, a influência de estruturas ovarianas (corpo lúteo *versus* folículo pré-ovulatório), bem como da sazonalidade reprodutiva (diestro *versus* anestro) foram avaliadas (ALVES et al., 2016b). Neste estudo, a presença de corpo lúteo teve efeito positivo na qualidade dos folículos pré-antrais, apresentando maior densidade folicular e de células do estroma. Além disso, o diestro apresentou maior percentagem de folículos morfologicamente normais, folículos em desenvolvimento e densidade de células estromais quando comparado ao anestro (ALVES et a., 2016b).

Por fim, um estudo avaliou o efeito do tempo de exposição do tecido ovariano equino a diferentes agentes crioprotetores (dimetilsufóxido, DMSO; etilenoglicol, EG; e propanodiol, PROH). Como principal resultado, o etilenoglicol (EG) demonstrou ser o agente crioprotetor menos prejudicial ao tecido ovariano equino nos diferentes tempos de exposição avaliados (0, 10, 15 e 20 minutos), não afetando a densidade celular (GASTAL et al., 2016).

2.3.2 Cultivo *in vitro* de folículos pré-antrais

O cultivo *in vitro* de folículos ovarianos é uma importante etapa da biotécnica de MOIFOPA, e tem por objetivo assegurar um ambiente ideal capaz de proporcionar o desenvolvimento *in vitro* dos oócitos até a produção de um oócito maturo, capaz de ser utilizado na produção de embriões em larga escala. Através do cultivo *in vitro* pode-se avaliar o efeito de diferentes substâncias, em concentrações variáveis durante as diferentes fases do desenvolvimento folicular. Existem basicamente dois tipos de sistemas de cultivo onde os folículos pré-antrais

podem ser cultivados: inclusos no fragmento de córtex ovariano (cultivo *in situ*), ou na forma isolada (cultivo de folículos isolados) (ARAUJO et al., 2014).

O cultivo *in situ* tem a vantagem de promover a manutenção do contato de diferentes folículos entre si, bem como com o estroma circundante. Além disso, permite a investigação do efeito de diversas substâncias sobre a ativação e crescimento folicular, até o estádio de folículo secundário (PENG, 2010). Uma das desvantagens deste modelo é que os folículos não conseguem crescer até a fase final da foliculogênese, sendo que para esta finalidade, tais folículos devem ser isolados do tecido, e cultivados na forma isolada até a formação de antro e maturação oocitária (TELFER e ZELINSKI, 2013). Neste sentido, o cultivo de folículos na forma isolada são previamente isolados de forma mecânica (utilizando-se um *Tissue chopper*, um homogeneizador ou microdissecção utilizando-se agulhas de seringa), ou ainda na forma enzimática, utilizando-se de enzimas como a colagenase e DNase, e cuidados de forma bidimensional, diretamente sobre a superfície plástica da placa de cultivo, ou sobre uma matrix (composta por exemplo de células fibroblásticas), e ainda na forma tridimensional sendo inserido em uma matrix, como por exemplo o gel de agarose (FIGUEIREDO et al., 2008).

Grandes progressos já foram obtidos com o cultivo *in vitro* de folículos pré-antrais em folículos em diferentes espécies animais. Em felinos (JEWGENOW, 1998) e marsupiais (BUTCHER E ULLMAN, 1996) foi observado o crescimento de folículos ovarianos pré-antrais isolados após o cultivo *in vitro*, porém, sem a formação de antro. Nas espécies bovina (GUTIERREZ et al., 2000; MCCAFFERY et al., 2000), canina (SERAFIM et al., 2010) e humana (ROY e TREACY, 1993), os folículos pré-antrais isolados desenvolveram-se *in vitro* até o estádio antral. Em suínos, os folículos secundários crescidos *in vitro* chegaram até a maturação e tiveram seus oócitos fecundados *in vitro* (HIRAO et al., 1994) com desenvolvimento até o estádio de blastocisto (WU et al., 2001). Mais recentemente, foram obtidos embriões de búfalos (GUPTA et al., 2008), cabras (SARAIVA et al., 2010; MAGALHÃES et al., 2011) e ovelhas (ARUNAKUMARI et al., 2010) a partir de folículos pré-antrais cultivados *in vitro*.

Na espécie equina, somente três estudos realizaram cultivo *in vitro* de folículos pré-antrais. Em estudo prévio (SZLACHTA e TISCHNER, 2000), avaliou-se o efeito da suplementação de FSH em folículos isolados enzimaticamente. Como resultado, houve aumento na taxa de atresia observada após quatro dias de cultivo. Posteriormente, testou-se a eficácia de dois diferentes meios de cultivo (Menezo B2 e Waymouth MB 752/1), com ou sem a suplementação de FSH no cultivo

in vitro de folículos pré-antrais isolados enzimaticamente (SZLACHTA e TISCHNER, 2004). O meio Menezo B2 apresentou melhor resultado de sobrevivência folicular quando comparado ao meio Waymouth MB 752/1. Contudo, a suplementação de FSH não beneficiou a taxa de crescimento e de sobrevivência folicular durante os quatro dias de cultivo.

Em outro estudo realizado por Haag et al. (2013c), utilizando tecido ovariano recuperado pela técnica de BPU, investigou o efeito de dois meios de base (α-MEM e TCM-199) após cultivo de 1 e 7 dias. Neste trabalho, o meio α-MEM obteve maior taxa de ativação folicular (27%) após 7 dias de cultivo, não sendo observados folículos viáveis no cultivo em TCM-199 no final do cultivo.

Em estudo mais recente, avaliou-se o papel da concentração-dependente (0, 10, 50 e 100 ng/mL) do ácido ascórbico sob tecido ovariano equino oriundo de abatedouro (GOMES et al., 2015). As concentrações de 50 ng/mL e 100 ng/mL de ácido ascórbico apresentaram maiores percentagens de folículos em desenvolvimento após 6 dias de cultivo em comparação com as concentrações de 0 e 25 ng/mL de ácido ascórbico.

Assim, a realização de mais trabalhos referentes ao cultivo *in situ* de folículos pré-antrais equinos são de grande importância, especialmente devido à escassez de resultados relativos à essa espécie. Com o objetivo de obter-se melhores resultados para o cultivo *in vitro*, diversos suplementos vêm sendo adicionados aos meios de cultivo de base. A seguir, será realizada uma breve abordagem da importância da composição do meio de base e de alguns suplementos para esse meio como a insulina, hormônio folículo estimulante (FSH) e o fator de crescimento epidermal (EGF).

2.4 A composição do meio e o desenvolvimento folicular *in vitro*

A composição do meio é um importante fator para o sucesso do cultivo *in vitro* de folículos ovarianos. Estudos prévios demonstraram que a sobrevivência de folículos pré-antrais bovinos *in vitro* foi reduzida na ausência de hipoxantina e substratos energéticos, tais como piruvato e glutamina (FIGUEIREDO et al., 1994). Adicionalmente, a suplementação de insulina, transferrina e selênio (ITS) têm assegurado o crescimento oocitário e a formação de antro (GORE-LANGTON e DANIEL, 1990). Desta forma, a adição de piruvato, glutamina, ITS e hipoxantina ao meio de

cultivo tem sido essencial para o crescimento de folículos pré-antrais *in vitro* (JEWGENOW et al., 1998).

Os antioxidantes, selênio e transferrina, são relatados como substâncias importantes a serem adicionadas ao meio de cultivo. Alguns autores sugerem que o processo de maturação folicular está relacionado aos altos níveis de transferrina e seus receptores na célula, e que o selênio pode ser adicionado ao meio de cultivo para ativar enzimas envolvidas na detoxificação e eliminação de radicais livres (DEMEESTERE et al., 2005). O desenvolvimento de um sistema de cultivo básico que garanta a ativação e o crescimento folicular até um estádio em que os oócitos possam ser maturados e fecundados *in vitro* é importante para estudar os fatores que controlam o crescimento oocitário e a multiplicação das células da granulosa (CORTVRINDT et al., 1996). Além disso, sabe-se que o crescimento dos folículos presentes no ovário mamífero é regulado por gonadotrofinas e por fatores intra-ovarianos (FORTUNE, 1998). Assim, estudos têm investigado o efeito de vários componentes no cultivo *in vitro* de folículos pré-antrais, tanto de animais de laboratórios como animais domésticos (SMITZ e CORTVRINDT, 2002). A seguir, serão descritos como os hormônios (insulina e FSH) e do fator de crescimento epidermal (EGF) influenciam no cultivo *in vitro* de folículos pré-antrais.

2.4.1 Insulina

A insulina é um hormônio fundamentalmente relacionado com a regulação da concentração circulante de glicose, tendo seu papel no crescimento e na diferenciação de diversos tipos celulares, e atuando como regulador da atividade ovariana (HERNANDEZ et al., 1988). A insulina no ovário estimula a proliferação das células da granulosa, a produção de esteróides (SPICER et al., 1993), a atividade da aromatase (GARZO e DORRINGTON, 1984), bem como regula a maturação oocitária (PAWSHE et al., 1998).

O efeito direto da insulina no ovário pode ser comprovado pela presença de seus receptores. O receptor de insulina pertence a uma família de receptores de fatores de crescimento que têm atividade tirosina-quinase (CARVALHEIRA et al., 2002). Este receptor específico de membrana é uma proteína heterotetramérica com atividade quinase, composta por duas subunidades α (massa molecular 135 kDa) e duas subunidades β (massa molecular 95 kDa), unidas por uma ponte dissulfídica (LAWRENCE et al., 2007). A subunidade α dos receptores de insulina são estruturas

extracelulares que servem como sítio para ligação deste hormônio. Já a subunidade β do receptor possui um domínio transmembranário e um intracelular, o qual é responsável pela transmissão do sinal (BELFIORE et al., 2009). A ligação da insulina à subunidade α permite que a subunidade β adquira atividade quinase, levando a alteração conformacional (IRS-1/-2: Insulin receptor substrate-1/-2) que aumenta ainda mais a atividade quinase do receptor (LAWRENCE et al., 2007). Uma vez fosforilado, o IRS- 1/-2 interage com uma série de proteínas intracelulares, desencadeando uma cascata complexa de reações de fosforilação e desfosforilação (CHEATHAM e KAHN, 1995). Em adição à ativação da fosfatidilinositol 3-quinase (PI-3 quinase), a proteína quinase mitogenicamente ativada (MAPK) também é fosforilada após a ligação da insulina ao seu receptor (CHEATHAM e KAHN, 1995; WHITE, 1996). A ativação do MAPK é responsável pelos efeitos no crescimento promovidos pela insulina (LAWRENCE et al., 2007). Tanto em modelos humanos como animais, os receptores de insulina são amplamente distribuídos em todos os compartimentos ovarianos, incluindo células da granulosa, células da teca, estroma e oócito (PORETSKY et al., 1988). No entanto, a expressão de RNAm para receptor de insulina nas células da granulosa e teca de folículos pré-ovulatórios foi maior do que em todos os outros estágios de desenvolvimento (SHIMIZU et al., 2008).

Quando a insulina liga-se ao seu receptor, esta promove uma série de efeitos metabólicos, destacando-se a estimulação do transporte de glicose para o interior das células, a principal fonte energética para o ovário (SHIMIZU et al., 2008). Estudos mostram que a insulina possui importante papel na regulação da responsividade do ovário, podendo também atuar sinergicamente junto às gonadotrofinas hipofisárias (LH e FSH), aumentando a produção de hormônio do crescimento e estimulando a proliferação e diferenciação das células da granulosa juntamente com o FSH (KAWAUCHI e SOWER, 2006).

Com relação ao papel da insulina na função ovariana, especificamente na foliculogênese *in vitro*, consiste na manutenção da viabilidade e crescimento dos folículos primordiais e primários, aumentando a formação de folículos primários em baixas concentrações (LOUHIO et al., 2000; CHAVES et al., 2011). Evidências apontam para o fato da insulina estimular o fator inibidor de leucemia, Kit Ligand e IGF-I, atuando como co-reguladora no padrão de sinalização da transição de folículos primordiais para primários durante a foliculogênese inicial (VAN DEN HURK e ZHAO, 2005). Estudos *in vitro* têm mostrado que a insulina estimulou a formação de folículos

primários em tecido ovariano cultivado em diferentes espécies, como em humanos (LOUHIO et al., 2000) e murinos neonatais (KEZELE et al., 2002).

A insulina quando utilizada em concentrações fisiológicas (10-20 ng/mL), atuou no crescimento folicular e oocitário de folículos pré-antrais bovinos, com alta porcentagem (acima de 60%) de formação de antro após 13 dias de cultivo (MCLAUGHLIN et al., 2010). Em ovinos, a suplementação de insulina na concentração de 10 ng/mL contribuiu para o desenvolvimento de folículos secundários (ARUNAKUMARI et al., 2010). Em caprinos reportou-se que folículos pré-antrais cultivados *in vitro* quando inseridos *in situ* ou isolados mecanicamente, apresentaram maior crescimento e sobrevivência com a utilização de insulina 10-ng/mL associada ao FSH em concentrações crescentes (sequencial: Dia 0 = 100 ng/mL; Dia 6 = 500 ng/mL; Dia 12 = 1000 ng/mL; CHAVES et al., 2012). Quando adicionada ao meio de cultivo juntamente com o piruvato, glutamina e hipoxantina, a insulina como componente do ITS (Insulina, Transferrina e Selênio) aumentou o percentual de folículos morfologicamente normais, e estimulou o crescimento folicular em roedores (DEMEESTERE et al., 2005). As ações da insulina quando adicionada ao meio de cultivo estão relacionadas possivelmente a um melhor aproveitamento das fontes energéticas presentes no meio, atuando assim como fator de sobrevivência, e garantindo aporte de precursores metabólicos como aminoácidos e glicose (CHAVES et al., 2011).

2.4.2 Hormônio Folículo Estimulante (FSH)

O FSH é uma gonadotrofina secretada pela hipófise anterior, sendo um dos principais hormônios adicionados ao meio de cultivo. Sua principal função é no desenvolvimento e maturação gonadal durante a puberdade, bem como no desenvolvimento de folículos durante a fase inicial da onda folicular (GINTHER et al., 1992). O receptor do FSH é composto de um grande domínio extracelular N-terminal, sete domínios transmembranários e um domínio C- terminal intracelular acoplado à proteína G (SALESSE et al., 1991). Após ligar-se ao receptor, ocorre a conversão de guanosina difosfato (GDP) em guanosina trifosfato (GTP), que se liga à subunidade α da proteína G, estimulando a adenilciclase (AC) a gerar AMP cíclico (cAMP). Este, por sua vez, aciona uma cascata de fosforilação nas proteínas quinases dependentes de cAMP (PK-A). Desta forma, a ativação da PK-A controla múltiplos aspectos da função celular por meio da fosforilação de substratos proteicos. Uma vez que a interação receptor-ligante tenha se estabelecido, o

complexo é internalizado por endocitose e degradado pelos lisossomos, sendo o receptor reciclado à membrana celular por exocitose (HILLIER, 1996). 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 e MAIZELS, 2006), tais como a estimulação da proliferação celular, a síntese de estereoides e a expressão de receptores para o Fator de Crescimento Epidermal (EGF), Fator de Crescimento Semelhante à Insulina 1 (IGF-1) e LH (VAN DEN HURK e ZHAO, 2005).

Desta forma, estudos *in vitro* da ação do FSH tem merecido atenção especial, principalmente pelo seu envolvimento na proliferação celular, síntese de esteroides e expressão de receptores para outras substâncias importantes, como por exemplo, o LH, o kit ligand (KL) e o GDF-9 (NILSSON e SKINNER, 2004). No ovário mamífero, embora os folículos pré-antrais sejam independentes de FSH para seu crescimento inicial, os receptores de FSH estão presentes nas células da granulosa foliculares (O'SHAUGHNESSY et al., 1996). Por conta disto, o FSH é capaz de exercer efeito benéfico sobre os folículos mesmo quando adicionado ao cultivo antes da formação da cavidade antral (ADRIENS et al., 2004). Desta forma, estudos têm demonstrado que o FSH promove aumento no crescimento folicular *in vitro* (WU e TIAN; 2007).

Os melhores resultados relatados na literatura relativo ao cultivo de FOPAs tem em comum a presença de FSH no meio de desenvolvimento, como por exemplo, o nascimento de animais vivos em camundongos a partir de folículos primordiais (EPPIG et al., 1996), bem como a produção de embriões oriundos de folículos pré-antrais em ovinos (ARUNAKUMARI et al., 2010), caprinos (SARAIVA et al., 2010) e búfalas (GUPTA et al., 2008). No cultivo de folículos primários e secundários isolados enzimaticamente, o FSH promoveu aumento no diâmetro, sobrevivência folicular, e secreção de hormônios esteroides e aumento na taxa de formação de antro (GUTIERREZ et al., 2000; ITOH et al., 2002). Estudos relataram que o FSH promove a formação de antro em diferentes espécies (Ovinos: ARUNAKUMARI et al., 2010; Bovinos: GUTIERREZ et al., 2000; Suínos: WU e TIAN., 2007; Caprinos: MATOS et al., 2007). Em equinos, trabalhos avaliando a adição de FSH ao meio de cultivo são escassos. No cultivo *in vitro* de folículos isolados em meio na presença de FSH, observou-se que após 4 dias de cultivo, o FSH não afetou as taxas de crescimento ou sobrevivência folicular (SZLACHTA e TISCHNER, 2000, 2004).

2.4.3 Fator de Crescimento Epidermal (EGF)

O EGF é considerado um fator de crescimento proteico pertencente à família EGF, a qual consiste de no mínimo oito membros (CONTI et al., 2006). Sua atividade biológica é mediada por receptores de membrana EGF-R do tipo tirosina-quinase, pertencentes à superfamília ErbB (YARDEN, 2001). Na década de 80, estudos já demonstravam que o EGF atua sobre o crescimento de folículos ovarianos, além de modular a função das células da granulosa (KNECHT e CATT, 1983; FENG et al., 1986). É considerado um potente fator mitogênico que estimula a proliferação de diferentes tipos celulares (DAS, 1984; MULLIN e MCGINNET, 1988). Tem sido demonstrado que o EGF atua como importante regulador da fisiologia ovariana, estando envolvido na regulação de diversos processos, incluindo a ativação folicular, a proliferação e diferenciação das células da granulosa, esteroidogênese e maturação oocitária (CELESTINO et al., 2009). Além disso, o EGF tem recebido notável atenção por inibir a apoptose, garantindo assim maior sobrevivência folicular em condições *in vitro* (MARKSTRÖM et al., 2002).

O EGF é conhecido como um fator de sobrevivência tanto *in vivo* como *in vitro* (SILVA et al., 2006). Quando utilizado *in vitro* na concentração de 10 ng/mL, o EGF inibiu apoptose das células da granulosa e proporcionou aumento da formação de antro após o cultivo de folículos isolados suínos (MAO et al., 2004). Porém, em folículos pré-antrais de camundongos, a utilização de EGF não demonstrou nenhum efeito na supressão da apoptose (DEMEESTERE et al., 2005). Em bovinos, quando o EGF foi utilizado em uma concentração de 0,5 ng/mL, resultou na redução dos níveis de atresia em folículos pré-antrais cultivados *in vitro* (GUTIERREZ et al., 2000). Por outro lado, nesta mesma espécie, utilizando a concentração de 10 ng/mL, o EGF não influenciou na sobrevivência de folículos primários e secundários cultivados *in vitro* (DERRAR et al., 2000).

Posteriormente, estudos de ZHOU e ZHANG (2005a,b) relataram que o EGF na concentração de 50 ng/mL estimulou a sobrevivência de oócitos caprinos após o cultivo *in vitro*. Além disso, baixas concentrações de EGF (1 ou 10 ng/mL) no cultivo *in vitro* de folículos pré-antrais caprinos mantiveram a morfologia e a ultraestrutura folicular após 7 dias de cultivo (CELESTINO et al. 2009). No entanto, em altas concentrações (100 ng/mL), não houve efeito do EGF sobre a sobrevivência de folículos pré-antrais caprinos após 5

dias de cultivo (SILVA et al. 2004). Na espécie ovina investigou-se o efeito de diferentes concentrações de EGF (0, 25, 50, 75 ou 100 ng/mL) sobre folículos pré-antrais cultivados *in vitro*, demonstrando que maiores concentrações (75 e 100 ng/mL) induziram a degeneração de todos os folículos pré- antrais após cultivo de 6 dias (TALMIMANI et al. 2005). Resultados similares foram descritos em folículos pré-antrais caprinos cultivados *in vitro* por 7 dias, onde altas concentrações de EGF (100 ou 200 ng/mL) não induziram ativação e apresentaram altas taxas de degeneração folicular (CELESTINO et al. 2009).

A ação do EGF sobre a ativação, diferenciação, proliferação e esteroidogênese das células da granulosa tem se mostrado controversa na literatura. Alguns estudos têm mostrado que embora o EGF não seja essencial para ativação de folículos primordiais (BRAW-TAL e YOSSEFI, 1997; FORTUNE et al., 1998), o mesmo parece ser importante para os estádios mais avançados de desenvolvimento folicular (GUTIERREZ et al., 2000; PENG et al., 2010). Em caprinos, o EGF na concentração de 100 ng/mL promoveu a ativação de folículos primordiais após cultivo *in situ* após 3 dias, bem como promoveu efeito benéfico no crescimento de oócitos de folículos primários (SILVA et al. 2004). Em ovinos, sua utilização nesta mesma concentração promoveu a ativação de folículos primordiais *in situ* e a manutenção da viabilidade por até 6 dias de cultivo (ANDRADE et al., 2005). Em pequenos e médios folículos pré-antrais bovinos (60-179 μm), a utilização de 50 ng/mL de EGF foi capaz de garantir a sobrevivência e promover o crescimento e produção de progesterona, bem como estimulou a formação de antro em grandes folículos pré-antrais (166 μm), mas não o crescimento do oócito (GUTIERREZ et al., 2000). Em suínos, o EGF em baixas concentrações (0,75, 1,5 ou 3 ng/mL) quando associado ao FSH, melhorou a qualidade dos oócitos, levando a maior taxa de desenvolvimento embrionário (WU e TIAN, 2007).

2.5 Avaliação da morfologia e funcionalidade folicular após cultivo *in vitro*

Diversas técnicas podem ser empregadas para análise folicular após o cultivo *in vitro* de folículos pré-antrais. Os parâmetros mais utilizados nesta análise são aqueles inerentes à avaliação da morfologia e funcionalidade folicular.

Avaliação morfológica

A histologia clássica (HC) constitui-se de uma importante ferramenta para avaliação de folículos pré-antrais *in vitro* permitindo uma análise quantitativa de um grande número de folículos cultivados, e a verificação da morfologia e número de células foliculares, bem como integridade e viabilidade das mesmas após ativação (normais ou atrésicos). Diante destes parâmetros, pode-se também classificar os folículos com relação ao seu estádio de desenvolvimento (primordial, transição, primário ou secundário). Todavia, a HC possui como limitação a incapacidade de avaliar a integridade de membranas e organelas citoplasmáticas (MATOS et al., 2007).

A técnica de microscopia eletrônica de transmissão pussui um sistema de captação de imagens de altíssima resolução (0,1 nm), e se vale de parâmetros morfológicos para visualização de estruturas biológicas detalhadas não visíveis por histologia clássica, com o auxílio de um microscópio eletrônico (SALEHNIA et al., 2002). As mudanças ultraestruturais ocorridas após cultivo *in vitro*, como por exemplo, pequenos danos em membranas nucleares (oocitária e das células da granulosa), podem ser identificados através da interação entre elétrons e átomos presentes nas células. Entretanto, essa técnica é por vezes laboriosa quando comparada a histologia clássica, limitando o número de folículos a serem analisados, sendo assim uma técnica essencialmente qualitativa.

A técnica de TUNEL (terminal deoxynucleotidil transferase-mediated deoxyuridine triphosphate biotin nick end-labeling) utiliza uma enzima (tranferase deoxynucleotidil terminal) para adicionar nucleotídeos aos fragmentos das fitas de DNA quebradas nas células apoptóticas. Essa técnica é utilizada para avaliar a fragmentação do DNA em secções histológicas, permitindo a avaliação histológica, bem como a detecção do grau de apoptose (LOO et al., 2011).

De maneira geral, ao longo do cultivo *in vitro*, a taxa de células apoptóticas aumenta ao longo do cultivo. MAO et al., 2004 relataram que a percentagem de células da granulosa apoptóticas em folículos frescos foi 0,1%, contudo após quatro dias de cultivo, essa porcentagem aumentou significativamente para 3,4%. Em bovinos, também se observou que não havia células da granulosa coradas por *TUNEL* em folículos isolados frescos ou em folículos cultivados na presença de ácido ascórbico. Por outro lado, havia um número significativamente maior de células da granulosa apoptóticas em folículos cultivados na ausência do ácido ascórbico (THOMAS et al., 2001).

Avaliação da funcionalidade

A funcionalidade dos folículos ovarianos pode ser avaliada *in vitro* de diferentes formas, como por exemplo, através da proliferação celular de células da granulosa (CUILING et al., 2005), da atividade esteroidogênica (BILLIG et al., 1993), espécies reativas de oxigênio (BEHRMAN et al., 2001), avaliação do perfil metabolômico (GOOK et al., 2014), e da expressão gênica (OKTAY et al., 1997).

No tocante à proliferação de células da granulosa, a proteína Ki-67 é fortemente associada com a proliferação celular (SCHOLZEN e GERDES, 2000). Seu antígeno pode ser identificado exclusivamente no núcleo. Esta proteína está presente durante todas as fases do ciclo celular (G(1), S, G (2), e mitose, mas está ausente na fase G (0), tornando esta proteína um excelente marcador para determinar a fração em crescimento de uma dada população celular. Sua aplicação na avaliação de folículos pré-antrais já foi demonstrada em estudos prévios (SCALERCIO et al., 2015; PAULINI et al., 2016).

