UNIVERSIDADE FEDERAL FLUMINENSE
FACULDADE DE VETERINÁRIA
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA VETERINÁRIA – CLÍNICA E REPRODUÇÃO ANIMAL

GLÁUCIA MOTA BRAGANÇA

ESTIMULAÇÃO HORMONAL OVARIANA EM OVELHAS DOADORAS DE OÓCITOS DA RAÇA SANTA INÊS

Niterói, 2019

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Tese apresentada à universidade Federal Fluminense, como requisito do Programa de Pós-graduação em Medicina Veterinária, área de concentração Clínica e Reprodução Animal, para obtenção do título de Doutor.

Orientador:
Prof. Dr. Felipe Zandonadi Brandão

Coorientadores:
Profª.Dra. Joanna Maria G. de Souza-Fabjan
Dr. Jeferson Ferreira da Fonseca

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Niterói, 2019

“A ciência humana de maneira nenhuma nega a existência de Deus. Quando considero quantas e quão maravilhosas coisas o homem compreende, pesquisa e consegue realizar, então reconheço claramente que o espírito humano é obra de Deus, e a mais notável.”

(Galileu Galilei)

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da escola e chamava de doutora desde que eu era uma criança,
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liberdade, coragem e ética.
Dedico*.*

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LISTA DE ABREVIATURAS E SÍMBOLOS

ACB / BCB	Azul cresil brilhante / *Brilliant cresyl blue*
ARTs	*Assisted reproductive technology*
Bax	*Bcl2-associated protein X*
Bcl2	*B-cell lymphoma protein 2*
BMPs	Bone morphogenetic proteins
^{O}C	Graus celsius
CCO / COC	Complexo cumulus-oócito */ Cumulus-oocyte complex*
E2	17β-estradiol
eCG	*Equine chorionic gonadotropin*
ERα	*Estradiol receptor alpha*
FSH	*Follicle stimulating hormone*
FSHr	*Follicle stimulating hormone receptor*
GDF9	*Growth differentiation factor 9*
G6PDH	*Glucose-6-phosphate deydrogenase*
GI, II, III e IV	Grau I, II, III e IV
H	Hora
Im	Intra muscular
KL	*Kit ligand*
LH	*Luteininzing hormone*
LHr	*Luteininzing hormone receptor*
LOPU	*Laparoscopic ovum pick up* (laparoscopia)
MAP	Acetato de medroxiprogesterona
MATER	*Maternal antigen that embryo requires*
MD	*Multiple dose* (doses múltiplas)
Mg	Miligrama
MOTE / MOET	Multipla ovulação e transferencia de embriões / *Multiple ovulation and embryo transfer*
ng⁄dL	Nanograma por decilitro
OS	*One-shot* (dose única)
P4	Progesterona
PGF2α	Prostaglandina F2-alfa
PIVE / IVEP	Produção *in vitro* de embriões / *In vitro embryo production*
PVA	Polyvinil alcohol
STAR	Steroidogenic acute regulatory protein
ZAR1	*Zygote arrest 1*