A atividade esteroidogênica ou produção de hormônios esteroides tem sido amplamente utilizada para avaliar a funcionalidade do folículo cultivados *in vitro* (SONGSASEN et al., 2011). Os hormônios esteróides são originários do colesterol por meio de uma série clássica de reações enzimáticas. O colesterol é transportado para a membrana interna da mitocôndria sendo alvo da enzima citocromo P450 *side chain cleavage* (P450scc) nas células da teca, e alvo do citocromo P450 aromatase (P450arom) nas células da granulosa (XU et al., 1995; TAMURA et al., 2007). A P450scc converte o colesterol em pregnenolona, a qual pode ser convertida em progesterona ou em andrógeno. O andrógeno pode ser convertido, formando androstenediona, a qual pode ser convertida no andrógeno mais ativo, a testosterona, pela enzima 17β hidroxisteróide desidrogenase (17β-HSD). Finalmente, a testosterona pode ser convertida a estradiol pela ação da enzima P450arom (CONLEY e BIRD, 1997). De uma forma geral, o produto esteróide final secretado pelos folículos depende do perfil das enzimas esteroidogênicas expressas pelo mesmo. Além disso, o estradiol é requerido para a indução da expressão de receptores para o hormônio luteinizante (LH) nas células da granulosa, o que é um pré-requisito para a ovulação. Assim, a alta atividade estrogênica associada com a alta atividade da aromatase é um bom indicador da dominância fisiológica folicular, do mesmo modo que a inibição da atividade da aromatase pode resultar na atresia folicular (BERGFELT et al., 1999).

As ERO's incluem todos os radicais e não radicais derivados do oxigênio, os quais são eletronicamente instáveis e, por isso, altamente reativos, tendo a capacidade de reagir com um grande número de compostos que estejam próximos. Eles podem exercer a função de agentes oxidantes, atuando como receptores de elétrons, ou de agentes redutores, atuando como doadores de elétrons (AGARWAL et al., 2005). Quando há um desequilíbrio entre a produção e a degradação de espécies reativas de oxigênio, ocorre o fenômeno chamado de estresse oxidativo (BEHRMAN, 2001).

O papel das ERO's no estresse oxidativo das fêmeas domésticas permanece incerto. Acredita-se que a atividade enzimática antioxidante esteja relacionada, por exemplo, com a esteroidogênese folícular, uma vez que o folículo dentro do ambiente ovariano encontra-se naturalmente exposto a um certo nível de estresse oxidativo durante a produção hormonal (SUGINO, 2005). Altas concentrações de estrógeno contribuem para uma maior atividade antioxidante, evidenciando que o estresse oxidativo apresenta impacto na produção de hormônios esteróides produzidos pelas células da granulosa, principalmente o estrógeno (LUND et al., 1999). A peroxidação lipídica parece estar envolvida nesse processo, influenciando também a produção de outras glicoproteínas produzidas pelas células da granulosa, como a inibina A, a inibina B, a ativina A e o hormônio antimuelleriano, os quais têm sido investigados como marcadores da resposta ovariana e sua reserva folicular (APPASAMY et al., 2008).

Durante o cultivo *in vitro*, diferentes metabólitos oriundos das células cultivadas podem ser monitorados através da técnica de metabolômica. Estas substâncias podem ser utilizadas como marcadores do perfil celular (GRIFFITHS e WANG, 2009). Os estudos na área de metabolômica fazem parte de uma pesquisa relativamente nova, e servem como um indicador da atividade celular. (PATTI et al., 2012). Aplicações desta técnica consistem em identificar patologias, alvos terapêuticos e investigar mecanismos de processos biológicos fundamentais. Assim, aplicações da metabolômica na biotécnica de MOIFOPA podem estar relacionadas a identificação de diferentes substâncias, as quais podem ser necessárias na suplementação do cultivo, sendo uma nova estratégia para os métodos tradicionais (GOOK et al., 2014).

A sobrevivência, o crescimento e a diferenciação celular podem também ser monitorados pela alteração dos padrões da expressão gênica utilizando-se a biologia molecular (MAZERBOURG e HSUEH, 2006). As técnicas de biologia molecular possibilitam a identificação da expressão de genes e/ou proteínas relacionados ao processo de foliculogênese.

Genes relacionados ao desenvolvimento folicular, como por exemplo, o GDF-9 e o BMP-15, os quais são apontados como importantes reguladores ovarianos (VITT et al., 2002, PENG et al., 2013), atuando no desenvolvimento folicular e oogênese (PAULINI e MELO, 2011). Assim, a quantificação dos níveis de transcrição de genes específicos é fundamental para garantir uma maior avaliação das funções foliculares.

3 JUSTIFICATIVA

A espécie equina possui uma grande importância econômica e social, por compartilhar diversas similaridades com a foliculogênese humana, no que tange a dinâmica das ondas foliculares, mudanças hormonais durante o ciclo estral, relação entre declínio da fertilidade e idade, bem como difunções anovulatórias (GINTHER et al., 2004a, GASTAL et al., 2011, GINTHER et al., 2012), e mais recentemente, em relação a dinâmica de folículos pré-antrais (ALVES et al., 2016b). Neste contexto, a espécie equina emerge como uma excelente alternativa de modelo animal visando auxiliar o desenvolvimento de protocolos no tratamento de infertilidade na espécie humana. Nesse sentido, torna-se de fundamental importância o emprego de biotécnicas que permitam um melhor entendimento do processo de foliculogênese equina, capaz de assegurar a otimização da utilização dos milhares de oócitos presentes no ovário. Além disso, o emprego da biotécnica de MOIFOPA em equinos poderá favorecer a multiplicação de animais de interesse econômico ou ameaçados de extinção.

Dentre as biotécnicas capazes de maximizar o potencial reprodutivo das fêmeas domésticas, a MOIFOPA disponta não somente pelos resultados promissores obtidos em camundongos e em outras espécies domésticas, mas também pela possibilidade de subsidiar outras biotécnicas reprodutivas, como a fertilização e produção *in vitro* de embriões (FIGUEIREDO et al., 2008). Na espécie equina, trabalhos relativos à manipulação *in vitro* de folículos pré-antrais podem ser considerados apenas insipientes. Uma etapa crucial para o sucesso da biotécnica de MOIFOPA é o cultivo *in vitro* de folículos pré-antrais. Neste sentido, a composição do meio incluindo as corretas concentrações dos diferentes suplementos adicionados ao meio de base é essencial para assegurar a sobrevivência e o crescimento folicular, dada a complexa interação entre concentrações, associações e tempos de adição dos fatores no meio de cultivo. Dentre as principais substâncias adicionadas ao meio de cultivo de base, merecem destaque a insulina, o FSH e o EGF, que vem sendo amplamente utilizadas por diferentes pesquisadores, entretanto, apresentando resultados variáveis entre as diferentes espécies (GUTIERREZ et al., 2000; GUPTA et al., 2002; MAO et al., 2004; MATOS et al., 2007; CHAVES et al., 2011; CELESTINO et al., 2009). Apesar da importância dos referidos suplementos, bem como dos achados da literatura referente a influência marcante concentração-dependente de diferentes suplementos utilizados, inexistia

trabalhos na literatura investigando curvas concentração-resposta de insulina, FSH e EGF no cultivo *in vitro* de folículos pré-antrais equinos inclusos em tecido ovariano, constituindo, portanto, a originalidade da presente tese.

Em conjunto, este trabalho se justificou pela necessidade de estabelecer qual as melhores concentrações de insulina, FSH e EGF, bem como a associação destes fatores, a fim de melhorar os resultados obtidos anteriormente na espécie equina, envolvendo o cultivo *in vitro* de folículos pré-antrais. A originalidade dos achados do presente trabalho e, consequentemente, sua importância científica, pode ser comprovada pela publicação de dois artigos científicos em relação ao emprego da insulina (Capítulo I) e FSH (Capítulo II).

4 HIPÓTESES CIENTÍFICAS

Diante do exposto, foram formuladas as seguintes hipóteses científicas:

- O uso de uma concentração de insulina similar a fisiológica (10 ng/mL), melhora a sobrevivência e o desenvolvimento de folículos pré-antrais equinos quando comparada a concentração (10 µg/mL) previamente utilizada na literatura (Fase I).

- A suplementação com FSH (Fase II) e EGF (Fase III) no cultivo *in vitro*, promove a manutenção da sobrevivência e desenvolvimento de folículos pré-antrais equinos de forma concentração-dependente.

- Um meio enriquecido, contendo a associação da insulina, FSH e EGF nas melhores concentrações definidas na presente tese, mantém a morfologia folicular e funcionalidade tecidual após o cultivo *in vitro* de longa duração de fragmento de biópsia ovariana equina (Fase IV).

5. OBJETIVOS

5.1 Objetivo Geral

Avaliar o efeito de diferentes concentrações de insulina, FSH e EGF, bem como da associação destes componentes no cultivo *in vitro* de folículos pré-antrais equinos inclusos em fragmentos de tecido ovariano.

5.2 Objetivos Específicos

1) Investigar o efeito da diferentes concentração fisiológica (10 ng/mL) e suprafisiológica (10 µg/mL) de insulina sobre o percentual de folículos pré-antrais morfologicamente normais, ativação folicular, diâmetro folicular e oocitário, produção hormonal (estradiol e progesterona) e de ROS após 1 e 7 dias de cultivo;

2) Avaliar o efeito concentração-dependente do FSH recombinante bovino (0, 10, 50 e 100 ng/mL) sobre o percentual de folículos pré-antrais morfologicamente normais, ativação folicular, diâmetro folicular e oocitário, produção hormonal (estradiol e progesterona) e de ROS após 1 e 7 dias de cultivo;

3) Estudar o efeito concentração-dependente do EGF (0, 10, 50 e 100 ng/mL) sobre o percentual de folículos pré-antrais morfologicamente normais, ativação folicular, diâmetro folicular e oocitário, produção hormonal (estradiol e progesterona) e de ROS após 1 e 7 dias de cultivo, e perfil metabolômico do meio de cultivo após 7 dias;

4) Avaliar o efeito de um meio de cultivo enriquecido contendo insulina (10 ng/mL) e EGF (50 ng/mL), suplementado ou não com FSH (50 ng/mL), no cultivo *in vitro* de tecido ovariano equino biopsado, tendo como parâmetros a morfologia folicular, produção de estradiol, níveis de

apoptose, expressão para o receptor de EGF e proteína Ki-67, e níveis de RNAm para os genes GDF-9, BMP-15 e Cyclin-D2 após 7 ou 15 dias de cultivo.

6 CAPÍTULO 1

Insulina melhora a sobrevivência *in vitro* de folículos pré-antrais inclusos em tecido ovariano e reduz a produção de espécies reativas de oxigênio após cultivo.

"Insulin improves *in vitro* survival of equine preantral follicles enclosed in ovarian tissue and reduces reactive oxygen species production after culture"

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RESUMO

Este estudo invetigou o efeito da concentração de insulina no cultivo *in vitro* de folículos pré-antrais equinos inclusos em tecido ovariano. Fragmentos ovarianos foram imediatamente fixados (controle não cultivado) ou cultivados por 1 ou 7 dias em α-MEM^{+}suplementado com 0 ng/mL, 10 ng/mL, or 10 µg/mL de insulina. Os fragmentos ovarianos foram processados e analisados para histologia clássica. Amostras de meio de cultivo foram coletadas após 1 e 7 dias para análise de esteróides e espécies reativas de oxigênio (EROs). A percentagem de folículos morfologicamente normais foi maior ($P < 0.001$) nos grupos tratados com insulina após 1 dia de cultivo; Similarmente, mais ($P < 0.02$) folículos normais foram observados após 7 dias de cultivo em meio suplementado com 10 ng/mL de insulina. Além disso, um aumento ($P < 0.01$) nos folículos em crescimento (transição, primários e secundários) entre os dias 1 e 7 de cultivo foi observado somente no tratamento 10 ng/mL de insulina. A produção de EROs após 1 ou 7 dias de cultivo foi menor ($P < 0.0001$) no meio com 10 ng/mL de insulina do que nos demais tratamentos. Os fragmentos ovarianos contendo folículos pré-antrais foram capazes de produzir estradiol e progesterona após 1 e 7 dias de cultivo; contudo os tratamentos não diferiram na produção de esteróides. Como conclusão, o uso de uma concentração fisiológica (10 ng/mL) de insulina, ao invés da concentração previamente relatada (10 µg/mL) para o cultivo *in vitro* de folículos pré-antrais equinos, melhorou a sobrevivência e o crescimento folicular, além de reduzir o estresse oxidativo. Os resultados deste estudo abre novas perspectivas para a produção de meios de cultivo apropriados capazes de incrementar a sobrevivência e o crescimento de folículos pré-antrais equinos.

Palavras - chave: Insulina. Cultivo *in vitro*, folículo pré–antral, espécies reativas de oxigênio, ovário equino.

Insulin improves *in vitro* survival of equine preantral follicles enclosed in ovarian tissue and reduces reactive oxygen species production afterculture

F.L.N. Aguiar [a], F.O. Lunardi[a], L.F. Lima [a], R.M.P. Rocha [a],J.B. Bruno [a], D.M. Magalhães-Padilha [b], F.W. Santos [c], A.P.R. Rodrigues [a], M.O. Gastal [d], E.L. Gastal[d,*], J.R. Figueiredo [a]

[a] *Laboratory of Manipulation of Oocytes and Preantral Follicles (LAMOFOPA), Faculty of Veterinary Medicine, State University of Ceará, Fortaleza, Ceará, Brazil*

[b] *Biotechnology Graduate School, Potiguar University/Laureate International Universities, Natal, Rio Grande do Norte, Brazil*

[c] *Federal University of Pampa, Uruguaiana, Rio Grande do Sul, Brazil*

[d] *Department of Animal Science, Food and Nutrition, Southern Illinois University, Carbondale, Illinois, USA*

*Corresponding author: Eduardo Gastal, Department of Animal Science, Food and Nutrition, Southern Illinois University, 1205 Lincoln Drive, MC 4417, Carbondale, IL, 62901, USA. Tel.: + 618 4531774; fax: + 618 453 5231. *E-mail address*: egastal@siu.edu (E.L. Gastal).

Abstract

This study investigated the effect of insulin concentration on *in vitro* culture of equine preantral follicles enclosed in ovarian tissue. Ovarian fragments were immediately fixed (non-cultured control) or cultured for 1 or 7 days in α-MEM$^+$ supplemented with 0 ng/mL, 10 ng/mL, or 10 µg/mL insulin. Ovarian fragments were processed and analyzed by classical histology. Culture medium samples were collected after 1 and 7 days of culture for steroid and reactive oxygen species (ROS) analyses. The percentage of morphologically normal follicles was greater ($P < 0.001$) in insulin-treated groups after 1 day of culture; likewise, more ($P < 0.02$) normal follicles were observed after 7 days of culture in medium supplemented with 10 ng/mL insulin. Furthermore, an increase ($P < 0.01$) in growing (transition, primary, and secondary) follicles between days 1 and 7 of culture was observed only with the 10 ng/mL insulin treatment. ROS production after 1 or 7 days of culture was lower ($P < 0.0001$) in medium with 10 ng/mL insulin than the other treatments. Ovarian fragments containing preantral follicles were able to produce estradiol and progesterone after 1 and 7 days of culture; however, treatments did not differ in steroid production. In conclusion, the use of a physiological concentration (10 ng/mL) of insulin rather than the previously reported concentration (10 µg/mL) for *in vitro* culture of equine preantral follicles improved follicular survival and growth, and reduced oxidative stress. Results from this study shed light on new perspectives for producing an appropriate medium to improve equine preantral follicle *in vitro* survival and growth.

Keywords: Insulin, *in vitro* culture, preantral follicle, ROS, equine ovary

1. Introduction

The progress of assisted reproductive biotechnologies in horses has been slower than in other domestic animals, mainly because of some technical barriers not present in other species and the deficient acceptance in many breed registries [1]. However, the high economic value of individual animals coupled with changing registry attitudes has resulted in a resurgence of interest and advances on horse assisted reproductive techniques in recent years [1,2]. Studies using equine oocytes have been done with limited numbers of oocytes because of the failure of mares to respond to superovulatory regimes and the scarce availability of horse abattoirs to collect ovaries for research projects. In this regard, the use of matured equine oocytes from *in vitro* cultured preantral follicles (the main oocyte reserve) will potentially contribute to the preservation of genetic material in horses [3] and an increase in the reproductive efficiency of genetically superior animals [4].

Contrary to what has been observed in mice, in which offspring have been produced from *in vitro* cultured preantral follicles [5,6], in farm domestic animals only the production of a few variable number of matured oocytes and embryos has been reported (rat: [7]; pig: [8]; buffalo: [9]; ewe: [10]; goat: [11]). In horses, there are only a few studies evaluating the *in vitro* culture of preantral follicles [12,13,14]. Recently, Haag et al. [14] used *in vitro* culture of ovarian biopsies in medium α-MEM^{+}containing10 µg/mL of insulin and reported that after 7 days of culture 27% of preantral follicleswere morphologically normal.

Insulin plays an important role in the regulation of ovarian function in several species. The presence of the insulin receptor has been identified in different cell types of the ovary [15]. This hormone maintains the viability and growth of ovarian follicles in humans [16], stimulates the production of steroids in ovarian interstitial cells of rats [17], and inhibits gene expressions

that induce apoptosis under conditions of oxidative stress in mice [18]. However, the concentration of insulin used in the medium to culture preantral follicles presents a large variation among species as well as research groups (mice: 0.2 and 0.5 µg/mL [19]; dogs:10 µg/mL [20]; goats:10 ng/mL [21] or 10 µg/mL [22,23]; sheep: 10 µg/mL [24,25]; cattle: 10 ng/mL [26]; and horses: 10 µg/mL [14]). In general, in the majority of studies, including those on horses, a supraphysiological concentration of insulin has routinely been added to a basic culture medium through the use of a commercial product called ITS (10 µg/mL insulin, 5.5 µg/mL transferrin, and 5 ng/mL sodium selenite).

In this context, we hypothesized that the use of lower concentrations of insulin similar to physiological concentrations found in the horse plasma [27] would improve the survival and development of equine preantral follicles. In addition, the effect of insulin on steroid (estradiol and progesterone) and reactive oxygen species (ROS) production after *in vitro* culture by equine preantral follicles enclosed in ovarian tissue has never been investigated up to now.

The aim of this study was to identify what concentration of insulin (10 ng/mL, physiological or 10 µg/mL, supraphysiological) is the most suitable for the *in vitro* culture of equine preantral follicles enclosed in ovarian tissue. To accomplish this goal, the following end points were evaluated: follicular survival, activation of primordial follicles, follicular and oocyte growth, and estradiol, progesterone, and ROS production.

2. Materials and methods

2.1. Chemicals

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

2.2. Animals and ovaries

The research protocol (#12637266-7) was approved by the Ethics and Animal Use Committee (CEUA) of State University of Ceará (UECE), Fortaleza, CE. The ovaries were obtained from euthanized mares (n = 5) positive for Equine Infectious Anemia (EIA). The mares were crossbreed, 3 to 11 years of age, and with body condition scores between 4 and 6 (1, emaciated to 9, obese; [28]).

Immediately after euthanasia, the ovaries were collected and washed in 70% alcohol, followed by two washes in minimum essential medium alpha (α-MEM) supplemented with 25 mM HEPES. The ovaries were placed into tubes containing 20 mL of α-MEM supplemented with 100 μg/mL penicillin and 100 μg/mL streptomycin and transported to the laboratory at 4°C [29] within 4 h. In the laboratory, both ovaries of each animal were stripped of surrounding fat tissue and ligaments. Subsequently, ovarian cortex tissue samples from each pair of ovaries were cut into 8 slices (approximate size, 3 x 3 x 1 mm) under sterile conditions using a scalpel blade.

2.3. Culture of preantral follicles and experimental design

Ovarian tissue was placed in 24-well culture plate containing 1 mL of culture media. Culture was performed at 39°C in a humidified atmosphere with 5% CO_2 in air. Fresh medium was prepared immediately before use and incubated for at least 1 h. The basic culture medium consisted of α-MEM (pH 7.2–7.4) supplemented with 5.5 μg/mL transferrin, 5.0 ng/mL sodium

selenite, 2 mM glutamine, 2 mM hypoxanthine, 1.25 mg/mL bovine serum albumin (BSA), and 100 μg/mL penicillin, and 100 μg/mL streptomycin, which was called α-MEM^{+}.

To test the effect of insulin in cultures of preantral follicles, the basic medium was supplemented with different concentrations of insulin, generating the following groups: 0ng/mL insulin, 10 ng/mL insulin, and 10 μg/mL insulin. The ovarian fragment pieces were then either fixed for histological analyses (fresh, non-cultured control group) or placed in culture for 1 or 7 days. Five replicates of each treatment were performed. The culture medium was replaced every other day, and before each replacement, 1mL of medium was collected at days 1 and 7 of culture and stored at -80°C until hormonal and ROS analyses.

2.4. Morphological evaluation and follicle development

Follicular morphology and development of preantral follicles were assessed *in situ* (ovarian fragments) before and after *in vitro* culture of ovarian tissue. Once harvested, ovarian tissue to be submitted to histological analysis was fixed in paraformaldehyde solution at 4ºC for 12 h and then kept in 70% alcohol. Ovarian fragments were embedded in paraffin wax and cut into serial sections of 7 μm. Samples were stained with periodic acid-Schiff (PAS) and counterstained with hematoxylin. Histology slides were analyzed using light microscopy (Nikon, Tokyo, Japan) at 400X magnification. The end points evaluated were number of follicles, follicle class (primordial, one layer of flattened granulosa cells surrounding the oocyte; transitional, one layer of flattened and cuboidal granulosa cells; primary, one layer of cuboidal granulosa cells; and secondary, two or more layers of cuboidal granulosa cells), follicle morphology, and diameter of follicles and oocytes. To evaluate follicular development, the percentages of normal primordial and developing follicles (transitional, primary, and secondary) were calculated before

(fresh non-cultured control group) and after culture for each treatment. Regarding morphology, follicles were classified as normal (follicle containing an intact oocyte and granulosa cells well organized in layers without pyknotic nucleus) or abnormal (oocyte with pyknotic nucleus, retracted cytoplasm, or disorganized granulosa cells detached from the basement membrane). Only sections where the oocyte nucleus was visualized were considered. Every follicle was examined in each section in which it appeared and matched with the same follicle on adjacent sections to avoid double counting, thereby ensuring that each follicle was counted only once. Follicle and oocyte diameters were measured only in morphologically normal follicles using software (Nis-Element AR 3.0) coupled with an epifluorescence microscope (Zeiss, Cologne, Germany).

2.5. Hormone analyses

To evaluate follicular steroidogenesis in vitro, concentrations of estradiol and progesterone were measured in reserved culture media against standard dilutions using competitive immunoassay commercial kits: IMMULITE 2000 Estradiol (catalog No: L2KE22; Siemens Medical Solutions Diagnostics, 5210 Pacific Concourse Drive Los Angeles, CA, 90045-6900 USA) and IMMULITE 2000 Progesterone (catalog No: L2KPW2; Siemens Medical Solutions Diagnostics, 5210 Pacific Concourse Drive Los Angeles, CA, 90045-6900 USA). The analytical sensitivity of the assay was 15 pg/mL (assay range, 20-2000 pg/mL) for estradiol and 0.1 ng/mL (assay range, 0.2 to 40 ng/mL) for progesterone.

2.6. ROS analysis

The levels of reactive oxygen species were determined in thawed cultured media using a spectrofluorimetric method [30]. Culture media from fresh control and treated groups were incubated with 10 μL of 2',7'-dihidrodiclorofluorescein diacetate (DCHF-DA; 1 mM). The oxidation of DCHF-DA to dichlorofluorescein was measured for detection of reactive species in the medium. The intensity of fluorescence emission was recorded at 520 nm (with 480 nm excitation) for 2 h after addition of DA-DCHF to the medium.

2.7. Statistical analyses

Data for follicular and hormonal end points that were not normally distributed according to a Kolmogorov–Smirnov test were transformed to natural logarithms or rank. Diameters of preantral follicles and oocytes, and concentrations of hormones and ROS were analyzed to determine the main effects of treatment groups, day and for their interaction. The SAS MIXED procedure was used (9.3 Version; SAS Institute Inc., Cary, NC, USA). If a significant effect of treatment or treatment-by-day interaction was detected, the Duncan test for multiple comparisons was used to locate differences in means among groups. If a significant day effect was obtained, differences between means within a group were examined by unpaired Student's t-tests. Chi-square analysis was used to compare the percentage of morphologically normal and growing preantral follicles among groups within each day, and between days within each group. A probability of $P \leq 0.05$ indicated that a difference was significant, and probabilities between $P > 0.05$ and ≤ 0.1 indicated that a difference approached significance. Data are presented as the mean ± SEM, unless otherwise indicated.

3. Results

3.1. Number of follicles evaluated

A total of 167, 385, 444, and 341 preantral follicles were evaluated on the fresh non-cultured control, 0 ng/mL insulin, 10 ng/mL insulin, and 10 µg/mL insulin treatment groups, respectively. On average, 267.4 ± 23.5 follicles were evaluated per replicate.

3.2. Effect of insulin on follicular morphology

The percentage of morphologically normal follicles in the 0 ng/mL insulin treatment was reduced ($P < 0.05$) after 1 or 7 days of culture when compared to the non-cultured control group (Table 1). Regarding the 10 ng/mL and 10 µg/mL insulin treatments, a similar reduction ($P < 0.05$) was observed only on day 7 of culture. After 1 day of culture, both 10 ng/mL and 10 µg/mL insulin-treated groups had a greater ($P < 0.002$) percentage of normal follicles than the 0 ng/mL insulin treatment. After 7 days of culture, the 10 ng/mL insulin treatment had a greater ($P < 0.002$) percentage of normal follicles than the other two treatments tested. A reduction ($P < 0.05$) in the percentage of normal follicles between days 1 and 7 of culture was observed in all treatments tested.

Table 1
Percentage of morphologically normal equine preantral follicles in fresh noncultured control group and after *in vitro* culture for 1 or 7 days in the absence or presence of different concentrations of insulin.

	Percentage of normal follicles (n)	
Fresh noncultured control	64.1 (107/167)	
Insulin treatments	Day 1	Day 7
0 ng/mL	32.2 (64/199)^{c,A,a}	15.1 (28/186)^{c,A,b}
10 ng/mL	55.1 (113/205)^{d,B,a}	36.8 (88/239)^{c,B,b}
10 μg/mL	55.8 (92/165)^{d,B,a}	22.2 (39/176)^{c,A,C,b}

^{A,B,C}Within a column, uncommon superscripts differed ($P < 0.001$–0.0001).
^{a,b}Within a row, uncommon superscripts differed ($P < 0.0001$).
^{c}Insulin treatment differed ($P < 0.001$) from the fresh noncultured control. Furthermore, on Day 7, the 10-μg/mL insulin treatment tended ($P < 0.08$) to be different from the 0-ng/mL insulin treatment.
^{d}Insulin treatment approached ($P < 0.08$–0.1) to be different from the fresh noncultured control.

3.3. Follicular development after in vitro culture

The percentages of primordial and developing follicles are shown (Fig. 1). After 1 and 7 days of culture, all treatments had a reduction ($P < 0.0001$) in the percentage of primordial follicles and an increase ($P < 0.0001$) in the percentage of growing follicles when compared to the fresh non-cultured control. However, no difference ($P > 0.05$) was observed within days for the percentage of primordial and growing follicles among the treatments tested. An increase ($P < 0.01$) in the percentage of developing follicles from day 1 to day 7 of culture was observed only with the 10 ng/mL insulin treatment.

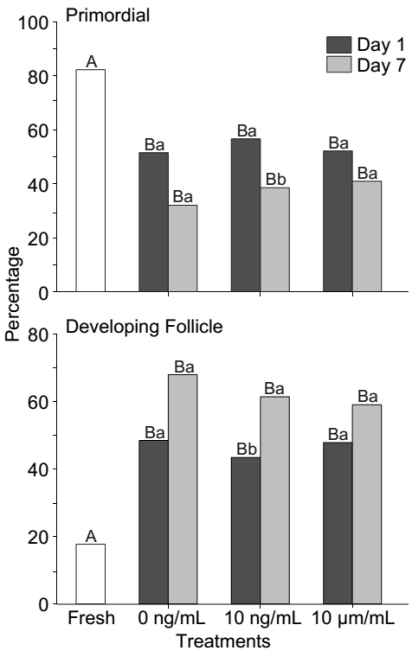

Figure 1. Percentage of primordial and developing follicles (transitional, primary, and secondary) in a fresh non-cultured control group and after *in vitro* culture for 1 or 7 days in the absence or presence of different concentrations of insulin (0 ng/mL, 10 ng/mL, or 10 µg/mL). [a,b]Within each treatment, values without a common letter differed ($P < 0.02$). [A,B]Within days (day 0 =fresh non-cultured control group; days 1 and 7 = insulin treated groups), values without a common letter differed ($P < 0.05$).

After days 1 and 7 of culture, the follicular and oocyte diameters were smaller ($P < 0.0001$) for all treatments when compared to the fresh non-cultured control group, except for the 10 µg/mL insulin treatment on day 1 of culture (Fig. 2). The 10 µg/mL insulin group also had greater ($P < 0.05$) follicle and oocyte diameters than the other treatments on day 1 of culture. However, at day 7of culture the 10 ng/mL and 10 µg/mL insulin treatments had greater ($P < 0.0001$) follicular diameter when compared with the 0 ng/mL insulin treatment. When comparing days 1 and 7 of culture, the 10 ng/mL insulin treatment was the only one that maintained the

follicular diameter; however, the oocyte diameter decreased ($P < 0.05$) only in the 10 µg/mL insulin treatment between days 1 and 7 of culture.

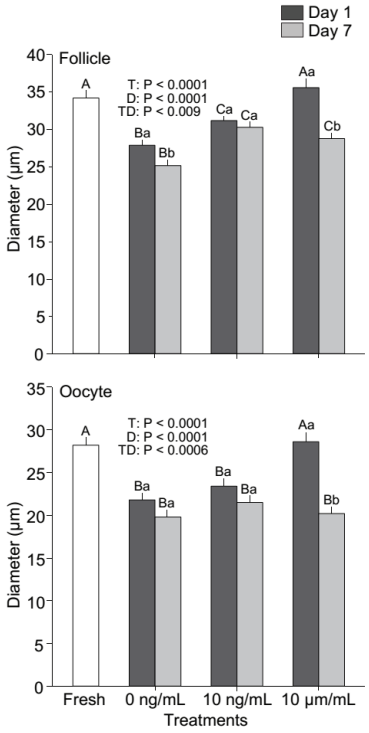

Figure 2. Mean (± SEM) diameters (µm) of preantral follicles (primordial and primary combined) and oocytes in a fresh non-cultured control group and after *in vitro* culture for 1 or 7 days in the absence or presence of different concentrations of insulin (0 ng/mL, 10 ng/mL, or 10 µg/mL). [a,b]Within each treatment, values without a common letter differed ($P < 0.03$-0.0001). [A,B]Within days (day 0 = fresh non-cultured control group; days 1 and 7 = insulin treated groups), values without a common letter differed ($P < 0.0001$).

3.4. Hormone production

Estradiol and progesterone production obtained from the culture media after days 1 and 7 of culture of equine preantral follicles enclosed in ovarian tissue are shown (Fig. 3). Concentrations of estradiol and progesterone were not different ($P > 0.05$) within each day of culture for all treatments evaluated. However, a significant reduction ($P < 0.0001$) of both hormones was observed between days 1 and 7 of culture in all treatments.

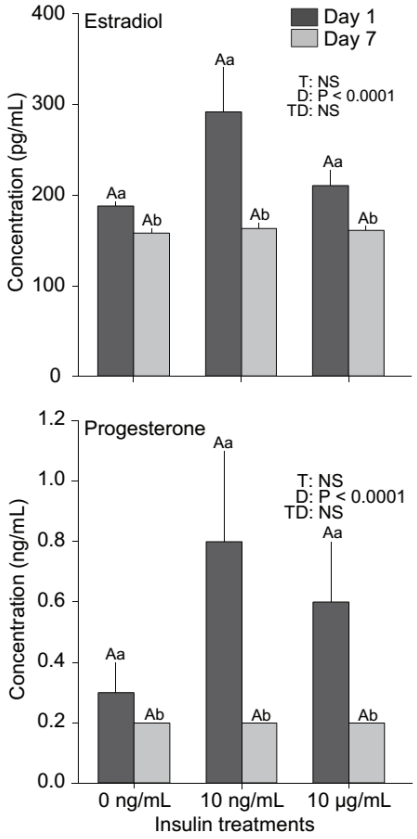

Figure 3. Mean (± SEM) concentrations of estradiol (pg/mL) or progesterone (ng/mL) produced in culture medium after 1 or 7 days of culture of equine ovarian follicles enclosed in ovarian tissue in the absence or presence of different concentrations of insulin. [a,b]Within each treatment, values without a common letter differed ($P < 0.05$). [A]Within each day, no difference was observed among treatments for estradiol and progesterone.

3.5. Reactive oxygen species (ROS) production

A decrease (P < 0.0001) in ROS production was observed between days 1 and 7 of culture in all treatments (Fig. 4). However, on days 1 and 7 of culture, the 10 ng/mL insulin treatment showed a lower (P < 0.0001) production of ROS when compared to the other treatments. Moreover, at day 1 of culture, the 10 µg/mL insulin treatment had a higher (P < 0.0001) production of ROS when compared to the other treatments.

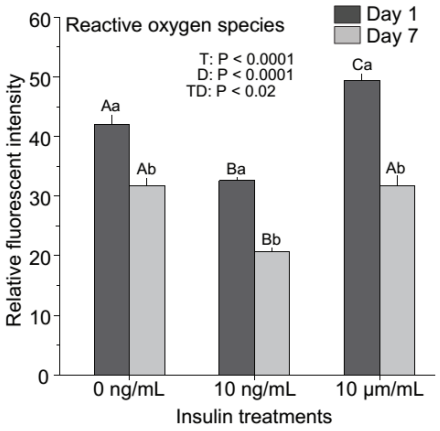

Figure 4. Mean (± SEM) production of reactive oxygen species (relative fluorescence units) produced in cultured medium after 1 or 7 days of culture of equine preantral follicles enclosed in ovarian tissue in the absence or presence of different concentrations of insulin. [a,b]Within each treatment, values without a common letter differed (P < 0.0001). [A,B]Within days, values without a common letter differed (P < 0.0001).

4. Discussion

This study showed for the first time that the concentration of insulin affects the *in vitro* culture of equine preantral follicles enclosed in ovarian tissue. Moreover, it revealed that equine

ovarian cortex containing preantral follicles, when cultured in vitro, had steroidogenic activity (estradiol and progesterone production).

The addition of a lower (physiological; 10 ng/mL) concentration of insulin to the culture medium induced greater follicular survival when compared to the treatment without insulin and the treatment with higher (supraphysiological; 10 µg/mL) concentration of insulin. Even though insulin has been used as an important constituent of culture medium for several studies in different species [31,32,33,34,35], the suitable concentration of this hormone is still controversial. In mares, Haag et al. [14] obtained only a 27% of follicular survival rate after 7 days of culture using a similar type of medium of the current study but with insulin at a concentration of 10 µg/mL. The present study improved the follicular survival rate (37%) and reduced the oxidative stress (ROS) using a lower concentration (physiological) of insulin. This finding corroborates previous reported results in goats [21] and cattle [36], in which the *in vitro* culture of preantral follicles in the presence of 10 ng/mL insulin resulted in higher rates of follicular survival. Sun et al. [19] demonstrated that insulin concentrations ranging from 0.2 – 0.5 µg/mL favored follicular survival and growth *in vitro*, and higher insulin concentrations (>1 µg/mL) resulted in deleterious effects on folliculogenesis and oogenesis in mice. However, in dogs [20], a 10µg/mL insulin concentration increased the percentage of viable follicles when compared to the medium without insulin or with lower (5 and 10 ng/mL) concentrations of insulin. Therefore, as stated above, the results of an ideal concentration of insulin for culture of preantral follicles have been controversial and might be influenced by several factors, such as *in vitro* culture conditions.

In the present study, the highest concentration of insulin tested (10 µg/mL) only tended to improve follicular survival. The binding of insulin to its receptors decreases in the presence of

high concentrations of this hormone [37]. Thus, high concentrations of insulin may reduce the ability for insulin-receptors binding in cultured ovarian tissue and consequently reduce follicular survival. Therefore, the addition of an appropriate concentration of insulin to the culture medium is important because it promotes cell survival through the modulation of intracellular kinases, such as Akt [38,39]. The Akt phosphorylates members of the forkheadbox protein transcription factor (FOXO), which inhibits the transcription of genes involved in apoptosis (e.g., Bim, Trail, FasL, Caspase-3; [18]).

Herein, the maintenance of follicular survival seemed to be related to a decrease in production of ROS, because ROS production was lower in the 10 ng/mL insulin treatment when compared to the other treatments. This novel finding might be explained because moderate concentrations of ROS have been able to stimulate the proliferation of theca and interstitial ovarian cells [40]. The production of high concentrations of ROS in the *in vitro* culture environment, as observed in the 10 µg/mL insulin treatment in the present study, might have caused deleterious effects to the follicles [41,42]. Furthermore, as previously reported [43], in cases of hyperinsulinemia there is an increase of ROS production with a reduction in the production of glutathione and induction of apoptosis in oocytes.

After 7 days of culture, all treatments had a significant reduction in the percentage of primordial follicles and a concomitant significant increase in the percentage of developing follicles. However, no difference was detected within days 1 and 7 for the concentrations of insulin tested, demonstrating that insulin did not affect follicular development. It has been known that *in vivo* follicular development is a process modulated by inhibitory factors that keep the preantral follicles dormant [44]. However, in *in vitro* culture, a reduction of the inhibitory action of some growth factors and/or hormones, such as the anti-Müllerian hormone, can occur and lead

to spontaneous preantral follicle activation by the action of autocrine and paracrine mechanisms [45]. Moreover, the medium used in the current experiment (α-MEM^{+}) contained a rich composition of vitamins, inorganic salts, amino acids [46], and essential substances (e.g., glutamine, hypoxanthine, BSA, transferrin, and selenium) for follicular development. This complex supplementation may also have been responsible for follicular development observed in all treatments.

This study showed for the first time that fragments of equine ovarian tissue containing preantral follicles when cultured *in vitro* exhibited steroidogenic activity. In the present study, after *in vitro* culture for 7 days, there was a significant reduction in the concentration of these hormones in all treatments. This finding might be explained by the fact that larger follicles, which have a higher steroidogenic activity, degenerate before smaller follicles, which have a lower steroidogenic capacity. Although aromatase activity has been reported in small preantral follicles, estradiol production at this developmental stage is limited by the inability of those follicles to produce substrates required for androgen aromatization for estradiol [47]. In addition, it has been reported [48] that cells of the ovarian stroma are capable of producing steroids. However, some stromal cells may degenerate during the culture period, decreasing the production of steroids.

In conclusion, the use of a physiological concentration (10 ng/mL) of insulin rather than the previously reported concentration (10 µg/mL) for *in vitro* culture of equine preantral follicles improved follicular survival and growth, and reduced oxidative stress. Results from this study shed light on new perspectives for producing an appropriate medium to improve equine preantral follicle *in vitro* survival and growth.

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7. CAPÍTULO 2

Hormônio folículo estimulante mantém a sobrevivência e promove o desenvolvimento de folículos pré-antrais equinos inclusos em tecido ovariano.

"Follicle stimulating hormone maintains *in vitro* survival and promotes development of equine preantral follicles enclosed in ovarian tissue"

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RESUMO

Este estudo investigou o efeito da adição de diferentes concentrações de hormônio folículo estimulante bovino no cultivo *in vitro* de folículos pré-antrais equinos inclusos em fragmentos de tecido ovariano. Fragmentos ovarianos aleatórios foram fixados imediatamente (controle fresco não cultivado) ou cultivados por 1 ou 7 dias em α-MEM^{+} suplementado com 0, 10, 50, e 100 ng/mL de FSH, os quais foram posteriormente analisados por histologia clássica. O meio de cultivo coletado no dia 1 ou 7 foi analizado para os esteróides estradiol e progesterona e para as espécies reativas de oxigênio (EROs). Após o dia 1 e dia 7 de cultivo, 50 ng/mL de FSH teve uma maior ($P < 0.05$) percentagem de folículos morfologicamente normais quando comparado com os outros grupos, exceto o tratamento 10 ng/mL de FSH no dia 1 de cultivo. A percentagem de folículos em desenvolvimento (transição, primário e secundário) e o diâmetro folicular e oocitário foram maiores ($P < 0.05$) no tratamento 50 ng/mL de FSH quando comparados com de outros grupos após 7 dias de cultivo. Adicionalmente, a secreção de estradiol e a produção de espécies reativas de oxigênio foram mantidas ($P > 0.05$) ao longo do cultivo no tratamento 50 ng/mL de FSH. Como conclusão, a adição de 50 ng/mL de FSH promoveu a ativação de folículos primordiais para folículos em desenvolvimento, aumentou a sobrevivência de folículos pré-antrais e manteve a produção de estradiol e EROs de tecido ovariano equino após 7 dias de cultivo.

Palavras - Chave: Ovário equino, FSH, Cultivo in vitro, folículo pré–antral.

FSH supplementation to culture medium is beneficial for activation and survival of preantral follicles enclosed in equine ovarian tissue

F.L.N. Aguiar [a*], F.O. Lunardi [a], L.F. Lima [a], R.M.P. Rocha [a]; J.B. Bruno [a], D.M. Magalhães-Padilha [b], F.W.S. Cibin [c], D.C.S Nunes-Pinheiro [a], M.O. Gastal [d], A.P.R. Rodrigues [a], G.A. Apgar [d], E.L. Gastal [d,*], J.R. Figueiredo [a]

[a] *Laboratory of Manipulation of Oocytes and Preantral Follicles (LAMOFOPA), State University of Ceará, Fortaleza, Ceará, Brazil*

[b] *Postgraduate biotechnology, Potiguar University/Laureate International Universities, Natal, RN, Brazil.*

[c] *Federal Universityof Pampa, Uruguaiana, RS, Brazil.*

[d] *Department of Animal Science, Food and Nutrition, Southern Illinois University, Carbondale, Illinois, USA*

*Corresponding author: Eduardo Gastal, Department of Animal Science, Food and Nutrition, Southern Illinois University, 1205 Lincoln Drive, MC 4417, Carbondale, IL, 62901, USA. Tel.: + 618 453 1774; fax: + 618 453 5231. *E-mail address*: egastal@siu.edu (E.L. Gastal).

Abstract

This study investigated the effect of adding different concentrations of bovine recombinant follicle stimulating hormone (rbFSH) on the *in vitro* culture of equine preantral follicles enclosed in ovarian tissue fragments. Randomized ovarian fragments were fixed immediately (fresh non-cultured control) or cultured for one or seven days in α-MEM^{+}supplemented with 0, 10, 50, and 100 ng/mL FSH, and subsequently analyzed by classical histology. Culture media collected on Day 1 or Day 7 and were analyzed for steroids (estradiol and progesterone) and reactive oxygen species (ROS). After Day 1 and Day 7 of culture, FSH 50 ng/mL treatment had a greater ($P < 0.05$) percentage of morphologically normal follicles when compared to the other groups, except the FSH 10 ng/mL treatment at Day 1 of culture. The percentage of developing follicles (transition, primary, and secondary), and follicular and oocyte diameters were higher ($P < 0.05$) in the 50 ng/mL FSH treatment compared to the other groups after Day 7 of culture. Furthermore, estradiol secretion and ROS production were maintained ($P > 0.05$) throughout the culture in the 50 ng/mL FSH treatment. In conclusion, the addition of 50 ng/mL of FSH promoted activation of primordial follicles to developing follicles, improved survival of preantral follicles, and maintained estradiol and ROS production of equine ovarian tissue after seven days of culture.

Keywords: FSH, *in vitro* culture, preantral follicles, equine ovary

1. Introduction

Assisted reproductive biotechnologies in horses have largely been used to maximize reproductive potential in valuable or endangered horses [1,2]. However, the efficiency of assisted reproduction is hampered due to reduced recovery of fertilizable oocytes. One approach to improve reproductive efficiency is the use of *in vitro* culture of ovarian preantral follicles [3].

Several substances have been shown to improve *in vitro* survival and development of preantral follicles in domestic animals, including hormones (FSH [4]; insulin [5]) and growth factors (growth differentiation factor-9 (GDF-9[6]); bone morphogenetic protein-15 (BMP-15[7]); epidermal growth factor (EGF[8]). Among these substances, the role of FSH in *in vitro* follicle culture [9,10,11] is remarkable.

FSH elicits effects by binding to its receptors located in primordial and primary preantral follicles in goats [12] and sheep [13], and in horses, in primordial, primary, secondary, and tertiary folliclesas well as in corpus luteum [14]. These findings suggest that FSH seems to be important even during early folliculogenesis (preantral follicular phase). Reports have shown that FSH added to culture medium maintains follicle viability and promotes the development of isolated preantral follicles and antrum formation in a concentration-dependent manner in different species (primate:[15]; bovine:[16]; human:[17]; caprine:[18]; ovine:[19]). Furthermore, FSH can act indirectly by stimulating the expression of kit-ligand (KL), GDF-9 and BMP-15 [20,21] which play important roles in folliculogenesis.

In vitro culture studies of preantral follicles enclosed in ovarian tissue fragments (*in situ* culture) have shown that the ideal concentration of FSH to be added in culture to improve follicle survival and development varies according to species (50 ng/mL, caprine:[22,23]; 100 ng/mL, ovine:[24]; 100 ng/mL, canine:[25]; 50 ng/mL, bovine:[26]; 25 mIU/mL, murine:[27]). However,

despite the importance of FSH on early folliculogenesis in many species, the impact of FSH on *in vitro* development of equine preantral follicles enclosed in ovarian tissue remains unknown.

Therefore, the objective of this paper was to evaluate the effect of different concentrations of FSH (0, 10, 50, and 100 ng/mL) on the survival, activation, hormone production (estradiol and progesterone), and reactive oxygen species (ROS) generation after *in vitro* culture of equine preantral follicles enclosed in ovarian tissue fragments.

2. Materials and methods

2.1. Chemicals

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

2.2. Animals and ovaries

This research protocol (#12637266-7) was approved by the Ethics and Animal Use Committee of the State University of Ceará (UECE), Fortaleza, CE. The ovaries were harvested from euthanized mares (n=5) positive but asymptomatic for Equine Infectious Anemia (EIA), during September to January, in the state of Ceará, Brazil. The mares were crossbred, 6 to 10 years old (mean, 7.2 ± 1.9 years), and had body condition scores between 4 and 6 (1, emaciated to 9, obese [28]). Dental characteristics [29] were used to estimate the age of the mares. At the time of ovary harvesting, mares were non-pregnant and cycling, based on the presence of large (maturing/growing) or small (regressing) corpus luteum and preovulatory (> 30 mm) follicle in one or both ovaries.

Immediately after euthanasia, the ovaries were collected and washed in 70% alcohol, followed by two washes in minimum essential medium (MEM) supplemented with 25 mM HEPES. The ovaries were placed into tubes containing 100 mL of MEM supplemented with 100 µg/mL penicillin and 100 µg/mL streptomycin and transported to the laboratory at 4°C within 4 h [30]. In the laboratory, the ovaries of each animal were stripped of surrounding fat tissue and ligaments. Subsequently, 72 ovarian tissue samples (approximate size, 3 x 3 x 1 mm) were obtained from each pair of ovaries under sterile conditions using a scalpel blade. Eight ovarian tissues samples were distributed for each treatment per day in five replicates.

2.3. Culture of preantral follicles and experimental design

Ovarian tissue was placed in 24-well culture plate containing 1 mL of culture media. Culture was performed at 39°C in a humidified atmosphere with 5%CO_2 in air. Fresh medium was prepared and incubated for at least 1 h before use. The basic culture medium consisted of α-MEM (pH 7.2–7.4) supplemented with 2 mM glutamine, 2 mM hypoxanthine, 1.25 mg/mL bovine serum albumin (BSA), 10 ng/mL insulin, 5.5 µg/mL transferrin, 5 ng/mL selenium, 100 µg/mL penicillin, and 100 µg/mL streptomycin, which is called α-MEM$^+$.

To test the effect of FSH in the culture of preantral follicles, the basic medium (control) was supplemented with increasing concentrations of bovine recombinant FSH (rbFSH®; NANOCORE, Campinas, SP, Brazil) generating the following treatments: 0 ng/mL, 10 ng/mL, 50 ng/mL, and 100 ng/mL. The ovarian fragment pieces were then either fixed for histological analyses (fresh, non-cultured control group) or placed in culture for one or seven days. Five replicates were performed for each treatment. The culture media were replaced every other day,

and before each replacement 1 mL of medium was collected at Day 1 and Day 7 of culture, and stored at -80°C for hormonal and ROS analyses.

2.4. Morphological evaluation and follicle development

Follicular morphology and development of preantral follicles were assessed *in situ* (ovarian fragments) before and after *in vitro* culture of ovarian tissue. Once harvested, ovarian tissue submitted to histological analysis was fixed in paraformaldehyde solution at 4°C for 12 h and kept in 70% alcohol. Ovarian fragments were dehydrated by use of a graded series of alcohol, embedded in paraffin wax, and cut into serial sections at 10 μm. Samples were stained with periodic acid-Schiff (PAS) and counterstained with hematoxylin. Histology slides were analyzed using light microscopy (Nikon, Tokyo, Japan) at × 400 magnification. The histological evaluation and classification of the follicles were performed by only one operator who was blind to the treatments, although other five trained helpers, also blind to the treatments, have been used to find preantral follicles on the histological sections of each fragment.

The end points evaluated were number of follicles, follicle class (primordial, one layer of flattened pre-granulosa cells surrounding the oocyte; transitional, one layer of flattened and cuboidal granulosa cells; primary, one layer of cuboidal granulosa cells; and secondary, two or more layers of cuboidal granulosa cells;[17]), follicle morphology, and diameter of follicles and oocytes.

To evaluate follicular development, the percentage of both normal primordial and developing follicles (transitional, primary, and secondary; Fig. 1) were calculated before (fresh non-cultured control group) and after culture for each treatment. Regarding morphology, follicles were classified as normal (follicle containing an intact oocyte and granulosa cells well-organized

in layers without pyknotic nucleus) or abnormal (oocyte with pyknotic nucleus, retracted cytoplasm, or disorganized granulosa cells detached from the basement membrane). Every follicle was examined in each section in which it appeared and matched with the same follicle on adjacent sections to avoid double counting, thereby ensuring that each follicle was counted only once.