Capítulo 1

Revisão de literatura

RESUMO

A manipulação hormonal das funções ovarianas usando associação de gonadotrofinas e progestágenos é amplamente aplicada nas tecnologias de reprodução assistida para aumentar a obtenção de oócitos em ovelhas vivas. O objetivo deste estudo foi avaliar o efeito de protocolos estimulatórios, considerando a resposta ovariana, a modulação gênica e seu impacto sobre a quantidade e qualidade dos complexos *cumulus* oócitos (CCOs) imaturos em ovelhas da raça Santa Inês. No experimento 1, avaliou-se o uso de duas doses (80 e 120 mg) e dois regimes de administração (única ou múltipla) do FSH. A população folicular, taxa de recuperação, quantidade de CCOs viáveis e CCOs azul cresil brilhante positivo (ACB+) não diferiram ($P>0.05$), mas houve efeito do regime em múltipla aplicação sobre a proporção de CCOs GI/II. A expressão de genes marcadores de qualidade e da via esteroidogênica foi afetada ($P<0.05$) pelo FSH. A abundância na via esteroidogênica foi reduzida com o aumento da dose de FSH nos protocolos de única aplicação, mas em múltipla somente o LHr foi afetado. Comparando-se a mesma dose de FSH em diferentes regimes; 80 mg em única aplicação reduziu a expressão de FSHr e ERα, porém em 120 mg apenas o LHr foi reduzido. No experimento 2, foi avaliado o uso da progesterona (P4) e acetato de medroxiprogesterona (MAP) durante a estimulação com 80 mg de FSH em múltipla administração previamente estabelecida no experimento 1. A resposta ovariana e os parâmetros oocitários não diferiram entre os grupos. Contudo, a expressão gênica foi alterada pelos progestágenos. Receptores da via esteroidogênica (FSHr, LHr, Erα) e marcadores de qualidade (ZAR1, GDF9 e Bcl-2) foram abundantes em P4 ($P <0,05$) em relação ao controle não tratado. RELN foi subexpresso e Bcl-2 superexpresso em MAP ($P <0,05$) em relação ao controle. FSHr, LHr e RELN foram superexpressos ($P <0,05$) em P4 (*vs* MAP). Em conclusão, 80 mg de FSH em ambos os regimes foi eficiente no incremento da população folicular, obtenção de CCOs com crescimento exponencial finalizados, o regime de múltipla administração apresentou melhor modulação de genes marcadores de competência oocitária. P4 e MAP não alteraram a dinâmica folicular, número de folículos, número de CCOs e nem a qualidade morfológica dos mesmos. A P4 exógena modulou positivamente a abundância dos genes com importância na aquisição da competência. Dessa forma, o uso de 80 mg de FSH associado a um dispositivo intravaginal contendo P4 promove a obtenção de CCOs imaturos potencialmente mais competentes em ovelhas para uso em tecnologias da reprodução.

Palavras-chave: FSH, progestágeno, progesterona, oócito, expressão gênica, ACB, ovinos.

ABSTRACT

Hormonal manipulation of ovarian function using an association of gonadotropins and progestogens are widely applied in the assisted reproductive technologies (ARTs) to enhance oocyte collection in live small ruminants donors. The aim of this study was to evaluate the effect of stimulatory protocols, considering ovarian response, gene modulation and its impact on the quality of immature cumulus oocytes complexes (COCs) in Santa Inês sheep. At experiment 1, doses (80 and 120 mg) and treatment regimen (single or multiple) of FSH were evaluated. Follicular population, recovery rate, number of viable COCs and brilliant cresyl blue positive (BCB+) COCs did not differ among groups, but there was an effect on the proportion of GI / II COCs in the multiple injection regimen. FSH affected the gene expression of quality markers and steroidogenic pathway ($P<0.05$). Abundance in the steroidogenic pathway was reduced with increasing of the FSH dose in single treatment, but in multiple only LHr was affected. When comparing the same FSH dose in different regimen, 80 mg in single treatment reduced the expression of FSHr and ERα, but in 120 mg only the LHr was reduced. In experiment 2, the effect of progesterone (P4) and medroxyprogesterone acetate (MAP) used during FSH stimulation were evaluated. Ovarian response and oocyte parameters did not differ among groups. However, gene expression was altered by progestogens: steroidogenic pathway receptors (FSHr, LHr, Erα) and quality markers (ZAR1, GDF9 and Bcl-2) were up-regulated in P4. RELN was down-regulated and Bcl-2 up-regulated in MAP ($P <0.05$). FSHr, LHr and RELN were up-regulated ($P <0.05$) in P4 (vs MAP). In conclusion, 80 mg of FSH in both regimen was efficient to increase follicular population, collection of fully grown COCs, the multiple injection regimen presented better modulation of markers of oocyte competence. P4 and MAP did not alter follicular dynamics, number of follicles, number of COCs or their morphological quality. Exogenous P4 positively modulated the abundance of genes important for oocyte competence. Thus, the use of 80 mg of FSH associated with an intravaginal device containing P4 improves the collection of immature COCs potentially more competent for use in reproduction technologies in sheep.

Key words: FSH, progestogen, progesterone, oocyte, gene expression, BCB.