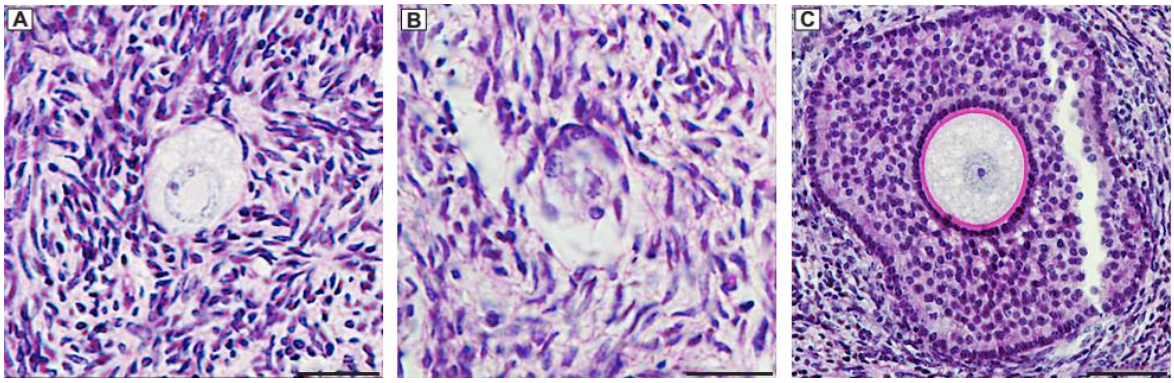

Figure 1. Morphological aspects of preantral follicles after seven days of culture in FSH 50 ng/mL treatment. (A) normal primordial follicle, (B) abnormal transitional follicle, and (C) secondary normal follicle. Bars = 25 µm (A, B) and 50 µm (C).

Follicle and oocyte diameters were measured only in morphologically normal follicles using software (Nis-Element AR 3.0) coupled with an epifluorescence microscope (Zeiss, Cologne, Germany). Follicle diameter was measured from one edge to the other edge of the outermost layer of granulosa cells. Oocyte diameter was measured from one edge of the oocyte membrane to the other. Two perpendicular diameters were recorded for each measurement and the average of those two values was calculated.

2.5. Hormone analyses

To evaluate follicular steroidogenesis *in vitro*, concentrations of estradiol and progesterone were measured in reserved culture media against standard dilutions, according to manufacturer's instructions, using competitive immunoassay commercial kits: IMMULITE 2000 Estradiol

(catalog No: L2KE22 Siemens Medical Solutions Diagnostics, 5210 Pacific Concourse Drive, Los Angeles, CA, USA) and IMMULITE 2000 Progesterone (catalog No: L2KPW2 Siemens Medical Solutions diagnostics, 5210 Pacific Concourse Drive, Los Angeles, CA, USA). The two assays have been previously used and validated for different species, including the horse [31,32]. The analytical sensitivity of the assay was 15 pg/mL (assay range, 20-2000 pg/mL) for estradiol and 0.1 ng/mL (assay range, 0.2 to 40 ng/mL) for progesterone.

2.6. ROS analysis

The levels of reactive oxygen species were determined in reserved culture media using a spectrofluorimetric method [33]. Culture media from all FSH-treated groups were incubated with 10 µl of 2', 7'-dichlorofluorescin diacetate (DCHF-DA; 1 mM). The oxidation of DCHF-DA to dichlorofluorescein was measured for detection of reactive species in the medium. The intensity of fluorescence emission was recorded at 520 nm (with 480 nm excitation) for 2 h after addition of the DCHF-DA to the medium.

2.7. Statistical analyses

Follicular and oocyte diameter end points were not normally distributed according to a Kolmogorov–Smirnov test and were transformed to natural logarithms and ranks, respectively. Diameters of preantral follicles and oocytes, and concentrations of hormones and ROS were analyzed to determine the main effects of treatment groups, day, and their interaction. One-way ANOVA and SAS PROC MIXED procedure were used (9.3 Version; SAS Institute Inc., Cary, NC, USA). If a significant effect of treatment or treatment-by-day interaction was detected, a Duncan test for multiple comparisons was used to discern differences in means among groups. If

a significant day effect was obtained, differences between means within a group were examined by unpaired Student's t-tests. Chi-square analysis was used to compare the percentage of morphologically normal and growing preantral follicles among groups within each day, and between days within each group. A probability of $P \leq 0.05$ indicated that a difference was significant, and probabilities between $P > 0.05$ and ≤ 0.1 indicated that a difference approached significance. Data are presented as the mean ± SEM, unless otherwise indicated.

3. Results

3.1. Number of fragments and follicles evaluated

A total of 3,600 slides with 36,000 tissue sections were produced from 360 ovarian fragments. At least 30 follicles (range, 30-54 follicles) were evaluated per treatment and per day in each replicate, allowing each animal to contribute in a similar way to the FSH treatments. A total of 206, 337, 373, 345, and 342 preantral follicles were evaluated on the fresh non-cultured control, 0 ng/mL FSH, 10 ng/mL FSH, 50 ng/mL FSH, and 100 ng/mL FSH treatment groups, respectively. Altogether, 1603 follicles were evaluated with an average, 320.6 ± 29.3 follicles were evaluated per treatment.

3.2. Effect of FSH on follicular morphology

The percentage of morphologically normal follicles was reduced after Day 1 and Day 7 of culture ($P < 0.01$) when compared to fresh non-cultured control (Table 1). Moreover, after Day 1 and Day 7 of culture, the 50 ng/mL FSH treatment had a higher ($P < 0.05$) percentage of normal follicles than the other treatments, except for the 10 ng/mL FSH at Day 1 of culture. After Day 7

of culture, FSH 50 ng/mL tended ($P < 0.07$) to differ from the 10 ng/mL treatment. A reduction ($P < 0.003$) in the percentage of normal follicles was observed in all treatments from Day 1 to Day 7 of culture.

Table 1
Percentage of morphologically normal equine preantral follicles in fresh noncultured ovarian tissue fragments and after IVC for 1 or 7 days in media supplemented with different concentrations of bovine recombinant follicle-stimulating hormone.

	Percentage of normal follicles (n)	
Fresh noncultured control	76.2 (157/206)	
FSH treatments	Day 1	Day 7
0 ng/mL	52.2 (94/180)[c,A,C,a]	31.2 (49/157)[c,A,b]
10 ng/mL	59.1 (110/186)[c,A,B,a]	32.6 (61/187)[c,A,b]
50 ng/mL	65.8 (100/152)[c,B,a]	41.4 (80/193)[c,B,d,b]
100 ng/mL	45.1 (78/173)[c,C,a]	29.6 (50/169)[c,A,b]

[A,B,C]Within days, uncommon uppercase superscripts differed ($P < 0.05$–0.008).
[a,b]Within treatments, uncommon lowercase superscripts differed ($P < 0.003$–0.0001).
[c] Values differed ($P < 0.01$) from the fresh noncultured control.
[d] Tended ($P < 0.07$) to differ from the 10 ng/mL treatment on Day 7.

3.3. Follicular development after in vitro culture

The percentages of primordial and developing follicles are shown (Fig. 2). After Day 1 of culture, the 10 ng/mL FSH and 50 ng/mL FSH treatments decreased ($P < 0.02$) the percentage of primordial follicles and increased the percentage of growing follicles when compared to the fresh non-cultured control group. In all treatments, after Day 7 of culture, the percentage of primordial follicles decreased ($P < 0.02$) and the percentage of growing follicles increased ($P < 0.02$) when compared to fresh non-cultured control. The 50 ng/mL FSH treatment had the highest ($P < 0.05$) rate of growing follicles on Day 7 of culture among all treatments. All treatments had a decrease ($P < 0.005$) in percentage of primordial follicles and an increase in developing follicles during *in vitro* culture.

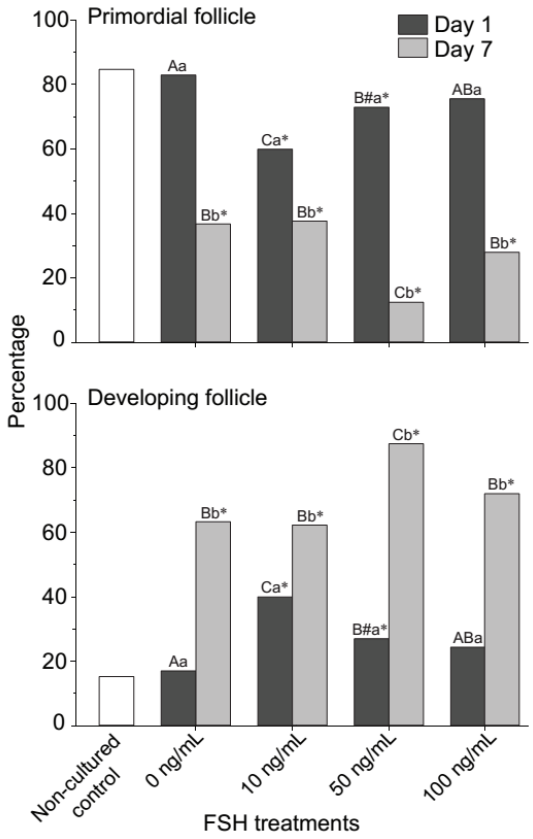

Figure 2. Percentage of primordial and developing follicles (transitional, primary, and secondary; n=779) in fresh non-cultured ovarian tissue fragments and after in vitro culture for one or seven days in media supplemented with different concentrations of rbFSH (mean, 86.5 follicles/treatment/day). [a,b]Within each treatment, values without a common letter differed ($P < 0.005$). [A,B,C]Within days, values without a common letter differed ($P < 0.05$). [#]Tended ($P < 0.09$) to differ from 0 ng/mL FSH treatment at one day. *Differed ($P < 0.02$) from fresh non-cultured control.

After Day 1 of culture, the 50 ng/mL FSH was the treatment that maintained follicular and oocyte diameters similar ($P > 0.05$) to fresh non-cultured control group. In addition, the 50 ng/mL FSH treatment showed greater ($P < 0.0001$) follicular and oocyte diameter than the other FSH treatments, except for the oocyte diameter in the 0 ng/mL FSH treatment (Table 2, 3). However, after Day 7 of culture, all treatments had lower ($P < 0.0001$) follicular and oocyte diameters when

compared to the fresh non-cultured control. After Day 7of culture, the FSH 50 ng/mL treatment had greater ($P < 0.05$) follicular and oocyte diameters than all other FSH treatments. Regardless of treatment, follicular and oocyte diameters decreased ($P < 0.03$) from Day 1 to Day 7 of culture.

Table 2
Mean (±standard error of the mean) diameters of equine preantral follicles (primordial, transitional, and primary combined) in fresh noncultured ovarian tissue fragments and after IVC for 1 or 7 days in media supplemented with different concentrations of bovine recombinant follicle-stimulating hormone.

	Diameter (μm)	
Fresh noncultured control	38.5 ± 0.3	
FSH treatments	Day 1	Day 7
0 ng/mL	35.1 ± 0.6^{c,B,a}	28.8 ± 0.6^{c,B,b}
10 ng/mL	34.8 ± 0.8^{c,B,a}	30.9 ± 0.7^{c,B,b}
50 ng/mL	39.1 ± 1.3^{A,a}	35.8 ± 1.0^{c,A,b}
100 ng/mL	33.0 ± 0.8^{c,B,a}	30.1 ± 0.6^{c,B,b}

^{A,B}Within days, uncommon uppercase superscripts differed ($P < 0.0001$).
^{a,b}Within treatments, uncommon lowercase superscripts differed ($P < 0.03$–0.0001).
^{c} Values differed ($P < 0.0001$) from the fresh noncultured control.

Table 3
Mean (±standard error of the mean) diameters of equine oocytes from preantral follicles (primordial, transitional, and primary combined) in fresh noncultured ovarian tissue fragments and after IVC for 1 or 7 days in media supplemented with different concentrations of bovine recombinant follicle-stimulating hormone.

	Diameter (μm)	
Fresh noncultured control	30.6 ± 0.4	
FSH treatments	Day 1	Day 7
0 ng/mL	28.8 ± 0.5^{A,a}	22.1 ± 0.6^{c,B,b}
10 ng/mL	26.7 ± 0.7^{c,B,a}	23.9 ± 0.7^{c,B,b}
50 ng/mL	30.9 ± 1.0^{A,a}	27.9 ± 0.9^{c,A,b}
100 ng/mL	26.3 ± 0.7^{c,B,a}	23.3 ± 0.5^{c,B,b}

^{A,B}Within days, uncommon uppercase superscripts differed ($P < 0.0001$).
^{a,b}Within treatments, uncommon lowercase superscripts differed ($P < 0.03$–0.0001).
^{c} Values differed ($P < 0.0001$) from the fresh noncultured control.

3.4. Estradiol and progesterone analyses

Estradiol production was not different among treatments ($P > 0.05$; Fig.3). However, estradiol concentration decreased ($P < 0.0001$) in all FSH treatments from Day 1 to Day 7 of culture, except for the 50 ng/mL treatment. Progesterone production in the culture media was

detected in a small number of samples (19 out of 160; data not shown). Therefore, data were not analyzed statistically.

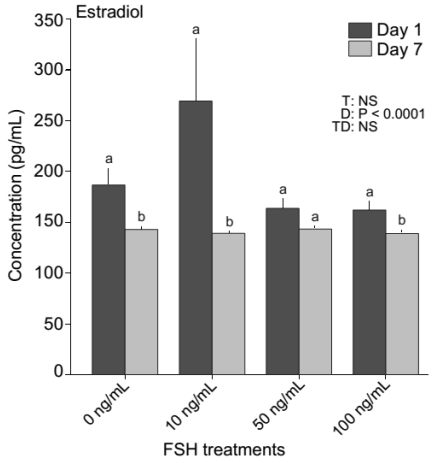

Figure 3. Mean (± SEM) concentrations of estradiol (pg/mL; n= 160 samples) produced by equine ovarian tissue fragments after in vitro culture for one or seven days in media supplemented with different concentrations of rbFSH (mean, 20 samples/treatment/day).[a,b] Within each treatment, non-common superscripts differed ($P < 0.0001$). No difference within days was observed among treatments.

3.5. Reactive oxygen species production

After Day 1 of culture, no differences ($P > 0.05$) among treatments were observed in ROS production. However, after Day 7 of culture, the 50 ng/mL FSH treatment produced more ($P < 0.05$) ROS only when compared to the 0 ng/mL FSH treatment (Table 4). The concentrations of ROS decreased from Day 1 to Day 7 of culture in the 0 ng/mL ($P < 0.07$) and in the 10 ng/mL ($P < 0.05$) FSH treatments. On the other hand, the 50 ng/mL and 100 ng/mL FSH treatments maintained ROS levels during the culture time.

Table 4
Mean (±standard error of the mean) reactive oxygen species (relative fluorescence units) produced by equine ovarian tissue fragments after IVC for 1 or 7 days in media supplemented with different concentrations of bovine recombinant follicle-stimulating hormone.

	Relative fluorescence units	
FSH treatments	Day 1	Day 7
0 ng/mL	32.4 ± 2.3^{A}	$26.9 \pm 1.3^{A,c}$
10 ng/mL	$34.6 \pm 2.0^{A,a}$	$28.0 \pm 1.3^{A,B,b}$
50 ng/mL	$30.8 \pm 2.1^{A,a}$	$31.1 \pm 1.6^{B,a}$
100 ng/mL	$29.2 \pm 2.3^{A,a}$	$30.6 \pm 1.9^{A,B,a}$

[A,B]Within days, uncommon uppercase superscripts differed ($P < 0.05$).
[a,b]Within treatments, uncommon lowercase superscripts differed ($P < 0.05$).
[c] ROS production tended ($P < 0.07$) to differ between Days 1 and 7.

4. Discussion

This study demonstrated for the first time that adding FSH in a concentration-dependent manner improved both *in vitro* follicular survival, and activation of equine primordial follicles enclosed in ovarian tissue.

The addition of 50 ng/mL of FSH was able to provide a greater percentage of morphologically normal follicles than all tested concentrations after Day 7 of culture. In goats, Magalhães et al. [23] used a concentration-response curve of rbFSH (0, 10, 50, 100, and 1000 ng/mL), and reported that 50 ng/mL maintained survival and follicular ultrastructure, and promoted the activation and growth of primordial follicles after Day 7 of culture. FSH receptors have been reported to be expressed in oocytes of primordial follicles of porcine and primary follicles in humans [34], and in granulosa cells from the primary follicle stage onward in horses [14]. The positive effect of FSH observed on *in vitro* follicle culture in the present study might have been due to its direct and/or indirect action. Among the direct effects is the activation of genes that code for the stimulation of cell proliferation and steroid synthesis [35]. Indirectly, FSH regulates the expression of some of the many important substances that play a role on folliculogenesis, such as KL, GDF-9, and BMP-15 [20].

The addition of 50 ng/mL of FSH to the culture medium reduced the proportion of primordial follicles and caused a concomitant increase in the proportion of growing follicles at Day 7of culture, indicating that primordial follicle activation had occurred. Such an effect is possibly due to the stimulatory effect of FSH on the expression of genes involved in proliferation and differentiation of granulosa cells [36], which in turn induce multiple signaling cascades [37] and can quickly stimulate the activation of MAPK pathways and phosphatidylinositol 3-kinase (PI3-K), which impact cell proliferation [38].

Follicular and oocyte diameters were greater after Day 7 of culture in the 50 ng/mL FSH treatment when compared to all other treatments. Similar results have been found for goat [22], and dog [25] preantral follicles. Compared with the fresh non-cultured control, FSH treatment in this study was not efficient in promoting follicular growth, which is in agreement with findings using horse preantral follicle culture without supplemental FSH [39]. In addition, a possible explanation for the decrease in follicle and oocyte diameters from Day 1 to Day 7 of culture was that primary and secondary follicles are more sensitive to degeneration than primordial follicles [40]. This could explain why only few primary and secondary follicles contributed to the mean follicle and oocyte diameters in this study. Thus, studies aimed toward developing a culture system for equine preantral follicles that promotes continuous follicular and oocyte growth to late stages of folliculogenesis are warranted.

This study showed that fragments of equine ovarian tissue containing preantral follicles, when cultured *in vitro*, exhibited steroidogenic activity. Irrespective of culture time, estradiol and progesterone production were not affected by FSH concentrations. However, after Day 7 of culture, while the 50 ng/ml FSH treatment maintained estradiol levels, all other FSH treatments significantly reduced estradiol concentration. This finding might be explained by the fact that larger follicles, which have a higher steroidogenic activity, degenerate before smaller follicles [40], which have a lower steroidogenic capacity [41]. In addition, we cannot rule out that other cell types such as ovarian stromal cells could contribute to estradiol production under our culture conditions, as it has been reported that these cells are capable of producing steroids [42,43].

In this study, 50 and 100 ng/mL FSH treatments maintained ROS production between Day 1 and Day 7of culture. However, the 50 ng/mL FSH treatment was the only one that differed (significant higher levels) from the 0 ng/mL FSH treatment (FSH control group). The evaluation of

ROS production has been an important parameter used to determine the presence of free radicals that might be deleterious to cells in culture [44,45]. Also, strong evidences demonstrate the toxicity role of ROS caused by several chemical and physical agents, in the initiation of apoptosis mainly on antral follicles, resulting in poor oocyte quality and possibly having noxious effects even in early preantral follicles [46]. Therefore, FSH was added to the culture media due to its important role in protecting follicles from apoptosis by increasing glutathione levels and suppressing ROS production as described previously [47]. Furthermore, appropriate levels of ROS are extremely important for the maintenance of cellular homeostasis to modulate physiological events (e.g., ovulation), possibly by its action in activation of the phosphokinase A (PKA) signaling [48] or cell growth and differentiation [49]. To date, it has been shown that FSH stimulates catalase activity in goat granulosa cells modulating intracellular ROS levels [50]. ROS inhibitors, in a concentration dependent manner, decreased oocyte maturation induced by FSH [51]. In our study, we suggest that the presence of antioxidants (e.g., transferring [52]; selenium [53]), insulin in physiological concentration (10 ng/mL [54]), along with an adequate FSH concentration (50 ng/mL), contributed to the maintenance of suitable levels of ROS after Day 7 of culture, resulting in higher rates of follicle survival and activation.

In conclusion, the addition of 50 ng/mL FSH promoted activation of primordial follicles to developing follicles, improved survival of preantral follicles, and maintained estradiol and ROS production of equine ovarian tissue after seven days of culture and can be recommended for *in vitro* culture of equine preantral follicles enclosed in ovarian tissue fragments. The novel findings of this study open the prospect for the use of FSH in the base culture medium and also to investigate the efficacy of other substances (such as growth factors) added to culture media to optimize equine *in vitro* follicular development.

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8. CAPÍTULO 3

Papel do EGF no cultivo *in situ* de folículos pré – antrais equinos e perfil metabolômico

"Role of EGF on *in situ* culture of equine preantral follicles and metabolomics profile"

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RESUMO

Os efeitos das concentrações do fator de crescimento epidermal (EGF - 0, 10, 50 e 100 ng/mL) foram avaliados no cultivo *in vitro* (CIV) de folículos pré – antrais equinos inclusos em tecido ovariano, usando histologia, produção hormonal, espécies reativas de oxigênio (EROs), e metabolômica. Após o CIV, a percentagem de folículos normais foi menor ($P < 0.05$) para todos os tratamentos, quando comparados com o controle não cultivado. O tratamento EGF 50 ng/mL teve maior ($P < 0.05$) número de folículos normais após 7 dias de cultivo quando comparado com o tratamento 0 ng/mL e 100 ng/mL de EGF. Os diâmetros foliculares e oocitários foram maiores ($P < 0.05$) com 50 ng/mL do que os outros tratamentos cultivados, mas similares ($P > 0.05$) ao controle não-cultivado. O tratamento 50 ng/mL de EGF foi o único tratamento que manteve a produção de EROs através do CIV. O perfil metabolômico do meio de cultivo indicou que onze íons avaliados por importância da projeção variável (VIP escores) foram hiper representados no tratamento 50 ng/mL de EGF. Em conclusão, o tratamento EGF 50 ng/mL manteve a sobrevivência folicular, a produção de EROs e promoveu a ativação folicular de folículos pré – antrais equinos inclusos em tecido ovariano.

Palavras - chave: Cultivo *in vitro*. Folículos Pré – Antrais Equinos, EGF, Metabolômica, Espécies Reativas de Oxigênio.

Role of EGF on *in situ* culture of equine preantral follicles and metabolomics profile

F.L.N. Aguiar[a], F.O. Lunardi[a], L.F. Lima[a], J.B. Bruno[a], B.G. Alves[a], D.M. Magalhães-Padilha[b], F.W.S. Cibin[c], L. Berioni[d], G.A. Apgar[e], E.G. Lo Turco[d], E.L. Gastal[e,*], J.R. Figueiredo[a]

[a] *Laboratory of Manipulation of Oocytes and Preantral Follicles (LAMOFOPA), State University of Ceará, Fortaleza, Ceará, Brazil*

[b] *Postgraduate Biotechnology, Potiguar University/Laureate International Universities, Av. Senador Salgado Filho, 1610, Lagoa Nova, Natal, 59056-000, CE, Brazil*

[c] *Federal University of Pampa, Uruguaiana, Rio Grande do Sul, Brazil.*

[d] *Department of Surgery, Division of Urology, Human Reproduction Section, Sao Paulo Federal University, Sao Paulo, SP, Brazil.*

[e] *Department of Animal Science, Food and Nutrition, Southern Illinois University, Carbondale, Illinois, USA*

Running head: EGF effects on equine preantral follicles

*Corresponding author: Eduardo Gastal, Department of Animal Science, Food and Nutrition, Southern Illinois University, 1205 Lincoln Drive, MC 4417, Carbondale, IL, 62901, USA. Telephone: (618) 453-1774; FAX: (618) 453-5231; e-mail: egastal@siu.edu

ABSTRACT

The effects of EGF concentrations (0, 10, 50, and 100 ng/ml) on *in vitro* culture (IVC) of equine preantral follicles were evaluated using histology, estradiol and reactive oxygen species (ROS) production and metabolomics. After IVC, the percentage of normal follicles was lower ($P < 0.05$) for all treatments when compared to non-cultured control. EGF 50 ng/ml treatment had more ($P < 0.05$) normal follicles after Day 7 of culture when compared with EGF 0 and 100 ng/ml. EGF 50 ng/ml had more ($P < 0.05$) developing follicles than the 0 ng/ml and 10 ng/ml EGF treatments. Follicular and oocyte diameters were greater ($P< 0.05$) with EGF 50 ng/ml than the other cultured treatments, but similar ($P > 0.05$) to the non-cultured control. From Day 1 to Day 7 estradiol production increased ($P < 0.05$) in all EGF treatments. EGF 50 ng/ml was the only treatment that maintained ROS production through IVC. Metabolomics profiles of the spent media indicated that eleven ions from VIP score were higher represented in the EGF 50 ng/ml treatment. In conclusion, EGF 50 ng/ml treatment maintained follicle survival and ROS production, and promoted activation of cultured equine preantral follicles enclosed in ovarian tissue.

Keywords: *In vitro* culture, equine preantral follicles, EGF, metabolomics, reactive oxygen species.

1. Introduction

An important strategy to maximize the reproductive potential of domestic animals consists of *in vitro* culture (IVC) of preantral follicles, aiming to preserve follicular survival and development, and minimize oocyte losses observed *in vivo* (Xu et al., 2013; Haag et al., 2013a; Araújo et al., 2014).

Considering the limited efficiency in assisted reproduction technologies in mares (e.g., superovulation and *in vitro* fertilization) studies using IVC of preantral follicles emerge as a future alternative source of meiotically competent oocytes. Moreover, mares share similarities with women in reproductive dynamic events, as previously reported for follicular waves, changes in hormonal levels, and age-related reduction in fertility (Ginther et al., 2004; Carnevale, 2008; Gastal, 2011; Cox et al., 2015). Particularly in horses, studies on IVC of preantral follicles are scarce because of the prohibition of slaughterhouses in some countries that could provide ovaries as a biological source. More recently, the mare has also been suggested as an interesting model for studies related to preantral follicles (Haag et al., 2013a, 2013b, 2013c; Alves et al., 2015). Previous reports on IVC of equine preantral follicles have produced limited results using follicles chemically isolated (Szlachta and Tischner, 2000, 2004) or enclosed in ovarian tissue (Haag et al., 2013a; Alves et al., 2015).

A base medium (α-MEM) has been used recently with limited success for culture of equine preantral follicles enclosed in ovarian tissue (Haag et al., 2013c). Furthermore, insulin at a physiological concentration (10 ng/ml) improved survival and development of equine preantral follicles (Aguiar et al., 2016a). In addition, 50 ng/ml of FSH promoted activation of primordial follicles, improved survival of preantral follicles, and maintained estradiol and ROS production of equine ovarian tissue after seven days of culture (Aguiar et al., 2016b). Ascorbic acid

supplementation (50 and 100 μg/ml) has also improved the development of equine preantral follicles after 6 days of IVC (Gomes et al., 2015). However, the role of different substances, including intraovarian factors such as epidermal growth factor (EGF), which has potent mitogen activity (Silva et al., 2004; Wu and Tian, 2007; Celestino et al., 2009; Fujihara et al., 2014), is still unknown on *in vitro* survival and development of equine preantral follicles. EGF belongs to the transforming growth factor β protein family (TGF-β; Silva et al., 2013). Once bound to its tyrosine kinase-like receptor, EGF induces a series of intracellular events, with an activation of second messengers responsible for regulating cell growth and differentiation (Ma et al., 2015).

Previous reports have shown that EGF, in a dose dependent manner (range, 0.5 to 200 ng/ml), affected survival, activation, and growth of IVC of preantral follicles [e.g., ovine: 100 ng/ml (Andrade et al., 2005), and 75 ng/ml (Peng et al., 2010), caprine: 1 to 200 ng/ml (Celestino et al., 2009), human: 50 ng/ml (Roy and Kole, 1998)], proliferation of granulosa cells [porcine: 10 ng/ml (Mao et al., 2004)], and reduced the rate of atresia of preantral follicles [bubaline: 50 ng/ml (Gupta et al., 2002), porcine: 1.5 ng/ml (Wu and Tian, 2007), and caprine: 100 ng/ml (Silva et al., 2004)].

Metabolomics may provide timely information about molecular interactions and metabolic signaling, indicating new biomarkers and specific supplies for successful IVC and physiological modulation (Montani et al., 2012). Molecules secreted in the medium during IVC have been investigated recently as an alternative approach (metabolomics) to determine oocyte maturation and embryo development potential in several species [e.g., humans (Wallace et al., 2012; Montani et al., 2014), murine (Preis et al., 2005), and bovine (Matoba et al., 2014). However, to date evaluation of metabolites from preantral follicles isolated or enclosed in ovarian tissue in spent culture medium has been performed only in a few species, demonstrating different

requirements of carbohydrates and amino acids for follicles cultured in different oxygen tensions (Gook et al., 2014). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been efficiently employed as a tool to analyze lipid composition, providing a study of highly complex structures (Schiller et al., 1999).

Studies investigating the dose-dependent effect of EGF on IVC of equine preantral follicles and the metabolic characterization of the spent culture media remain unknown. Thus, the aim of this study was to evaluate the effect of different concentrations of EGF (0, 10, 50 and 100 ng/ml) on IVC of preantral follicles enclosed in ovarian tissue fragments, using the following end points: (i) follicular survival, (ii) follicular growth, (iii) hormonal production, (iv) reactive oxygen species (ROS), and (v) metabolomics profile of the spent culture media.

2. Materials and methods

2.1. Chemicals

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

2.2. Animals and ovaries

The research protocol (#12637266-7) was approved by the Ethics and Animal Use Committee (CEUA) of State University of Ceará (UECE), Fortaleza, CE. The ovaries were harvested from euthanized mares (n = 5) positive but asymptomatic for Equine Infectious Anemia (EIA), during February to May, in the state of Ceará, Brazil. The mares were crossbred, 6 to 10 years old (mean, 7.2 ± 1.9 years), and had body condition scores between 4 and 6 [1, emaciated to 9, obese; Henneke et al., 1983]. Dental characteristics (American Association of

Equine Practitioners, 2002) were used to estimate the age of the mares. At the time of ovary harvesting, mares were non-pregnant and cycling, based on the presence of large (maturing/growing) or small (regressing) corpus luteum and a preovulatory (> 30 mm) follicle in one or both ovaries.

Immediately after euthanasia, the ovaries were collected and washed in 70% alcohol, followed by two washes in minimum essential medium (MEM) supplemented with 25 mM HEPES. The ovaries were placed into tubes containing 150 ml of MEM supplemented with 100 μg/ml penicillin and 100 μg/ml streptomycin and transported to the laboratory at 4°C (Chaves et al., 2008) within 4 hours. In the laboratory, both ovaries of each animal were stripped of surrounding fat tissue and ligaments. Subsequently, 54 ovarian cortical tissue samples (approximate size, 3 x 3 x 1 mm) were obtained from each pair of ovaries under sterile conditions using a scalpel blade. Cortical samples were recovered avoiding adjacent areas to a corpus luteum and preovulatory follicle. A pool of six similar ovarian fragments randomly chosen from the same animal were distributed in nine treatments. Five replicates (mares) were performed for each treatment.

2.3. Culture of preantral follicles and experimental design

Each ovarian tissue fragment was placed in a well of a 24-well culture plate containing 1 ml of culture media. Culture was performed at 39°C in a humidified atmosphere with 5% CO_2. Fresh medium was prepared and incubated for at least 1 h before use. The basic culture medium consisted of α-MEM (pH 7.2–7.4) supplemented with 2 mM glutamine, 2 mM hypoxanthine, 1.25 mg/ml bovine serum albumin (BSA), 10 ng/ml insulin, 5.5 μg/ml transferrin, 5.0 ng/ml sodium selenite, 100 μg/ml penicillin, and 100 μg/ml streptomycin, which was called α-MEM^{+}.

To test the effect of EGF in cultures of preantral follicles, the basic medium (control) was supplemented with increasing concentrations of EGF, generating the following treatments: EGF 0 ng/ml, EGF 10 ng/ml, EGF 50 ng/ml, and EGF 100 ng/ml.

The ovarian tissue fragments were then either fixed for histological analyses (fresh, non-cultured control group) or placed in culture for one (Day 1) or seven (Day 7) days. The whole culture medium was replaced every other day, and before each replacement, 1 ml of medium was collected after 24 h (Days 1 and Days 7 of culture), and stored at -80°C for hormonal and ROS analyses.

2.4. Follicle morphology and development

Follicular morphology and development of preantral follicles were assessed in ovarian fragments *in situ* before (fresh, non-cultured control group) and after IVC of ovarian tissue. Once harvested, ovarian tissue was fixed in paraformaldehyde solution at 4°C for 12 h and kept in 70% alcohol, and submitted to histological analysis. Ovarian fragments were dehydrated by use of a graded series of alcohol, embedded in paraffin wax, and cut into serial sections of 10 μm. Samples were stained with periodic acid-Schiff (PAS) and counterstained with hematoxylin. A total of 2,700 slides with 27,000 tissue sections were produced from 270 ovarian fragments. Histology slides were analyzed using light microscopy (Nikon, Tokyo, Japan) at 400X magnification. The histological evaluation and classification of the follicles were performed by only one operator, who was blind to the treatments. However, five other trained helpers, also blind to the treatments, were used to find preantral follicles on the histological sections of each fragment.

Once that equine preantral follicles had a similar morphology to other species (Haag et al., 2013a), the end points evaluated were number of follicles, follicle class (primordial, one layer of flattened pre-granulosa cells surrounding the oocyte; transitional, one layer of flattened and cuboidal granulosa cells; primary, one layer of cuboidal granulosa cells; and secondary, two or more layers of cuboidal granulosa cells; Maciel et al., 2004), follicle morphology, and diameter of follicles and oocytes.

To evaluate follicular development, the percentages of both normal primordial and developing follicles (transitional, primary, and secondary) were calculated before (fresh non-cultured control group) and after culture for each treatment. Regarding morphology, follicles were classified as normal (follicle containing an intact oocyte and granulosa cells well-organized in layers without pyknotic nucleus) or abnormal (oocyte with pyknotic nucleus, retracted cytoplasm, or disorganized granulosa cells detached from the basement membrane; Haag et al., 2013b). Every follicle was examined in each section in which it appeared and matched with the same follicle on adjacent sections to avoid double counting, thereby ensuring that each follicle was counted only once.

Follicle and oocyte diameters were measured only in morphologically normal follicles using software (Nis-Element AR 3.0) coupled with an epifluorescence microscope (Zeiss, Cologne, Germany). Follicle diameter was measured from one edge to the other edge of the outermost layer of granulosa cells. Oocyte diameter was measured from one edge of the oocyte membrane to the other. Two perpendicular diameters were recorded for each measurement and the average of those two values was calculated.

2.5. Hormone analyses

To evaluate follicular steroidogenesis *in vitro*, concentrations of estradiol and progesterone were measured in spent culture media against standard dilutions according to manufacturer's instructions using competitive immunoassay commercial kits: IMMULITE 2000 Estradiol (catalog No: L2KE22; Siemens Medical Solutions Diagnostics, 5210 Pacific Concourse Drive, Los Angeles, CA, USA) and IMMULITE 2000 Progesterone (catalog No: L2KPW2; Siemens Medical Solutions Diagnostics, 5210 Pacific Concourse Drive, Los Angeles, CA, USA). The two hormonal assay kits for estradiol and progesterone in this study have been used previously and validated for different species, including the horse (Relave et al., 2007; Claes et al., 2015). The analytical sensitivity of the assay was 15 pg/ml (range, 20 to 2000 pg/ml) for estradiol and 0.1 ng/ml (range, 0.2 to 40 ng/ml) for progesterone.

2.6. ROS analysis

The levels of reactive oxygen species were determined in spent cultured media using a spectrofluorimetric method (Loetchutinat et al., 2005). Culture media from all EGF-treated groups were incubated with 10 μl of 2',7'-dihidrodiclorofluorescein diacetate (DCHF-DA; 1 mM). The oxidation of DCHF-DA to dichlorofluorescein was measured for detection of reactive species in the medium. The intensity of fluorescence emission was recorded at 520 nm (with 480 nm excitation) for 2 h after addition of DCHF-DA to the medium.

2.7. Metabolomics analysis

Thirty-two samples of spent culture media (eight samples in each EGF treatment) were analyzed in four replicates. Mass spectra from the spent culture media of the different treatments at Day 7 were acquired using MALDI equipment. The mass spectra were acquired in positive ion

mode using a MALDI AUTO FLEX SPEEDY TOF/TOF (Bruker Daltonics, Billerica, Ma, USA) mass spectrometer equipped with a 1 kHz solid-state laser with a wavelength of 355 nm and a range of 600–1200 *m/z* in the reflectron mode. Typical operating conditions for both modes were as follows: laser energy, 750 (arbitrary units); sample plate, 22.5 Kv; and laser irradiation of 100 shots in the region where the sample had been placed on the target plate for 60–90 sec in the positive ion mode. The quality of the spectra was individually evaluated with respect to ion count. To avoid potential noise influence on the assays, only spectra presenting an ion count > 800 were included in the study. The mass spectrum of each sample was acquired using Flex Control software (Bruker Daltonics, Billerica, MA, USA) and the *m/z* values and ion intensities were exported to an Excel table. The ions presenting zero values in > 50% of the samples were removed, and the whole data set was standardized to a common peak (most intense) present in all samples. The ions were identified by using METLIN metabolite database from Scripps Center for Metabolomics (http://metlin.scripps.edu/index.php). The mass error was calculated and only assignments with an error < 50 ppm were considered.

2.8. Statistical analyses

Statistical analyses were carried out using R statistical software version 3.0.2 (R Foundation for Statistical Computing, Vienna, Austria). Data for end points that were not normally distributed according to a Kolmogorov–Smirnov test were transformed to natural logarithms or ranks. Chi-square test was used to compare the percentage of morphologically normal and growing preantral follicles among and within treatments. Follicle and oocyte diameters, estradiol, progesterone, and ROS were compared among treatments by one-way ANOVA. For metabolomics analyses of the spent culture media after seven days of culture, the data were

analyzed using the principal component analysis (PCA), followed by a partial least square discrimination analysis (PLS-DA), and combined with variable influence in the projection (VIP) scores. The statistical analysis was performed using the Metabo-Analyst 3.0 (http://www.metaboanalyst.ca). According to the results, one-way ANOVA and subsequently *t*- or Fisher-tests were used when appropriate. Thereafter, statistical ions were identified in the online database, through the *m/z* values detected by the equipment. A probability of $P < 0.05$ indicated that a difference was significant, and $P > 0.05$ and ≤ 0.1 indicated that a difference approached significance. Data are presented as the mean ± SEM, unless otherwise indicated.

3. Results

3.1. Number of follicles evaluated

A total of 207, 317, 373, 345, and 342 preantral follicles were evaluated in the fresh non-cultured control, EGF 0 ng/ml, EGF 10 ng/ml, EGF 50 ng/ml, and EGF 100 ng/ml treatment groups, respectively. Altogether, 1,584 follicles were evaluated, with an average of 236.0 ± 26.6 follicles evaluated per treatment.

3.2. Effect of EGF on follicular morphology

The percentage of morphologically normal follicles was reduced ($P < 0.05$) after one and seven days of culture when compared to the fresh non-cultured control group (Table 1). Overall, after each culture time, EGF 50 ng/ml treatment had a greater ($P < 0.05$) percentage of normal follicles than the other treatments, except for the EGF 100 ng/ml (Day 1) and EGF 10 ng/ml (Day 7) treatments. A reduction ($P < 0.05$) in the percentage of normal follicles was observed in all treatments from Day 1 to Day 7 of culture.

3.3. Follicular development after in vitro culture

The percentages of primordial and developing follicles are shown (Fig. 1 A,B). After one day of culture, only the EGF 0 ng/ml and 10 ng/ml treatments decreased ($P < 0.05$) the percentage of primordial follicles and increased ($P < 0.05$) the percentage of developing follicles when compared to the fresh non-cultured control group. In all treatments, after seven days of culture, the percentage of primordial follicles decreased ($P < 0.05$) and the percentage of developing follicles increased ($P < 0.05$) when compared to fresh non-cultured control group. The EGF 50 ng/ml treatment had a greater ($P < 0.05$) rate of developing follicles on Day 7 of culture, except when compared with the EGF 100 ng/ml treatment. Regarding to follicle activation, except for the EGF 0 ng/ml treatment, all EGF treatments had a reduction ($P < 0.05$) in the percentage of primordial follicles and an increase ($P < 0.05$) in developing follicles between Day 1 and Day 7 of culture.

After one and seven days of culture, the EGF 50 ng/ml treatment maintained follicular and oocyte diameters similar to fresh non-cultured control group ($P > 0.05$; Table 2). In addition, regardless of culture time, the EGF 50 ng/ml treatment had greater ($P < 0.05$) follicular and oocyte diameters than the other EGF treatments. Moreover, only the EGF 50 ng/ml treatment maintained ($P > 0.05$) follicular and oocyte diameters between Day 1 and Day 7 of culture.

3.4. Hormonal production

Estradiol production was not different ($P > 0.05$) among treatments (Table 3). However, estradiol concentration in the spent culture medium increased ($P < 0.05$) in the EGF 10 ng/ml, 50 ng/ml, and 100 ng/ml treatments between Day 1 and Day 7 of culture. Progesterone production

in the culture media was detected in a small number of samples (20 out of 158; data not shown). Therefore, data were not analyzed statistically.

3.5. Reactive oxygen species production

After one day of culture, no difference ($P > 0.05$) among treatments was observed in ROS production (Table 4). However, after seven days of culture, the EGF 50 ng/ml treatment produced more ($P < 0.05$) ROS than the other treatments, except when compared with the EGF 100 ng/ml treatment. After seven days of culture, the EGF 50 ng/ml treatment was the only treatment that maintained ($P > 0.05$) ROS production similar to Day 1 of culture.

3.6. Metabolomics analysis

A total of 32 samples of spent culture media were analyzed. These samples produced an average of 7,356 peaks, with 229.9 peaks per sample. Data submitted to PCA generated 32 components. Pairwise score plots providing overviews of the various separation patterns among the most significant PCs are presented (Fig. 2A). Furthermore, a scree plot showing the variances explained by the selected PCs is shown (Fig. 2B). The five principal components (PC1, PC2, PC3, PC4, and PC5; Fig. 2A) represented > 53.9% of the variance observed in the data (Fig. 2B). Two-dimensional score plots between selected PCs 1 and 2 using principal component analysis (PCAs; Fig. 3A), and partial least squares - discriminant analysis (PLS-DA; Fig. 3B) are shown. Important features identified with the partial least square discrimination analysis (PLS-DA) are shown by the variable importance in projection (VIP scores; Fig. 4). The colored boxes on the right indicate the relative concentrations of the corresponding metabolite on Day 7 in each EGF treatment (0 ng/ml, 10 ng/ml, 50 ng/ml, and 100 ng/ml). A complete separation of ions was

conducted using VIP scores to obtain the most important ions responsible for the variance among the groups (Table 5; Table S1). Ten out of 15 VIP ions were identified. For the 50 ng/ml treatment, 6 ions were listed by the METLIN metabolite database mainly as chemical groups of amino acids, carbohydrates, lipids, and steroids (Table S1)

After linear regression, PC5 showed a negative correlation with survival ($R^2 = 0.15$, r = -0.38, $P < 0.05$), follicle diameter ($R^2 = 0.19$, r = -0.44, $P < 0.01$), and oocyte diameter ($R^2 = 0.19$, r = -0.44, $P < 0.01$; Fig. 5). Because the other PCs (e.g., PC1, PC2, PC3, and PC4) were not correlated ($P > 0.05$) with survival rate, follicle diameter, and oocyte diameter, data were not further evaluated and presented. The factor loadings extracted by the principal component analysis of metabolites found 13 relevant ions for PC5 (Table S2), and six of those ions were identified by the METLIN database. From the six ions identified in PC5, three ions were considered the most representative (Table 6). Furthermore, from the three most representative ions, three metabolites belonging to the classes of phenols, Glycosyl, and Benzophenone were identified (Table 6; Table S3).

4. Discussion

To our knowledge, this is the first study to demonstrate the beneficial effect of EGF for maintenance of *in vitro* follicular survival (i.e., morphologically normal follicles), and activation of equine preantral follicles enclosed in ovarian tissue and its subsequent metabolomics profile in the spent media.

This study considered IVC *in situ* (i.e., preantral follicles enclosed in ovarian tissue) because of the low efficiency of isolating early preantral follicles (Telfer and Watson, 2000; Szlachta and Tischner, 2000, 2004; Haag et al., 2013a). Because of this, a “two-step culture system”, where

follicles are cultured *in situ* for some days until the secondary stage, and then are isolated mechanically or enzymatically, has been proposed for other species (Telfer and Zelinski, 2013). Therefore, knowledge regarding hormonal interactions, ROS production, and nutrient requirements for preantral follicles and stromal cells using an *in situ* model is imperative. In this study, because of ovarian fragments from the same animal were randomly allocated among treatments, a similar contribution of preantral follicles and stromal cells from each fragment (same number and comparable size in each treatment) was expected.

In the present study, greater follicular survival was obtained with EGF 50 ng/ml after seven days of culture, except for the EGF 10 ng/ml treatment. After binding to its receptor tyrosine kinase, EGF stimulates cell survival by acting on intracellular signaling pathways such as PIK3/AKT, MAPK/ERK, and JAK/STAT (Henson and Gibson, 2006). EGF reduces apoptosis of preantral follicles (porcine: Mao et al., 2004) and follicular cells (rats: Tilly et al., 1992), and inhibits spontaneous cleavage of DNA through stimulation of anti-apoptotic genes, such as BAD (Sastry et al., 2006), survivin (Peng et al., 2006), and NFkB (Sethi et al., 2006).

Previous studies demonstrated the importance of supplementation of an appropriate concentration of EGF on *in vitro* culture of preantral follicles either enclosed in ovarian tissue (caprine: 100 ng/ml, Silva et al., 2004, 1 and 10 ng/ml, Celestino et al., 2009; ovine: 100 ng/ml, Andrade et al., 2005) or in an isolated form (caprine: 50 ng/ml, Zhou and Zhang, 2005a, 50 ng/ml, Silva et al., 2013; ovine: 50 ng/ml, Hemamalini et al., 2003, 50 ng/ml, Santos et al., 2014; bubaline: 50 ng/ml, Gupta et al., 2002; bovine: 50 ng/ml, Wandji et al., 1996, 0.5 ng/ml, Gutierrez et al., 2000). In a previous study in goats (Celestino et al., 2009), the concentration of 1 and 10 ng/ml EGF maintained follicular survival similar to the control group after seven days of culture, but no differences were observed between the 10 and 50 ng/ml EGF treatments.

The percentage of developing follicles, as an indicative of follicle activation, was greater after seven days of culture on the EGF 50 ng/ml treatment, when compared with the other treatments, except for the EGF 100 ng/ml treatment. Similar results have been reported previously (caprine: Celestino et al., 2009, ovine: Andrade et al., 2005). In our study, follicle activation possibly occurred through EGF action in cell cycle progression (Luo et al., 2007; L'Hortet et al., 2012; Wee et al., 2015), promoting cell differentiation through the transcription factor stimulus such as activator protein 1 (AP-1), Cyclin D1, (Mitsudomi and Watanabe, 2010), and cAMP response element-binding protein (CREB; Rodrigues et al., 2010).

In this study, only the EGF 50 ng/ml treatment maintained follicular and oocyte diameters after seven days of culture. EGF has increased follicular diameters when associated with hormones (e.g., FSH: Wu and Tian, 2007; Celestino et al., 2011; LH: Saraiva et al., 2010) or growth factors (e.g., IGF-I: Zhou and Zhang, 2005b). Therefore, it seems reasonable to assume that the association of EGF with other growth factors or hormones is necessary to promote follicular growth in mares.

In our study, irrespective of culture time, estradiol production by cultured ovarian tissue was not affected by the different EGF concentrations. Moreover, it revealed that fragments of equine ovarian tissue containing preantral follicles when cultured *in vitro* exhibited steroidogenic activity (estradiol production). However, besides follicular cells, we cannot rule out the possibility that other cells such as ovarian stromal cells could have contributed to estradiol production under our culture conditions, since it has been reported that these cells are capable of producing steroids (McNatty et al., 1979; Qiu et al., 2014). Our results are in agreement with a previous study (Jones et al., 1982) reporting that the addition of EGF in different concentrations

(0.1, 1, and 10 ng/ml) to the culture medium did not affect estradiol production in rat granulosa cells.

The treatment EGF 50 ng/ml maintained ROS production throughout the culture period in our study. Production of ROS has been used to evaluate *in vitro* oocyte quality (Ou et al., 2012; Martinho et al., 2014), and the balance between the production and degradation of ROS is an indicator of oxidative control (Rizzo et al., 2012; Winterbourn, 2014). Furthermore, ROS might be important due to its modulatory role as a second messenger in intracellular pathways such as MAPK/PI3K (McCubrey et al., 2006), and blockage or inactivation of inhibitory phosphatases (Shkolnik et al., 2011).

This study shows for the first time the metabolomics profile in the spent culture medium obtained on Day 7 from equine preantral follicles enclosed in ovarian tissue cultured *in vitro*. In the current study, eleven ions from VIP score were higher represented in the EGF 50 ng/ml treatment, and three ions in the EGF 0 ng/ml treatment. Moreover, negative correlations with follicle survival and follicle and oocyte diameters were observed for the PC5 component. In addition, from the ions identified, chemical groups such as amino acids, carbohydrates, lipids, and steroids were listed.

To support the *in vitro* culture of preantral follicles, the base medium α-MEM with the most enriched formulation containing substances such as aminoacids, vitamins, and inorganic salts, was used in the present study. Previous studies demonstrated that aminoacids (Figueiredo et al., 1994), vitamins (Eppig and O'brien, 1996), and inorganic salts (Wright et al., 1997) are essential to promote the *in vitro* culture of preantral follicles in different species. However, in our study, the negative correlation observed in PC5 led us to hypothesize the potential substances present in the culture medium that could have been responsible for this effect. After a METLIN metabolite

database search, substances such as Dinex (Phenol group), Leonuriside A (Glycosyl group), and Avobenzone were identified as having a potential deleterious effect on *in vitro* cell culture assay systems. Dinex metabolite acts as an antagonist of steroidogenic receptors (PubChem AID=743078; National Center for Biotechnology Information, 2015a), Leonuriside A possesses an anti-steroidogenic activity that may lead to a reduction in cell proliferation (PubChem AID=470167; National Center for Biotechnology Information, 2015b). Avobenzone, a sunscreen blocker, has an antagonistic action on the estrogen alpha-receptor signaling pathway (PubChem AID=743091; National Center for Biotechnology Information, 2015c). This fact may support the findings that EGF supplementation in this study, regardless of day of culture, did not have a positive effect on estradiol production or follicular and oocyte growth.