1. INTRODUÇÃO

A criação de ovelhas foi uma das primeiras atividades pecuária praticada pelo homem, após a domesticação desta espécie ocorrida por volta do período neolítico (PEDROSA et al., 2005; CHESSA et al., 2009). Atualmente, a ovinocultura está presente em todos os continentes, exercendo importante papel na produção de alimentos de alto valor biológico e na geração de emprego e renda, especialmente em países em desenvolvimento (FAO, 2015; SKAPETAS e KALAITZUDOU, 2017). No Brasil, o rebanho efetivo ovino é de aproximadamente 18.500.000 cabeças, distribuídas em todo o território nacional (IBGE, 2015). Neste cenário, a raça Santa Inês se destaca, devido à sua alta adaptabilidade às condições tropicais hostis, boa habilidade materna e baixo grau de estacionalidade reprodutiva (BALARO et al., 2014; 2015).

A aplicação de tecnologias de reprodução assistida, como a produção *in vitro* de embriões (PIVE), permite o incremento da eficiência reprodutiva e acelera o ganho genético pela rápida propagação de embriões com características desejáveis para a indústria, além de fornecer material biológico para pesquisa básica (revisado por OROZCO-LUCERO e SIRARD 2014). Contudo, conforme dados reportados ao *Committee of the International Embryo Transfer Society* (IETS, 2017), a atividade comercial mundial de embriões ovinos e caprinos produzidos *in vivo* é baixíssima, já para embriões produzidos *in vitro* os dados são praticamente inexistentes, apenas os Estados Unidos reportou 207 embriões caprinos oriundos de PIVE em 2017.

A PIVE envolve quatro etapas: coleta de oócitos, maturação, fertilização e cultivo *in vitro* (MIV, FIV, CIV, respectivamente) em geral acompanhados até o estágio de blastocisto, pois nesta fase podem ser eficientemente criopreservados ou transferidos para o útero de fêmeas receptoras sincronizadas. Embora, a PIVE ovina tenha feito grandes progressos ao longo das últimas décadas, ainda apresenta resultados pouco consistentes e com grande variabilidade entre laboratórios quando comparado a outras espécies. O sucesso desta biotécnica depende da obtenção de oócitos de boa qualidade, capazes de suportar o desenvolvimento embrionário inicial até a ativação do genoma no estádio pré-implantacional (SOUZA-FABJAN et al., 2013, 2016; revisado por PARAMIO e IZQUIERDO, 2016).

A manipulação hormonal das funções ovarianas usando associação de gonadotrofinas e progestágenos é amplamente aplicada para incrementar a obtenção de oócitos para uso na PIVE (BERLINGER et al., 2007, PARAMIO e IZQUIERDO, 2016; THAMMISIRI et al., 2016).

Protocolos compostos por diferentes concentrações, origens (suína e ovina) e graus de pureza do hormônio folículo estimulante (FSH), em regime de múltiplas (três a seis) ou única administração, são utilizados com intuito de promover o desenvolvimento folicular ovariano (BALDASSARRE et al., 1996, 2002; BERLINGUER et al.. 2004; SOUSA et al., 2011; MENCHACA et al., 2018). Enquanto que, os progestágenos, são usados durante o tratamento com gonadotrofinas, para inibir a secreção pulsátil do hormônio luteinizante (LH), permitindo a regressão do(s) folículo(s) dominante(s) pela atresia, além de coibir a ovulação dos folículos em crescimento (revisado por SOUZA-FABJAN et al., 2014c). No entanto, existem controvérsias a respeito de como os protocolos hormonais impactam a qualidade do oócito e consequentemente a produção de embriões, efeitos benéficos e/ou deletérios já foram relacionados ao uso tanto do FSH, como a fonte de progestágeno (BERLINGUER et al., 2004; 2007; NIVET et al., 2012; MENCHACA et al., 2018; THAMISSIRI et al., 2018).

A competência ao desenvolvimento é adquirida ao longo do crescimento folicular, principalmente na fase final. Todavia, o crescimento total do oócito não garante que este, seja capaz de passar por todas as etapas do desenvolvimento embrionário a termo (SIRAD et al., 2006; 2011). Entretanto, evidências indicam que oócitos que já terminaram seu crescimento exponencial, selecionados pelo teste do azul cresil brilhante (ACB), são mais competentes e apresentam maior taxa de blastocistos (TORNER et al., 2008; SALVIANO et al., 2015). Assim, identificou-se um nicho de investigação científica a ser explorado, relacionado ao impacto molecular dos protocolos hormonais estimulatórios e implicações na qualidade oocitária.