5. Conclusions

In conclusion, 50 ng/ml of EGF maintained follicle survival and ROS production, and promoted follicle activation in equine preantral follicles enclosed in ovarian tissue after *in vitro* culture. In addition, a metabolomics profile for the spent culture media identified three substances (Dinex, Leonuriside A, and Avobenzene) that had a potential negative effect on follicle survival, and follicle and oocyte diameters at Day 7 of culture. Therefore, further research is warranted into the association between EGF with other growth factors and hormones to improve *in vitro* culture of equine preantral follicles.

Conflict of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Figure legends

Fig. 1. Percentage of primordial and developing follicles (transitional, primary, and secondary) in a fresh non-cultured control group and after *in vitro* culture for one or seven days using different concentrations of EGF. [a,b] Within each treatment, values without a common letter differed ($P < 0.05$). [A,B] Within days, values without a common letter differed ($P < 0.05$). * Differed ($P < 0.05$) from fresh non-cultured control.

Fig. 2. (A) Pairwise score plots between the selected principal compounds (PCs). The explained variance of each PC is presented in the corresponding diagonal cell. (B) Scree plot showing the variance explained by the PCs. The green line on top shows the accumulated variance explained; the blue line underneath shows the variance explained by individual PC.

Fig. 3. Two-dimensional score plots between selected PCs 1 and 2 using (A) principal component analysis (PCAs), and (B) partial least squares - discriminant analysis (PLS-DA). The explained variances are shown in parentheses in both axes. Color dots represent different treatments as indicated.

Fig. 4. Important features identified with the partial least square discrimination analysis (PLS-DA) are shown by variable importance in projection (VIP scores). The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in each experimental treatment by EGF (0 ng/ml, 10 ng/ml, 50 ng/ml, and 100 ng/ml) under study.

Fig. 5. Relationship of (A) normal preantral follicles, (B) follicle diameter, and (C) oocyte diameter with principal component 5 (scores). Regardless of treatment, each point on the graph represents a medium sample collected after seven days of *in vitro* culture (n = 32). A linear regression is represented by the equation and the line (black) for (A) [normal preantral follicles = 32.975 - (0.933 × component score), R2 = 0.15, r = -0.38, $P < 0.05$]; (B) [follicle diameter = 31.225 - (0.604 × component score), R2 = 0.19, r = -0.44, $P < 0.01$]; and (C) [oocyte diameter = 24.050 - (0.592 × component score), R2 = 0.19, r = -0.44, $P < 0.01$].

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Table 1

Percentage of morphologically normal equine preantral follicles in fresh non-cultured control group and after one and seven days of culture using different concentrations of EGF.

	Percentage of normal follicles (n)	
Non-cultured control	76.3 (158/207)	
EGF treatments	Day 1	Day 7
0 ng/ml	51.8 (83/160)^{*,A,a}	26.5 (49/157)^{*,A,b}
10 ng/ml	50.7 (110/186)^{*,A,a}	36.5 (61/187)^{*,A,B,b}
50 ng/ml	65.6 (100/152)^{*,B,a}	42.5 (80/193)^{*,B,b}
100 ng/ml	54.1 (78/173)^{*,A,B,a}	26.4 (50/169)^{*,A,b}

* Values differed ($P < 0.01$) from the fresh non-cultured control group.

^{A,B,} Within a column, uncommon superscripts differed ($P < 0.05$).

^{a,b,} Within a row, uncommon superscripts differed ($P < 0.05$).

Table 2

Mean (± SEM) diameter (µm) of preantral follicles and oocytes (primordial and primary combined) in fresh non-cultured group and after one and seven days of culture using different concentrations of EGF.

	Follicle diameter (µm)		Oocyte diameter (µm)	
Fresh Non-Cultured Control	38.8 ± 0.4		31.0 ± 0.4	
EGF treatments	Day 1	Day 7	Day 1	Day 7
0 ng/ml	$35.9 \pm 0.4^{*,B,a}$	$28.5 \pm 0.8^{*,B,b}$	$28.8 \pm 0.4^{*,B,a}$	$21.5 \pm 1.0^{*,B,b}$
10 ng/ml	$35.6 \pm 0.8^{*,B,a}$	$31.2 \pm 0.5^{*,B,b}$	$27.8 \pm 0.7^{*,B,C,a}$	$23.9 \pm 0.5^{*,B,b}$
50 ng/ml	$39.7 \pm 0.8^{A,a}$	$37.6 \pm 1.1^{A,a}$	$32.0 \pm 0.6^{A,a}$	$30.3 \pm 0.9^{A,a}$
100 ng/ml	$34.2 \pm 1.0^{*,B,a}$	$27.6 \pm 0.5^{*,B,b}$	$26.6 \pm 0.8^{*,C,a}$	$20.5 \pm 0.5^{*,B,b}$

* Values differed ($P < 0.05$) from the fresh non-cultured control group.

^{A,B,C} Within a column, uncommon superscripts differed ($P < 0.05$).

^{a,b} Within a row, uncommon superscript differed ($P < 0.05$).

Table 3

Mean (± SEM) concentrations of estradiol (pg/ml) produced in spent medium after one or seven days of culture of equine preantral follicles enclosed in ovarian tissue using different concentrations of EGF.

	Estradiol (pg/ml)	
EGF treatments	Day 1	Day 7
0 ng/ml	276.0 ± 50.9[a]	289.2 ± 3.7[a]
10 ng/ml	189.3 ± 20.4[a]	289.2 ± 5.7[b]
50 ng/ml	174.3 ± 7.6[a]	287.3 ± 4.8[b]
100 ng/ml	181.9 ± 11.6[a]	284.6 ± 4.4[b]

[a,b] Within a row, uncommon superscripts differed ($P < 0.05$).

Within a column, data were not different ($P > 0.05$).

Table 4

Mean (± SEM) reactive oxygen species (relative fluorescence units) produced in spent medium after one or seven days of culture of equine preantral follicles enclosed in ovarian tissue using different concentrations of EGF.

	Relative fluorescence units	
EGF treatments	Day 1	Day 7
0 ng/ml	$23.6 \pm 1.8^{A,a}$	$16.8 \pm 1.2^{A,C,b}$
10 ng/ml	$22.1 \pm 1.1^{A,a}$	$15.0 \pm 1.5^{A,b}$
50 ng/ml	$21.9 \pm 1.0^{A,a}$	$22.3 \pm 1.5^{B,a}$
100 ng/ml	$23.2 \pm 1.9^{A,a}$	$19.3 \pm 1.4^{B,C,b,\#}$

^{A,B,C} Within a column, uncommon superscripts differed ($P < 0.05$).

^{a,b} Within a row, uncommon superscripts differed ($P < 0.05$).

$^{\#}$ ROS production tended ($P < 0.08$) to differ between days one and seven of culture.

Table 5

Most representative ions identified in VIP score.

Mass (m/z)	Name	Formula	Ion	Delta Mass (ppm)	Category
395.28	Hydroxyprogesterone acetate	$C_{23}H_{32}O_4$	$M+Na^+$	-48.83	Steroids
223.96	L-Cysteinesulfonic acid	$C_{37}H_7NO_5S_2$	$M+Na^+$	36.02	Glycosyl
417.225	PA(P-16:0/0:0)	$C19H_{39}O_6P$	$M+H^+$	-30.20	Acid Phosphatidic
417.225	LPA(P-16:0e/0:0)	$C_{19}H_{39}O_6P$	$M+H^+$	-30.20	Ether Lipid
417.23	Stanolone Benzoate	$C_{26}H_{34}O_3$	$M+H^+$	-30.20	Steroid
417.23	CPA (18:2(9Z,12Z)/0:0	$C_{21}H_{37}O_6P$	$M+H^+$	-36.19	Acid cyclic phosphatidic
417.23	(6RS)-22-hydroxy-23,24,25,26,27-pentanorvitamin D3 6,19-sulfur dioxide adduct	$C_{22}H_{34}O_4S$	$M+Na^+$	43.14	Vitamine D derivate
311.17	Nafenopin	$C_{20}H_{22}O_3$	$M+H^+$	18.64	Acids
311.17	2-hydroxyestradiol	$C_{18}H_{24}O_3$	$M+Na^+$	26.35	Steroids
402.23	N-Didesmethylmifepristone (RU 42848)	$C_{27}H_{31}NO_2$	$M+H^+$	-31.82	Hormone

VIP score, variable influence in the projection scores.

Table 6

Most representative ions identified in PC5.

Mass (m/z)	Name	Formula	Ion	Delta Mass (ppm)	Category
289.08	Dinex	$C_{12}H_{14}N_2O_5$	M+Na	1.73	Phenols
333.13	Leonuriside A	$C_{14}H_{20}O_9$	M+H	36.02	Glycosyl
333.13	Avobenzone	$C_{20}H_{22}O_3$	M+Na	-48.33	Benzophenone

PC5, principal component 5.

Table S1: VIP score components listed in research database after PLS-DA

https://docs.google.com/spreadsheets/d/17hpEraVdEZbYlrlDUJspEy97a_CwiX2XBfkYa5BcuIo/edit?usp=sharing

Table S2: Factor loadings of five principal components (PCs) extracted by PCA showing the metabolites (mass) found in spent medium after seven days of *in vitro* culture of equine preantral follicles

https://docs.google.com/spreadsheets/d/148j3beQdBcAl1pLC63btPw7SE36Dv5aOL3JGFfHqzE4/edit?usp=sharing

Table S3: Components listed after research in database for PC5 m/z after regression analysis

https://docs.google.com/spreadsheets/d/1Uv0obztyFT-S0UkghXSPNNHUDK8zHs6nHYHwtdG8bYc/edit?usp=sharing

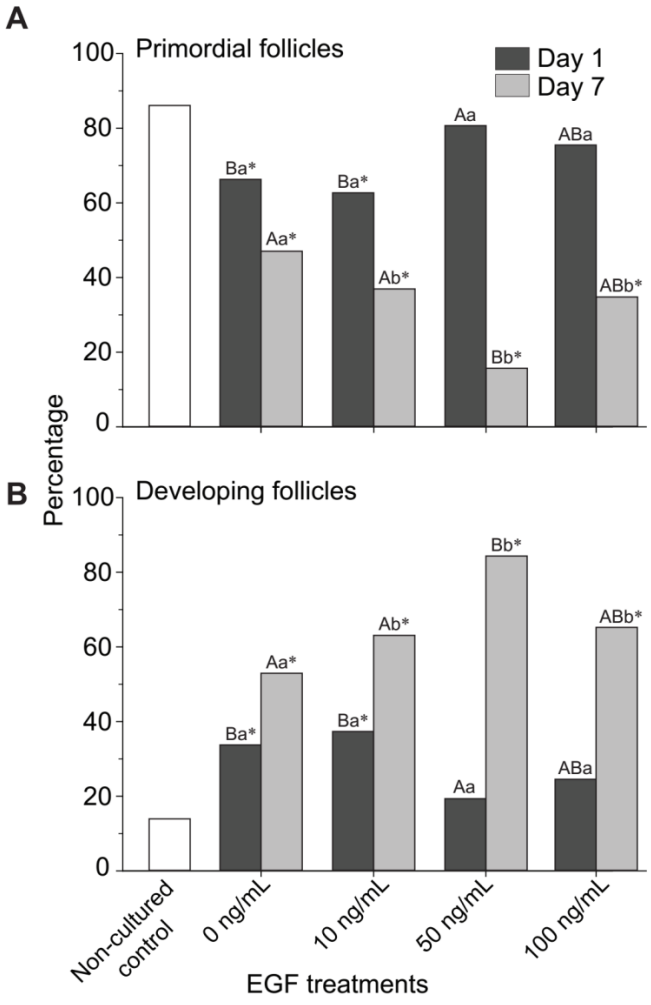

A
Primordial follicles
Day 1
Day 7
100
80
60
40
20
0
Ba*
Aa*
Ba*
Ab*
Aa
Bb*
ABa
ABb*
Percentage
B
Developing follicles
100
80
60
40
20
0
Ba*
Aa*
Ba*
Ab*
Aa
Bb*
ABa
ABb*
Non-cultured control
0 ng/mL
10 ng/mL
50 ng/mL
100 ng/mL
EGF treatments

Figure 1

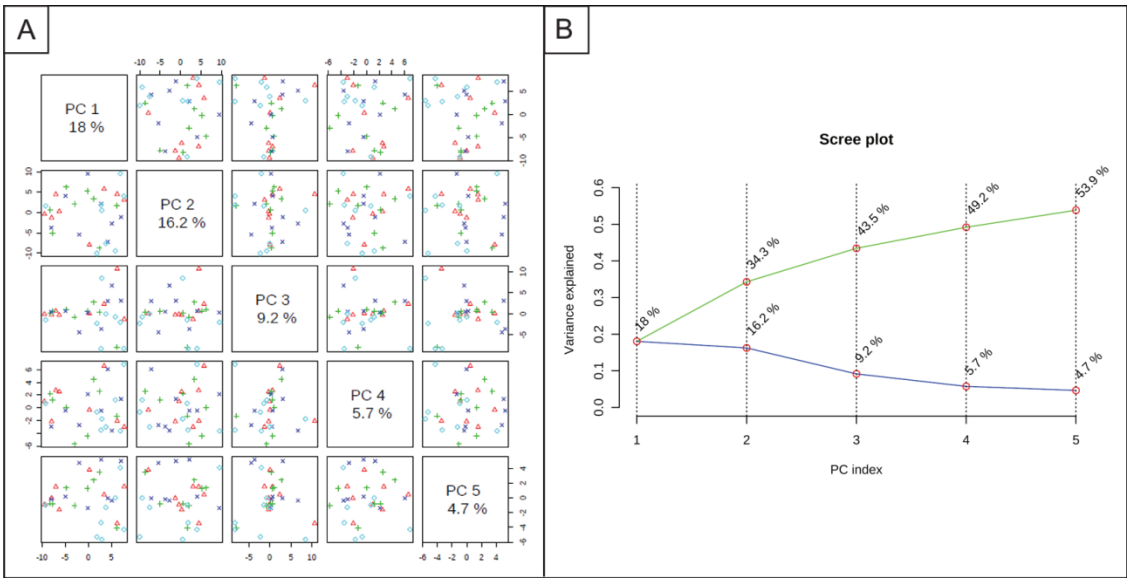

A
PC 1
18 %
PC 2
16.2 %
PC 3
9.2 %
PC 4
5.7 %
PC 5
4.7 %
B
Scree plot
Variance explained
PC index
18 %
34.3 %
43.5 %
49.2 %
53.9 %
16.2 %
9.2 %
5.7 %
4.7 %

Figure 2

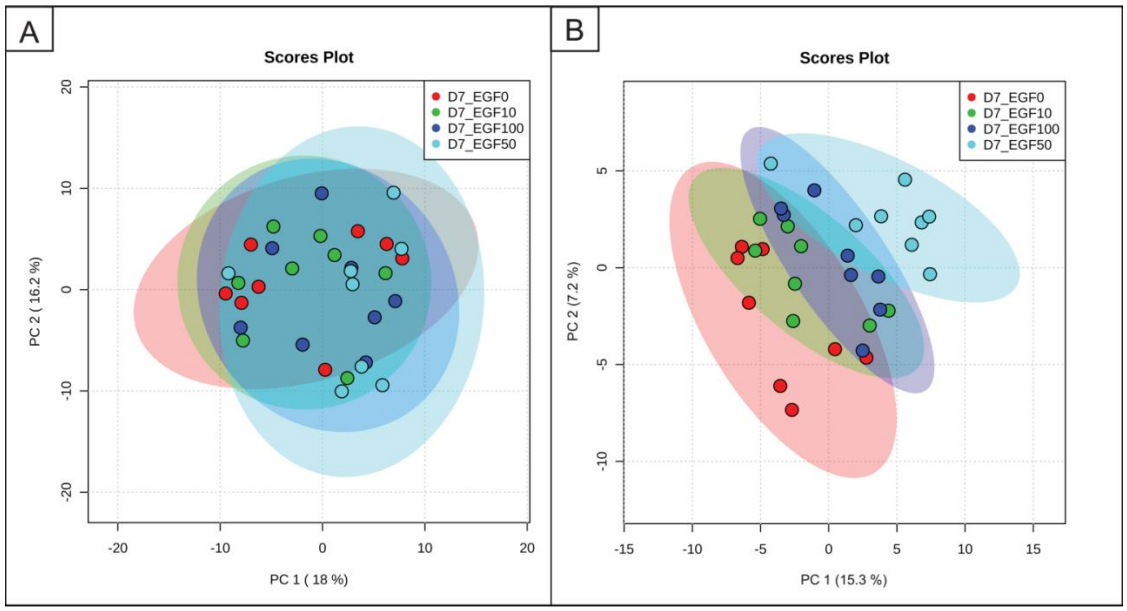

A
Scores Plot
D7_EGF0
D7_EGF10
D7_EGF100
D7_EGF50
PC 2 (16.2 %)
PC 1 (18 %)
B
Scores Plot
D7_EGF0
D7_EGF10
D7_EGF100
D7_EGF50
PC 2 (7.2 %)
PC 1 (15.3 %)

Figure 3

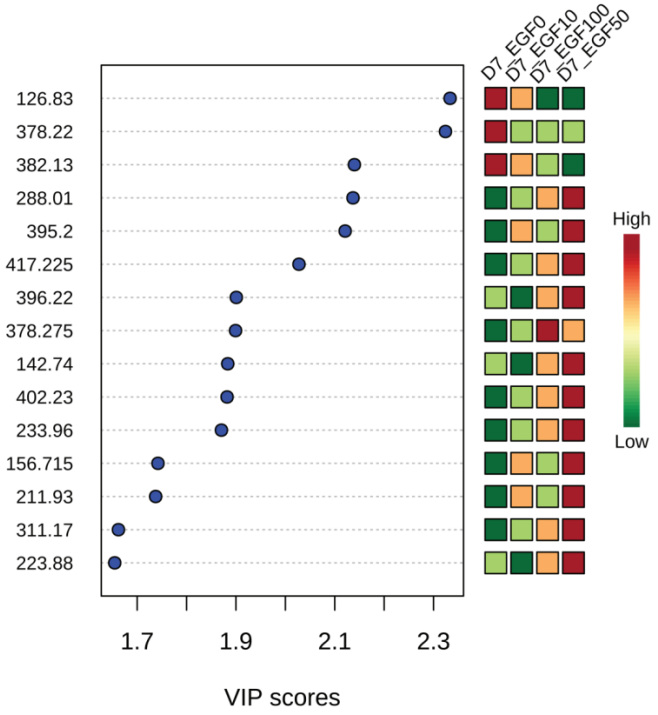
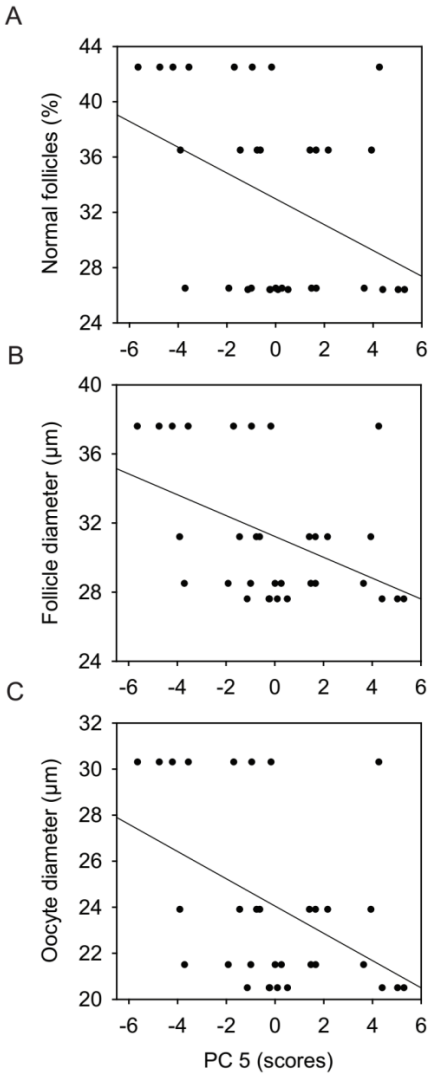

D7_EGF0
D7_EGF10
D7_EGF100
D7_EGF50
126.83
378.22
382.13
288.01
395.2
417.225
396.22
378.275
142.74
402.23
233.96
156.715
211.93
311.17
223.88
High
Low
1.7
1.9
2.1
2.3
VIP scores

Figure 4


A
Normal follicles (%)
44
40
36
32
28
24
B
Follicle diameter (µm)
40
36
32
28
24
C
Oocyte diameter (µm)
32
30
28
26
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9 CAPÍTULO 4

Efeitos da adição de FSH em um meio enriquecido contendo insulina e EGF após cultivo de longa duração na funcionalidade de biópsias de tecido ovariano equino.

"Effects of FSH addition to an enriched medium containing insulin and EGF after long-term culture on functionality of equine ovarian biopsy tissue"

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Co-Authors: Francisco L Aguiar, DVM, MS; Gustavo D Gastal, DVM, MS; Ghassan M Ishak, DVM, MS; Melba O Gastal, DVM, MS, PhD; Dárcio I Teixeira, DVM, MS, PhD; Jean M Feugang, DVM, MS, PhD; José R Figueiredo, DVM, MS, PhD;
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RESUMO

Os efeitos da suplementação de FSH (50 ng/mL) em um meio de cultivo enriquecido contendo insulina (10 ng/mL) e EGF (50 ng/mL) foi investigado no cultivo *in vitro* (CIV) de biópias de tecido ovariano. Dez éguas foram usadas para recuperar os tecidos ovarianos usando o método de biópsia por pick-up (BPU), e distribuído nos seguintes tratamentos: **1.** grupo Controle não cultivado, **2.** Controle cultivado e **3.** Cultivado + FSH. Ambos grupos tratados foram cultivados por 7 ou 15 dias. Os parâmetros avaliados foram: morfologia folicular, níveis de estradiol no meio de cultivo, intensidade de fluorescência para o TUNEL, detecção de EGFR, Ki-67 e expressão gênica do GDF-9, BMP-15, Cyclin-D2, no tecido ovariano. Após 7 dias de cultivo, o grupo suplementado com FSH teve similar ($P < 0.05$) percentagem de folículos morfologicamente normais quando comparado ao grupo Controle não cultivado. Os níveis de estradiol aumentaram ($P < 0.05$) do Dia 7 para o Dia 15 de cultivo em ambos os grupos tratados. Nenhuma diferença ($P > 0.05$) foi observada para a intensidade de TUNEL e EGFR entre os grupos controle Controle não cultivado e os grupos tratados após 15 dias de cultivo. A intensidade de Ki-67 não diferiu entre os grupos tratados após 15 dias de cultivo, mas diminuiu ($P < 0.05$) quando comparado com o grupo Controle não Cultivado. Similares ($P > 0.05$) níveis de mRNA foi encontrado para os genes GDF-9, BMP-15 e Cyclin-D2, entre todos os tratamentos após 15 dias de cultivo. Em conclusão, um meio enriquecido suplementado ou não com FSH foi capaz de manter a funcionalidade da biópsia de tecido ovariano equino após um CIV de longa duração.

Palavras - chave: biópsia ovariana, Células estromais ovarianas, Folículos pré-antrais, meio enriquecido, cultivo *in vitro* de longa duração.

Highlights

- *In vitro* culture (IVC) of equine ovarian biopsy tissue for 7 or 15 days
- Enriched medium supplemented or not with FSH sustained IVC for 15 days
- Apoptosis rate was similar to fresh control after IVC for 15 days in both treatments
- Ovarian tissue estradiol production and gene expression were preserved for 15 days

Effects of FSH addition to an enriched medium containing insulin and EGF after long-term culture on functionality of equine ovarian biopsy tissue

F.L.N. Aguiar[a], G.D.A. Gastal[b], G.M. Ishak[b], M.O. Gastal[b], D.I.A. Teixeira[a], J.M.N. Feugang[c], J.R. Figueiredo[a], E.L. Gastal[c,*]

[a] *Laboratory of Manipulation of Oocytes and Preantral Follicles (LAMOFOPA), State University of Ceará, Fortaleza, Ceará, Brazil*

[b] *Department of Animal Science, Food and Nutrition, Southern Illinois University, Carbondale, Illinois, USA*

[c] *Department of Animal and Dairy Sciences, Mississippi State University, Mississippi State, Mississippi, USA.*

Running head: Effects of FSH to an enriched medium on equine ovarian tissue

*Corresponding author: Eduardo Gastal, Department of Animal Science, Food and Nutrition, Southern Illinois University, 1205 Lincoln Drive, MC 4417, Carbondale, IL, 62901, USA. Telephone: (618) 453-1774; FAX: (618) 453-5231; e-mail: egastal@siu.edu

Abstract

The effect of FSH supplementation on an enriched cultured medium containing insulin (10 ng/mL) and EGF (50 ng/mL) was investigated on *in vitro* culture of equine ovarian biopsy tissue. Ovarian tissue fragments were collected from mares (n = 10) and distributed in the following treatments: noncultured control, cultured control, and cultured + FSH. Both treated groups were cultured for 7 or 15 days. The end points evaluated were: follicular morphology, estradiol levels in the culture medium, fluorescence intensity for TUNEL, EGFR and Ki-67 detection, and gene expression of GDF-9, BMP-15, and Cyclin-D2 in the ovarian tissue. After seven days of culture, medium supplemented with FSH had a similar ($P > 0.05$) percentage of morphologically normal follicles to the noncultured control group. Estradiol levels increased ($P < 0.05$) from Day 7 to Day 15 of culture for both treated groups. No difference ($P > 0.05$) was observed for TUNEL and EGFR intensity between the noncultured control group and the treated groups after 15 days of culture. Ki-67 intensity did not differ ($P > 0.05$) between treated groups after 15 days of culture, but decreased ($P < 0.05$) when compared with the noncultured control group. Similar ($P > 0.05$) mRNA expression for GDF-9, BMP-15, and Cyclin-D2 was observed among all treatments after 15 days of culture. In conclusion, an enriched medium supplemented or not with FSH was able to maintain the functionality of equine ovarian biopsy tissue after a long-term *in vitro* culture.

Keywords: Ovarian biopsy; Ovarian stromal cells; Preantral follicles; Enriched medium; Long term *in vitro* culture

1. Introduction

In vitro culture (IVC) of preantral follicles enclosed in ovarian stromal cells has been a major topic in the literature due to its potential to supply fertilizable oocytes [1,2,3,4,5]. Stromal cells have a crucial importance in supporting the architecture and plasticity of the ovary [6], and serve as a reserve for recruitment and differentiation of theca cells [7]. Stromal cells are responsible for the biosynthesis of hormones such as estradiol [8], and for interacting with the follicles to produce competent oocytes [9]. Few studies have investigated the requirements of ovarian stromal cells during *in vitro* culture [10,11]. Therefore, a suitable IVC condition that ensures the maintenance of stroma cell survival and proliferation is essential for new and established IVC of preantral follicles (PAFs) enclosed in ovarian tissue protocols.

The base culture medium to maintain the survivability of equine preantral follicles enclosed in ovarian tissue has been already reported [12]. However, additional studies to improve the culture condition of equine preantral follicles enclosed in the ovarian tissue are needed to allow more preantral follicles to grow and achieve later stages. For instance, the supplementation of the physiological insulin concentration (10 ng/mL) to the culture medium maintained high PAF survival rates [13]. Moreover, FSH supplementation (50 ng/mL) improved PAF viability and follicle development [14], and EGF supplementation (50 ng/mL) enhanced follicle survivability up to 7 days [15].

EGF and FSH have been proved to be important for proper *in vitro* folliculogenesis in several species [16,17,18,19]. Nevertheless, to the best of our knowledge, there is no information related to the association effect of EGF and FSH on *in vitro* survival and proliferation of equine ovarian tissue. Therefore, the comprehension of factors capable of supporting the survivability of ovarian

cells during IVC is crucial to allow advancements in knowledge regarding adequate development of equine PAFs [20].

Thus, the aim of this study was to evaluate the effect of FSH supplementation on an enriched culture medium containing insulin and EGF on the survival and proliferation of equine ovarian tissue for up to 15 days of IVC. The main end points evaluated were: (i) estradiol level in the culture medium, (ii) follicular morphology, (iii) rate of apoptosis, (iv) immunostaining of EGFR and Ki-67, and (v) gene expression of GDF-9, BMP-15, and Cyclin-D2 in ovarian tissue. The hypothesis tested in this study was that the association of FSH with EGF is not superior to treatment with EGF only to maintain ovarian tissue survival *in vitro*.

2. Materials and methods

2.1. Chemicals

Unless mentioned otherwise, medium and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA), and antibodies and mounting media from Santa Cruz Biotechnology (Santa Clara, CA, USA).

2.2. Animals and ovarian tissue collection

All experimental procedures were performed according to the United States Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training (http://grants.nih.gov/grants/olaw/references/phspol.htm) and were approved by the Institutional Animal Care and Use Committee of Southern Illinois University. Healthy reproductive light Quarter Horse mares (n = 10; 4 to 11 years old) were kept on pasture with

orchard grass/alfalfa mixed hay, with free access to mineral salt and fresh water. No hormonal treatments were administered during the experimental period.

Ovaries and uteri of mares were scanned using a transrectal ultrasound scanner (Aloka SSD-900, Aloka Co., LTD., Wallingford, CT, USA) equipped with a multi-frequency 5–10 MHz linear array transducer (Aloka UST-5821-7.5). Ovaries were biopsied and an average of nine ovarian fragments (size = 1.5 x 1.5 x10 mm) were obtained from each mare via the biopsy pick-up (BPU) method as previously described for horses [21]. Harvested biopsy fragments were immediately washed three times at room temperature (20°C) in 100 µL drops of α-MEM buffered with 25 mM HEPES, supplemented with 100 µg/mL penicillin and 100 µg/mL streptomycin. Biopsy fragments were cut into smaller pieces (1.5 x 1.5 x 2 mm), transferred into vials containing 1 mL α-MEM HEPES, and transported to the laboratory in a container at 20°C within 3 h. Each mare was considered a replicate.

2.3. Experimental design and in vitro culture

Small cut biopsy pieces (n = 40 per animal) were placed in a 24-well culture plate containing culture medium at 39°C in a humidified atmosphere with 5% CO_2. The control culture medium consisted of α-MEM (pH 7.2 – 7.4) supplemented with 10 ng/mL insulin, 5.5 µg/mL transferrin, 5 ng/mL selenium, 2 mM glutamine, 2 mM hypoxanthine, 1.25 mg/mL bovine serum albumin, 100 µg/mL penicillin, 100 µg/mL streptomycin, and 50 ng/mL of EGF, which was called α-MEM^{+}. To test the effect of FSH on ovarian tissue morphology and function, the following experimental groups were evaluated: fresh noncultured control; cultured control, and cultured + FSH. Ovarian fragments were immediately fixed (fresh noncultured control group) or cultured for 7 or 15 days (cultured treated groups). Every other day culture medium was completely renewed and stored at -80°C for hormonal analyses. The composition of the base control medium

[15] as well as the FSH concentration [14] chosen for the treated group have been recently reported.

2.4. Histology

At the end of IVC (Day 7 or Day 15), all ovarian biopsies were immediately fixed in 10% neutral buffered formalin (12 h) and kept in 70% alcohol until standard histological processing. Briefly, biopsies were embedded in paraffin wax, and cut in 7.0 µm serial sections, and placed on histology microscope slides. A subset of slides was stained with Periodic Acid-Schiff (PAS) and counterstained with hematoxylin, while the other subset was used for immunohistochemistry assays.

Histological slides were examined, and preantral follicles were categorized as morphologically normal (follicles containing an intact oocyte and granulosa cells well-organized in layers without pyknotic nucleus) or abnormal (oocytes with pyknotic nucleus, retracted cytoplasm, or disorganized granulosa cells detached from the basement membrane) [22]. Every follicle was examined in each section in which it appeared and matched with the same follicle on adjacent sections, thereby ensuring that each follicle was counted only once. Representative morphologically normal and abnormal follicles are shown (Fig. 1).

2.5. Estradiol analysis

On Days 2, 4, 7, 10, and 15 of culture, spent culture media from each treatment were collected and frozen at -80°C for estradiol assay using the commercial Estradiol (Horse) ELISA kit (Abnova Corporation**,** 1697 Walnut, CA, USA) as recommended by the manufacturer. The analytical sensitivity of the assay was 0.001 pg/mL (range, 0.001 to 0.219 pg/mL). The plate was

read using the SpectraMaxPlus Microplate Reader (Molecular Devices, LLC, CA USA), and the data were analyzed by the software SoftMax Pro 6.5.1.

2.6. Detection of apoptosis

The TUNEL detection kit of apoptosis and the positive and negative controls were used according to the manufacturer's instruction (*In Situ* Cell Death Detection kit, Fluorescein; Roche Applied Science, Mannheim, Germany). Tissue sections were counterstained using UltraCruz® mounting medium with DAPI. Samples were evaluated under fluorescence microscope (EVOS FLAuto Imaging System, Life Technologies, Grand Island, NY) and four to six images (magnification, 20X; 1228800 square pixels per image) of each section were randomly taken for image analysis using ImageJ software version 1.50i (imagej.nih.gov/ij).

2.7. Immunohistochemistry

The *in situ* immunofluorescence technique was used to assess the expression levels of EGFR and Ki-67 proteins. The employed protocols have been described previously [23]. Briefly, all slides were subjected to antigen retrieval (Dako North America, Carpinteria, CA, USA), followed by permeabilization (X-100 detergent diluted in PBS; 30 min), binding to non-specific sites (1% BSA in PBS; 1 h), and incubation with primary antibodies diluted 100x (anti-rabbit EGFR and anti-rabbit Ki-67; 1 h). Thereafter, samples were incubated with secondary antibody (anti-Rabbit IgG, FITC) conjugated for 1 h [dilution 1:200]. All procedures were undertaken at room temperature and samples were washed three times with PBS. The tissue sections were counterstained using UltraCruz® mounting medium with DAPI. Negative control was prepared

by incubating the tissue sections without the primary antibodies. Images capturing and analyses were performed as described above for the TUNEL analysis.

2.8. RNA isolation and real time-qPCR

Real time-qPCR analysis was performed as previously described [23] with some modifications. Briefly, samples were stored at -80°C in RNAlater®Stabilization Solution (Ambion™ ThermoFisher) until analysis. Total RNA samples were isolated after frozen-thawed samples using RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA), and quantified in Nanodrop 1000. Three pools were constituted according to the quality and quantity of RNA extracted from each sample. Subsequently, synthesis of cDNA of each pool was performed using Quantitect® Reverse Transcription Kit (Qiagen Inc., Valencia, CA, USA) and high quality samples (A260/A280) were used after normalization in a concentration of 250 µg/µL of total cDNA.

The real-time qPCR (2x Rotor-Gene SYBR® Green PCR Kit; Qiagen Inc., Valencia, CA, USA) was performed using 2.0 µL of cDNA, in a final concentration of 10 µM of each primer. The PCR conditions were the following: (1) initial denaturation of 5 min at 95°C; (2) two step cycling of 5 sec at 95°C for denaturation, and 10 sec at 60°C for the optimal annealing and extension. A total number of 40 cycles were performed. Relative gene expression was calculated using the $2^{(-\Delta\Delta Ct)}$ method and expressed in arbitrary units. Three replicates were performed and primers for the equine target genes GDF-9, BMP-15, SNRPD3 (housekeeping; [24]), and Cyclin–D2 [25] were used as previously designed.

2.9. Statistical analyses

Data on fluorescent intensities and mRNA expression were normalized after transformation to natural logarithms or ranks for statistical analyses. The SAS MIXED procedure was used (9.3 Version; SAS Institute Inc., Cary, NC, USA). If a significant effect of treatment or treatment-by-day interaction was detected, a Duncan test for multiple comparisons was used to distinguish differences in means among groups. Chi-square analysis was used to compare the percentage of morphologically normal follicles among groups within each day, and between days within each group. For estradiol analysis, data were rank transformed and tested using a two-way ANOVA comparing treatments, days, and the interaction of treatment by day of culture, followed by a Duncan test for multiple comparisons. Data are expressed as mean ± SEM, with $P < 0.05$ set as the threshold of significance and P values between 0.05 and 0.1 indicating tendency for a significance.

3. Results

3.1. Number of fragments and follicles evaluated

A total of 325 slides with 5,200 tissue sections were produced from 407 small cut ovarian fragments. Altogether, 947 preantral follicles were evaluated, with an average of 189.4 ± 60.1 follicles per treatment. The number of preantral follicles evaluated was 196, 419, 134, 95, and 103 for the fresh noncultured control group, cultured Day 7, cultured Day 15, cultured + FSH Day 7, and cultured + FSH Day 15 treatments, respectively.

3.2. Follicular morphology

A similar distribution of morphologically normal follicles was observed for both cultured treatments (48%) when compared with the noncultured control group (52%) regardless of culture

time (data not shown). After seven days of culture, medium supplemented with FSH was the only treatment that maintained a percentage of morphologically normal follicles similar ($P > 0.05$) to the noncultured control group. However, after 15 days of culture, both treated groups had a reduction ($P < 0.05$) in the percentage of normal follicles compared with the noncultured control group.

3.3. Estradiol production

Although estradiol production was detected in both cultured groups, no difference ($P > 0.05$) within days of culture was observed (Fig. 2). Within each cultured group, similar levels ($P > 0.05$) of estradiol were observed among Days 2, 4, and 7 of culture, as well as between Days 10 and 15 of culture. In the cultured control group, estradiol production increased ($P < 0.05$) from Day 4 to Day 10 and remained unchanged. An increase ($P < 0.05$) in estradiol production was observed between Days 7 and 10 of culture for the medium supplemented with FSH.

3.4. Incidence of apoptosis

The level of TUNEL fluorescence intensity was measured per pixel area for each image as represented (Fig. 3) and illustrated (Fig. 4). Apoptosis levels were similar ($P > 0.05$) among treatments and culture times.

3.5. Immunohistochemistry for EGFR and Ki-67

The staining of EGFR and Ki-67 was measured (Figs. 5-8) and expressed using fluorescence intensity per pixel area (10^6), as illustrated (Fig. 6 and Fig. 8). When compared with the noncultured control group, EGFR fluorescence intensity levels were similar ($P > 0.05$) to all

treated groups. However, the FSH supplemented medium after 15 days of culture tended ($P < 0.06$) to have higher fluorescence intensity than the noncultured control group. No difference ($P > 0.05$) was observed between the treated groups after 15 days of culture. However, the FSH supplemented medium was the only treatment in which an increase ($P < 0.05$) in fluorescence intensity was observed between Days 7 and 15 of culture.

Except for the cultured control medium at Day 7, a decrease in the expression ($P < 0.05$) of Ki-67 was observed for the treated groups when compared with the nonculture control group. Even though Ki-67 had lower levels of expression in the FSH treatment at Day 7 when compared to the cultured control medium, such a difference between these treatments was no longer observed at Day 15 of culture.

3.6. Gene expression

Gene expression of GDF-9, BMP-15, and Cyclin-D2 for the noncultured control group and treated groups are shown (Fig. 9). GDF-9 and Cyclin-D2 mRNA expression was observed in the noncultured control group and in all cultured treated groups; however, no difference ($P > 0.05$) was observed among groups. In contrast, BMP-15 gene expression was observed only in the non-cultured control group and in the cultured control treatment after 15 days of culture.

Discussion

The present study shows for the first time that equine ovarian biopsy tissue can be cultured successfully for up to 15 days, while maintaining the survival and functionality of enclosed ovarian stromal cells/preantral follicles. To the best of our knowledge, there is no study reporting the effect of FSH addition in a medium containing insulin plus EGF. Overall, the addition of

FSH to an enriched culture medium containing insulin and EGF did not affect the studied end points.

Estradiol production has been a viable tool to evaluate tissue functionality and differentiation for long-term culture in sheep [26]. In the present study, estradiol production from equine ovarian tissue was detected in every time point and increased significantly over time for up to 15 days of culture. However, estradiol production in every time point was not affected by the addition of FSH. The source of estradiol in our culture system was potentially the stromal cells, since preantral follicle number and density in equine ovarian biopsy fragments has been reported to be very low [12,21,27,28,29]. In addition, the estradiol production by stromal cells has previously been described in humans [8]. The estradiol levels found in the present study were lower than our previous results in *in vitro* culture of equine ovarian tissue [14,15]; this might have been due to the use of different types of estradiol assays in our previous studies. Furthermore, the current study was performed during the anestrous equine season, in which lower levels of steroids are expected to be produced [30,31]. Lastly, the lower levels of estradiol found in our study might have been caused by the smaller size (1.5 x 1.5 x 2 mm) of the cultured biopsied fragment compared to those used in our previous studies (3 x 3 x 1 mm) recovered from euthanatized animals [14,15].

Even though the addition of FSH to an enriched culture medium containing insulin and EGF did not improve the percentage of morphologically normal follicles, medium supplemented with FSH was the only treatment that maintained the percentage of morphologically normal follicles similar to the noncultured control group on Day 7 of culture. The best concentrations of insulin (10 ng/mL; [13]), EGF (50 ng/mL; [15]), and FSH (50 ng/mL; [14]) to improve the percentage of morphologically normal equine preantral follicles in culture have been established in previous

studies performed by our group. Nevertheless, to the best of our knowledge, there was no study reporting the effect of FSH addition in a medium containing insulin plus EGF in equine preantral follicles. Although the combination of insulin, EGF, and FSH has previously been used for some species, the benefit of this association has been controversial (mouse: [32]; bovine: [16,33]; bubaline: [34]; caprine: [17]; ovine: [35]. The effect of the addition of several factors to the culture medium can be strongly influenced by the concentration [36] and composition [37,38] of the base medium. Therefore, follicular cell survival and function depend on a fine balance among the substances present in the culture medium to control properly metabolic events, such as gene expression, and protein synthesis [15,36,38]. Our study supported the hypothesis that the association of FSH with EGF was not superior to treatment with EGF only to maintain ovarian tissue survival *in vitro*. However, further combinations of lower concentrations of insulin, EGF, and FSH should also be tested for the equine species.

An important finding in the present work was that the apoptotic levels detected by TUNEL assay and the expression of EGFR until 15 days of culture for both treated groups were statistically similar to the noncultured control group. The role of insulin [39], EGF [40], and FSH [41] on cell survival has previously been described. As a result, our findings clearly demonstrate that the concentrations of insulin, EGF, and FSH were appropriate for the survival and functionality of equine ovarian tissue even in the absence of FSH.

Despite the similarity of mRNA levels for Cyclin-D2 between treated groups and noncultured control, the expression for Ki-67 as a proliferative marker was lower in the treated groups at 15 days of culture. The later finding indicates a reduction in proliferative activity of cultured ovarian cells, suggesting that mRNA level does not necessarily correlate with its protein production [42]. These results clearly demonstrate that cell survival requirements differ from cell

proliferation. Hence, we have postulated that ovarian tissue cells decrease their metabolism, and consequently proliferation, with the final goal of surviving without jeopardizing cell functionality. Previous study [43] has reported that under certain culture conditions, i.e. after vitrification of mouse ovarian tissue, cell survival was maintained with a decrease in cell proliferation.

GDF-9 and BMP-15, belonging to the TGF-β family [44], are oocyte–specific proteins secreted by growing oocytes of several species [45,46,47], and have been highly correlated with follicle development [48,49]. However, BMP-15 was only expressed in the noncultured control and cultured control Day 15 groups. This finding most likely was due to the low relative mRNA expression of BMP-15 in primordial and primary follicles [50], which represent the majority of the follicular population in equine ovaries [6,12,21,27,29,51]. Contrary to the BMP-15 findings, the mRNA levels for GDF-9 were detected in all groups after culture and were similar to the noncultured control group. This finding indicates the presence of oocytes enclosed in preantral follicles after the culture periods. This is the first description of mRNA expression for GDF-9 and BMP-15 in equine ovarian tissue. In humans, the expression of GDF-9 and BMP-15 in primordial and primary follicles has previously been reported [52,53].

In conclusion, an enriched medium with insulin and EGF, supplemented or not with FSH, was able to maintain the functionality of equine ovarian biopsy tissue for up to 15 days of culture. The medium composition of both treated groups was beneficial to increase estradiol production, modulate cell proliferation to avoid a significant rise of apoptosis, and sustain EGFR and mRNA expression for GDF-9, BMP-15, and Cyclin-D2 genes. The findings of this study warrant future investigations on the evaluation of longer culture periods, as well as new culture systems with isolated ovarian cells, and the use of different concentrations of combined

supplemented substances to enhance knowledge in ovarian tissue manipulation of equine preantral follicles.

Conflict of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Figure legends

Fig. 1. Morphological representative aspects of preantral follicles in equine ovarian tissue after treatment with an enriched medium with or without FSH during seven and fifteen days of culture. (A) normal primordial follicle, (B) abnormal transitional follicle, Bars = 25 µm.

Fig. 2. Mean (± SEM) estradiol concentrations (pg/mL x 100; n = 60 samples) produced by equine ovarian biopsy tissue after *in vitro* culture for two, four, seven, ten, and fifteen days in spent enriched media with or without FSH (n = 3 pooled samples/treatment/day; 9 mares). [a,b,c] Within each treatment, values without a common letter differed ($P < 0.002$). No difference ($P > 0.05$) within days was observed between treatments.

Fig. 3. Mean (± SEM) fluorescence intensity detected by TUNEL analysis in equine ovarian biopsy tissue in the noncultured control group, and treated groups with or without FSH during seven and fifteen days of culture. No difference ($P > 0.05$) was observed between treatments.

Fig. 4. Representative immunofluorescence staining of TUNEL in equine ovarian biopsy tissue in the noncultured control group, and treated groups with or without FSH during seven and fifteen days of culture. (A) noncultured control, (B) cultured Day 7, (C) cultured Day 15, (D) cultured + FSH Day 7, (E) cultured + FSH Day 15, (F) positive control, (G) negative control. FITC revealed TUNEL positive nuclei in green and DAPI, counterstained all nuclei in blue. Bars = 200 µm.

Fig. 5. Mean (± SEM) detection of epidermal growth factor receptor (EGFR) in equine ovarian biopsy tissue in the noncultured control group, and treated groups with or without FSH during seven and fifteen days of culture. [a,b] Values without a common letter differed ($P < 0.05$). [†] Tended to differ ($P < 0.07$) from noncultured control group.

Fig. 6. Representative immunofluorescence staining of epidermal growth factor receptor in equine ovarian biopsy tissue in the noncultured control group, and treated groups with or without FSH during seven and fifteen days of culture. (A) noncultured control, (B) negative control, (C) cultured Day 7, (D) cultured Day 15, (E) cultured + FSH Day 7, (F) cultured + FSH Day 15. FITC revealed TUNEL positive nuclei in green and DAPI, counterstained all nuclei in blue. Bars = 200 µm.

Fig. 7. Mean (± SEM) fluorescence detection of Ki-67 in equine ovarian biopsy tissue in the noncultured control group, and treated groups with or without FSH during seven and fifteen days of culture. [a,b,c] Values without a common letter differed ($P < 0.05$).

Fig. 8. Representative immunofluorescence staining of Ki-67 in equine ovarian biopsy tissue in the noncultured control group, and treated groups with or without FSH during seven and fifteen days of culture. (A) noncultured control, (B) negative control, (C) cultured Day 7, (D) cultured Day 15, (E) cultured + FSH Day 7, (F) cultured + FSH Day 15. FITC revealed TUNEL positive nuclei in green and DAPI, counterstained all nuclei in blue. Bars = 200 µm.

Fig. 9. Mean (± SEM) relative amounts of mRNA for (A) GDF-9, (B) BMP-15, and (C) Cyclin-D2 (Ccnd2) in equine ovarian biopsy tissue in the noncultured control group and treated groups with or without FSH during seven and fifteen days of culture. No difference ($P > 0.05$) was observed among treatments in the GDF-9, BMP-15, and Cyclin-D2 genes.

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Table 1

Primers sequences and characteristics.

Gene	NCBI Acc.#	Primer sequences (5-3)	Amplicon size (bp)
Ccnd2	XM_001494152.3	S: TCACGACTTCATCGAGCACATC AS: GGCGAACTTAAAGTCAGTGGCA	118
BMP-15	XM_001496223.2	S: AGCCCTTGACCAATGTAGCAA AS: CGGTTGGATCTCAGAGGAAAGT	79
GDF-9	XM_001504427.2	S: GGCCACCTCTACAACACTGTCC AS:CCAGGTTAAACAGCAGGTCCAC	113
SNRPD3	XM_001489060.4 XM_008511652.1	S: ACGCACCTATGTTAAAGAGCATG AS: CACGTCCCATTCCACGTC	120

S: Sense, AS: Antisense; Ccnd2, G1/S-specific cyclin-D2; BMP-15, bone morphogenetic protein-15; GDF-9, growth differentiation factor-9; SNRPD3, small nuclear ribonucleoprotein D3.