Delineou-se três experimentos visando investigar os efeitos da dose de FSH (80 e 120 mg), regime de administração (único e múltiplo) e fontes exógenas de progestágenos (progesterona-P4 e acetato de medroxiprogesterona-MAP) sobre a resposta ovariana e modulação de genes conhecidos por exercerem funções importantes na aquisição da competência, tais como: fatores de crescimento (BMP15 e GDF9), genes de efeito materno (MATER e ZAR1), via esteroidogênica (FSHr, LHr, STAR e ERα), apoptose (Bcl-2 e BAX) e dois genes recentemente relacionados com a regulação do crescimento folicular final (RELN e LRP8). Ressalta-se que avaliar os impactos moleculares no oócito promovidos pelos protocolos hormais de estimulação ovariana é de grande importância para desenvolver estratégias de uso desses hormônios como potenciais melhoradores da qualidade oocitária. Visto que a obtenção de oócitos em fêmeas vivas deve ser tratada com a mesma importância que as demais etapas da PIVE, visando maximizar os resultados dados os custos envolvidos.

2. REVISÃO DE LITERATURA

2.1 Princípios gerais da oogênese e folículogênese

Desde de os primeiros estudos anatômicos e especulações científicas sobre a função ovariana realizados por Jones (1836, 1843), a contribuição de cientistas do mundo inteiro, através da publicação de seus estudos, proporcionou a ampliação dos conhecimentos e compreensão da biologia reprodutiva humana e animal. Dessa forma, hoje sabe-se que os ovários dos mamíferos possuem função gametogênica e esteroidogênica. O folículo é a unidade morfológica funcional dos ovários e é estruturalmente composto por um oócito interno circundado pelas células somáticas epiteliais da granulosa (CG) e da teca (CT), mais externamente. As CG em conjunto com o oócito formam o complexo cumulus-oócito, cuja a comunicação ocorre através de uma rede de canais transmembranas. As CG e CT agem de forma cooperativa entre si na produção de esteroides sexuais, maturação e nutrição do oócito (revisado por VAN DEN HURK e ZHAO, 2006; LI e ALBERTINI, 2013; KEMILÄINEN et al., 2016). Nos mamíferos placentários, a oogênese e a foliculogênese são eventos que se iniciam previamente ao nascimento, estes eventos seguem um padrão bifásico de crescimento sincrônico entre folículo e oócito (TISDALL et al., 1995; CARABATSOS et al., 1998; BENDSEN et al., 2006).

A oogênese é o processo de formação e diferenciação das células germinativas primordiais até a formação do oócito haploide fecundado. Desta forma, durante a vida fetal, as células germinativas primordiais sofrem sucessivas divisões mitóticas e se tornam oogônias. Estas por sua vez, se diferenciam em oócito primário ao iniciarem a primeira divisão meiótica, onde passam pelos estádios de leptóteno, zigóteno e paquíteno, estacionando em diplóteno da prófase I. Os oócitos primários (no folículo de Graaf) retomam a meiose já na puberdade, por ocasião dos picos transitórios de FSH e LH, o núcleo então progride do estádio de vesícula germinativa para diacinese. Sequencialmente, ocorre a progressão para metáfase I, anáfase I, telófase I, seguida da extrusão do primeiro corpúsculo polar, culminando na sua diferenciação em oócito secundário. Por fim, a segunda divisão meiótica acontece, desta vez estacionando no estádio de metáfase II, onde permanece até ser fecundado pelo espermatozoide, que promove a extrusão do segundo corpúsculo polar completando a meiose, e resultando na formação do oócito haploide fecundado (revisado por HIRSHIFIELD 1991; revisado por MAGALHÃES et al., 2009a).