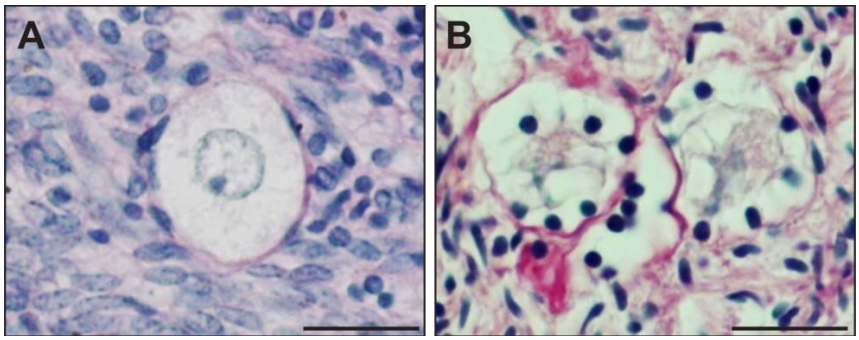

Figure 1

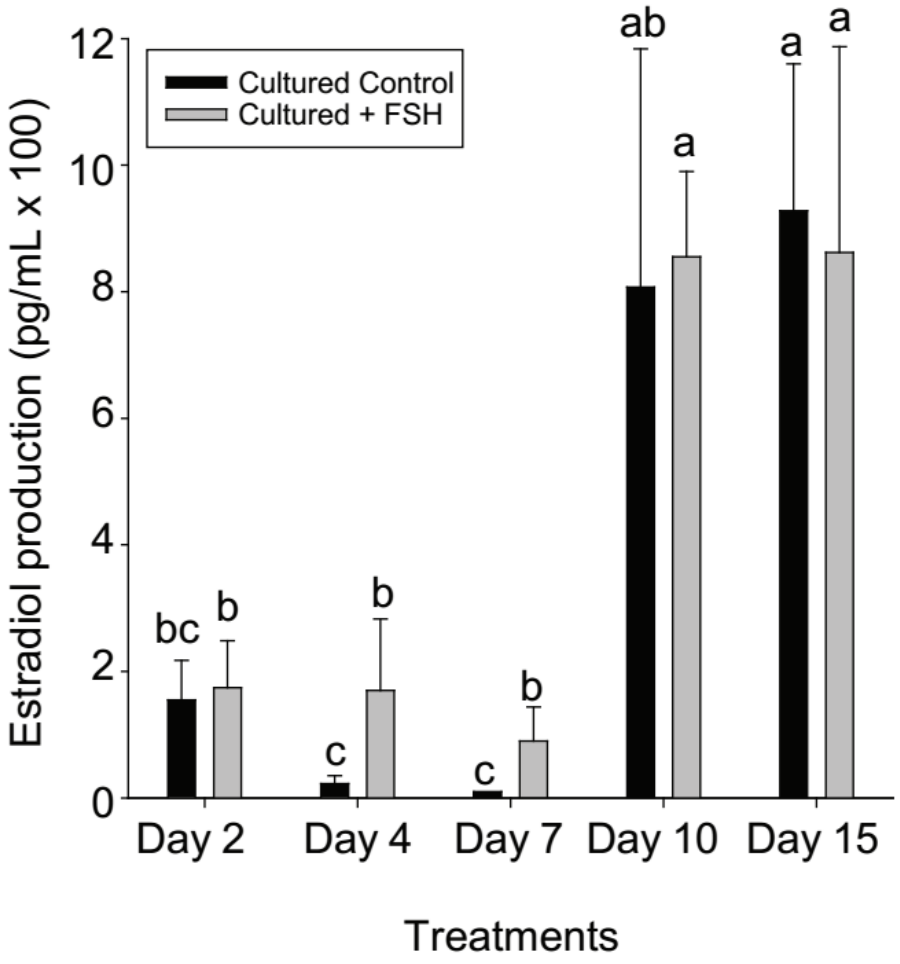
Cultured Control
Cultured + FSH
Estradiol production (pg/mL x 100)
0
2
4
6
8
10
12
bc
b
c
b
c
b
ab
a
a
a
Day 2
Day 4
Day 7
Day 10
Day 15
Treatments

Figure 2

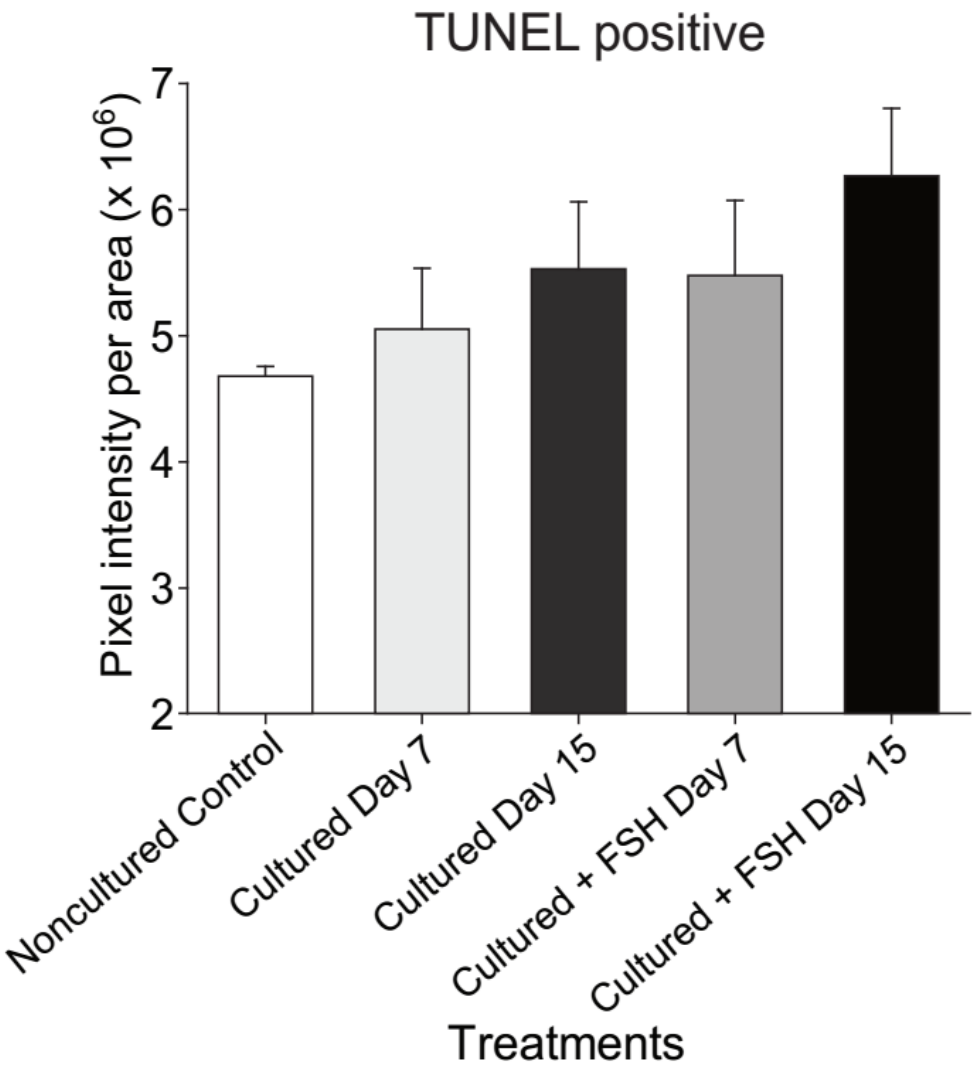
TUNEL positive
Pixel intensity per area (x 10^6)
7
6
5
4
3
2
Noncultured Control
Cultured Day 7
Cultured Day 15
Cultured + FSH Day 7
Cultured + FSH Day 15
Treatments

Figure 3

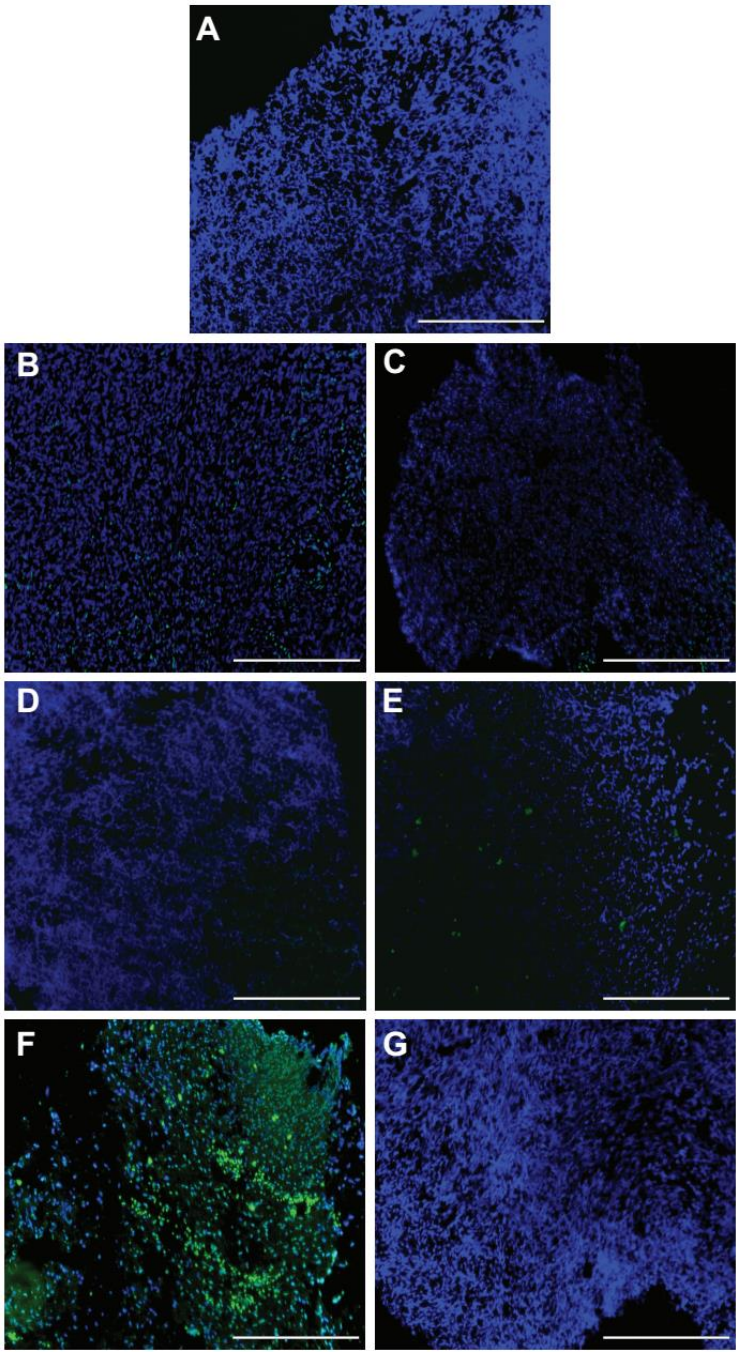

Figure 4

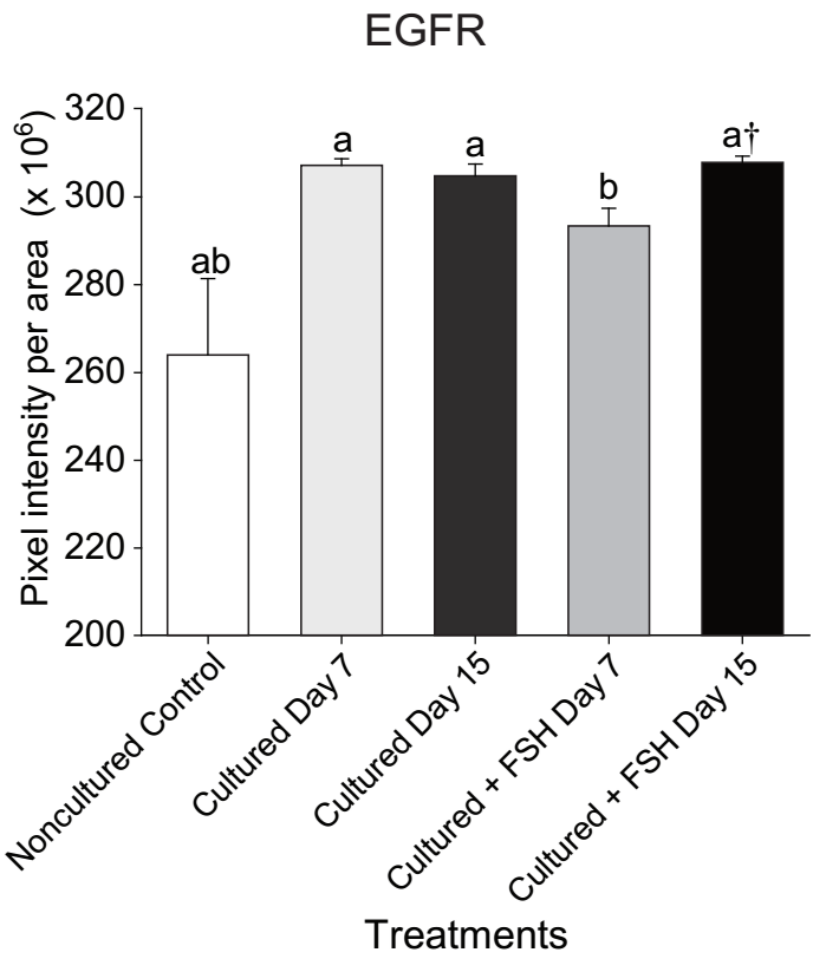
EGFR
Pixel intensity per area (x 10^6)
320
300
280
260
240
220
200
ab
a
a
b
a†
Noncultured Control
Cultured Day 7
Cultured Day 15
Cultured + FSH Day 7
Cultured + FSH Day 15
Treatments

Figure 5

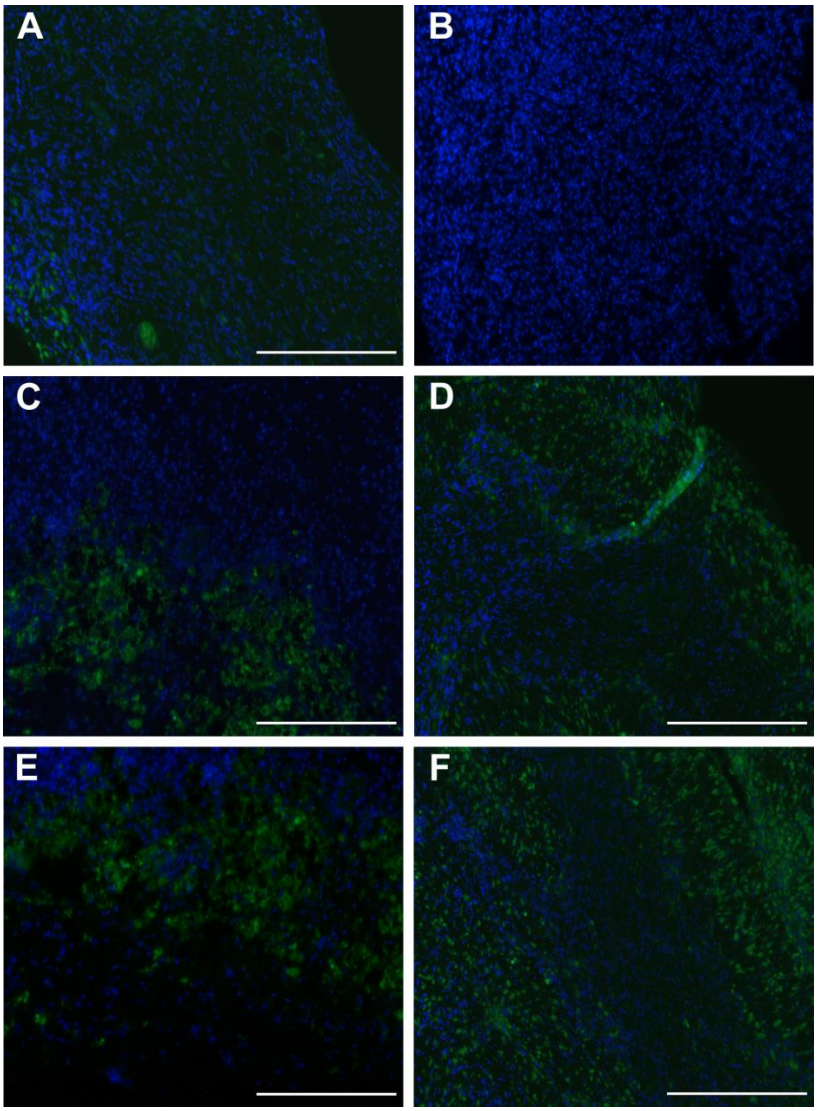

Figure 6

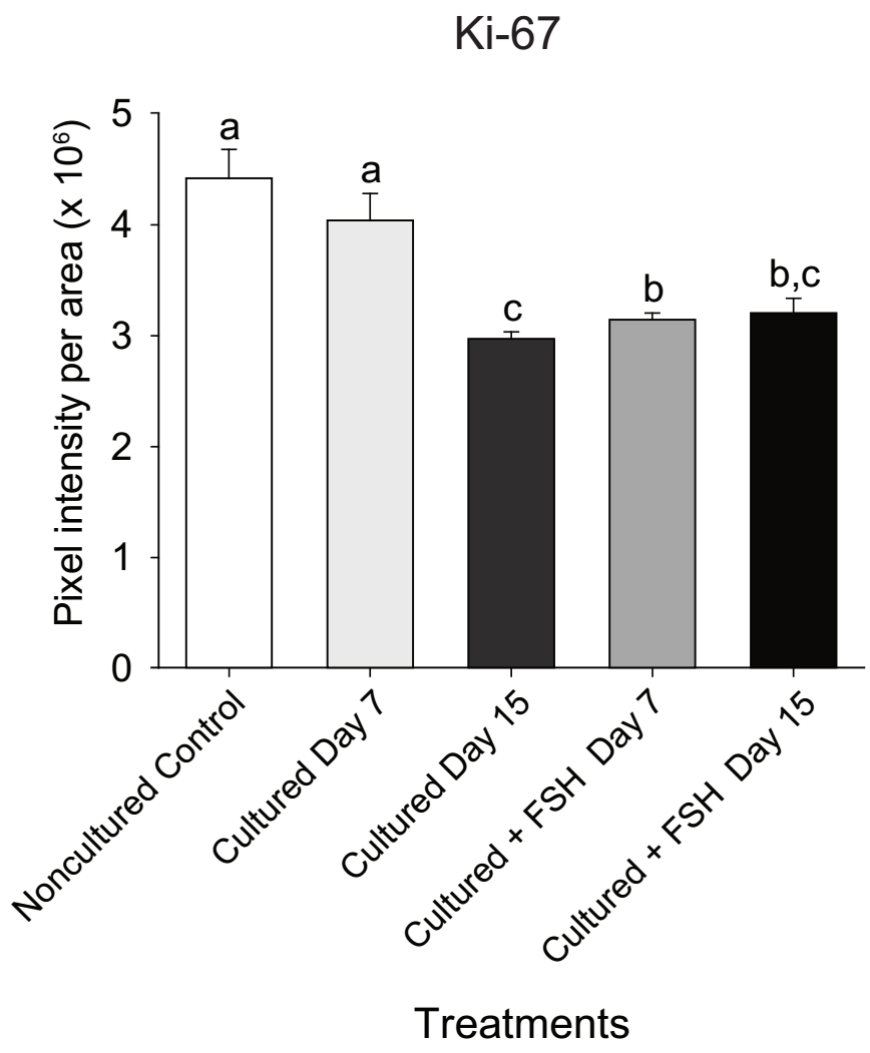
Ki-67
Pixel intensity per area (x 10⁶)
0
1
2
3
4
5
a
a
c
b
b,c
Noncultured Control
Cultured Day 7
Cultured Day 15
Cultured + FSH Day 7
Cultured + FSH Day 15
Treatments

Figure 7

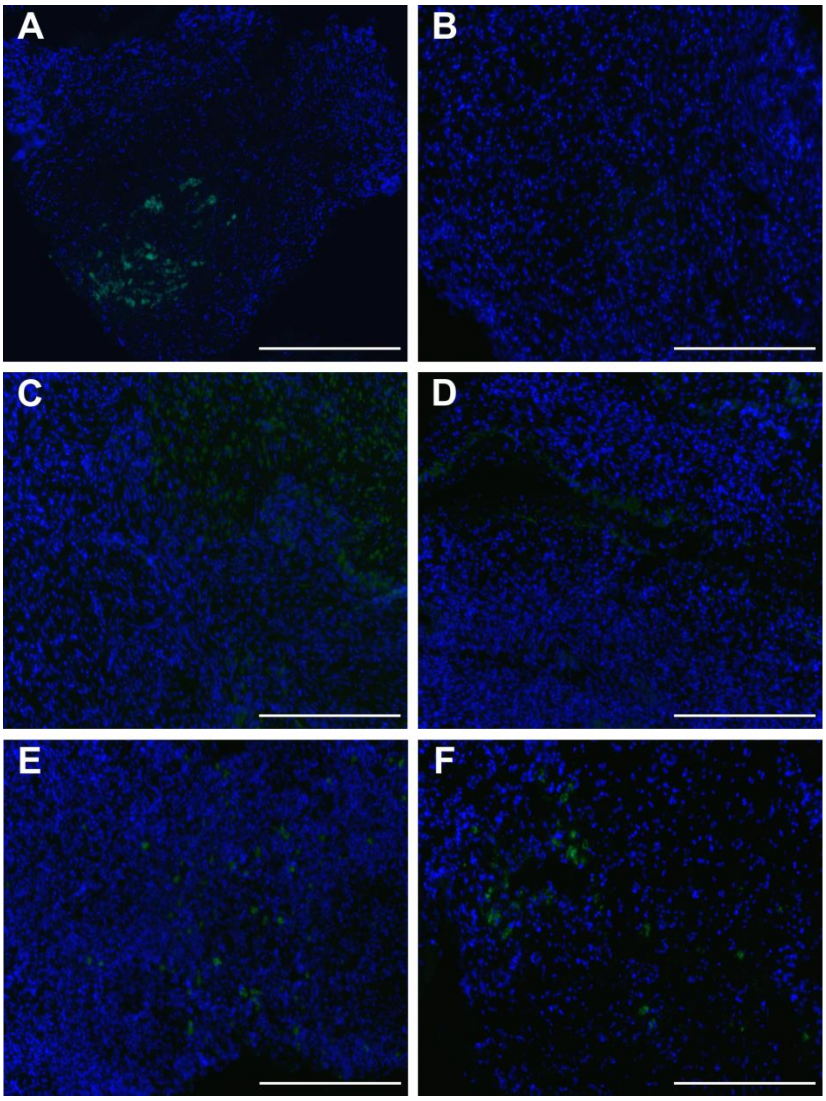

Figure 8

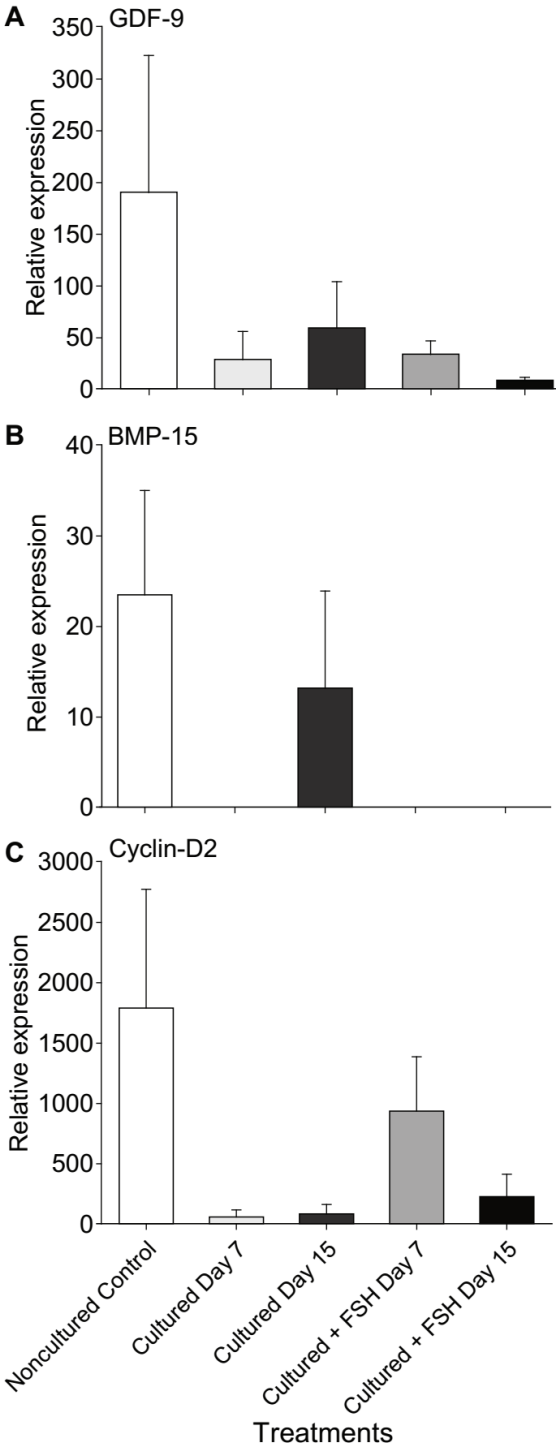
A
GDF-9
Relative expression
350
300
250
200
150
100
50
0
B
BMP-15
Relative expression
40
30
20
10
0
C
Cyclin-D2
Relative expression
3000
2500
2000
1500
1000
500
0
Noncultured Control
Cultured Day 7
Cultured Day 15
Cultured + FSH Day 7
Cultured + FSH Day 15
Treatments

Figure 9

10 CONCLUSÕES

- O uso de uma concentração fisiológica (10 ng/mL) de insulina para o cultivo *in vitro* de folículos pré-antrais equinos melhorou a sobrevivência e o desenvolvimento folicular, com uma redução no estresse oxidativo (Fase I);

- A adição de 50 ng/mL de FSH promoveu a ativação de folículos primordiais, aumentou a sobrevivência de folículos pré-antrais e manteve a produção de estradiol e de ROS do tecido ovariano equino do dia 1 para 7 de cultivo (Fase II);

- A concentração de 50 ng/mL de EGF manteve a sobrevivência folicular e a produção de ROS do dia 1 para o dia 7 de cultivo, bem como promoveu a ativação de folículos pré-antrais equinos inclusos em tecido ovariano após cultivo *in vitro.* Além disso, a análise do perfil metabolômico do meio de cultivo revelou a presença de três substâncias (Dinex, Leonuriside A e Avobenzene) com potencial efeito negativo sobre a sobrevivência, bem como sobre diâmetro folicular e oocitário após 7 dias de cultivo (Fase III).

- Um meio enriquecido com EGF (50 ng/mL) e insulina (10 ng/mL), suplementado ou não com FSH (50 ng/mL), foi capaz de manter a sobrevivência e a funcionalidade do tecido ovariano equino biopsado, após cultivo *in vitro* de longa duração (15 dias) (Fase IV).

11 PERSPECTIVAS

Este trabalho definiu uma suplementação ao meio de cultivo de base capaz de promover a sobrevivência e desenvolvimento de folículos pré-antrais iniciais inclusos em fragmentos de tecido ovariano equino cultivados *in vitro*. Isso representou um marco importante no desenvolvimento da biotécnica de MOIFOPA na espécie equina, a qual possui grande importância socioeconômica, além de constituir um valioso modelo animal para o estudo da foliculogênese humana.

Apesar dos avanços relatados na espécie equina relativos ao cultivo *in vitro* de folículos pré-antrais, esses resultados podem ser considerados modestos quando comparados aos relatados em outras espécies domésticas, como por exemplo, ovinos e caprinos. Nesse sentido, em função da complexidade do cultivo *in vitro* de tecido ovariano equino, devido às características inerentes à sua população folicular, a avaliação de novas combinações de fatores em diferentes concentrações e associações deve ser realizada visando no futuro incrementar a obtenção de oócitos maturos e, posteriormente, a produção *in vitro* de embriões a partir de folículos pré-antrais.

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