A foliculogênese corresponde a formação, crescimento e maturação folicular, abrangendo os estádios de desenvolvimento pré-antral e antral. O estádio pré-antral consiste na

formação do folículo primordial, primário e secundário, enquanto o estádio antral é composto pelo folículo terciário e pré-ovulatório (revisado por SARAIVA et al., 2010). A atuação das gonadotrofinas hipofisárias durante o estádio antral é fundamental para o crescimento folicular (EVANS et al., 2000; GINTHER et al., 2016a). Entretanto, o estádio pré-antral parece ser pouco dependente deste processo para se desenvolver, visto que a adição de FSH ao meio de cultivo *in vitro* ativou o crescimento de folículos pré-antrais em caprinos e inibiu a apoptose (MATOS et al., 2007), mas a adição de LH sozinho ou em associação com FSH resultou na degeneração destes (SARAIVA et al., 2008). Contudo, um estudo em ovinos reportou a detecção de receptores de FSH (FSHr) em ovários de fetos de 100, 120 e 135 dias de gestação, bem como em 79% dos folículos primários de fêmeas adultas (TISDALL et al., 1995). O surgimento dos receptores de FSH a partir dos folículos primários permite uma sensibilidade ao hormônio, porém os folículos se tornam dependentes do FSH somente após o seu recrutamento (progressão a fase antral), o que ocorre quando o folículo atinge aproximadamente 2 mm de diâmetro em ovelhas. Esses folículos pré-antrais permanecem em estado de quiescência até o recrutamento (revisado por DRIANCOURT, 2001).

O estádio antral é caracterizado pela formação do antro, que é uma cavidade preenchida por um fluido rico em substâncias originárias do sangue e secreções das células foliculares, como gonadotrofinas, esteroides e fatores de crescimento, fruto da intensa proliferação das CG. A produção desse fluido se intensifica progressivamente com o aumento da vascularização e da permeabilidade vascular durante o crescimento folicular, a partir da ativação e recrutamento até a formação do grande folículo pré-ovulatório (revisador por VAN DEN HURK e ZHAO, 2005).

2.2 Dinâmica folicular em ovelhas

Denomina-se dinâmica folicular o processo contínuo de crescimento e regressão de folículos antrais com consecutivo desenvolvimento de um folículo pré-ovulatório. O padrão pode ser organizado no surgimento de um *pool* de folículos que crescem sincronicamente caracterizando uma onda folicular. Em ovelhas, pode ocorrer de 2-4 ondas, com predominância de três ondas dentro de um ciclo estral, o qual dura aproximadamente 17 dias (BARTLEWSKI et al., 1998; EVANS et al., 2000). A ação das gonadotrofinas sobre a onda folicular envolve três fenômenos: emergência ou recrutamento, seleção ou desvio e dominância. Logo, o destino do folículo é controlado por fatores endócrinos e parácrinos que desencadeiam estímulos à expressão de genes que modulam o seu crescimento até atingir o platô, seguindo para a ovulação ou desviando à atresia (GIRARD et al., 2015; GINTHER et al., 2016ab).

Deste modo, a emergência folicular está correlacionada com o aumento nas concentrações circulantes de FSH, que promove o surgimento de um grupo de folículos na superfície ovariana originando a onda. Em ovelhas, os picos de flutuações do FSH não são afetados pelo anestro e são associados com o crescimento de folículos de 3-5 mm da mesma forma que ocorre durante a estação reprodutiva (BARTLEWSKI et al., 1998; 2000). Por outro lado, um estudo em bovinos demonstrou que a seleção é um evento FSH-LH dependente e ocorre quando um folículo se diferencia dos demais, adquirindo competência potencial para ovular. Os mecanismos de seleção não variam entre as ondas 1 e 2, porém a elevação transitória do LH é menos pronunciada na onda dois, possivelmente devido à alta concentração de progesterona durante esta última (KULIK et al., 2001). O folículo selecionado parece ser o primeiro a adquirir receptores de LH (LHr) nas células da granulosa (BEG et al., 2001; LUO et al., 2011) e, em ovelhas, isso ocorre quando o folículo atinge o diâmetro de 4 mm (revisado por DRIANCOURT, 2001). A aquisição de LHr habilita o folículo a continuar seu desenvolvimento quando a concentração de FSH diminui a concentrações basais pós-seleção. Em seguida, o folículo aumenta a secreção de estradiol e inibina que via *feedback* negativo suprime a liberação de FSH pela hipófise, restringindo o crescimento dos demais folículos dessa onda. Assim, o folículo selecionado assume a dominância e segue seu crescimento, já os folículos subordinados entram em atresia (BEG et al., 2001; SARTORI et al., 2001; GINTHER et al., 2016ab).

O folículo dominante estrogênio-ativo aumenta seu diâmetro enquanto as concentrações séricas de LH aumentan, pois este depende do LH para terminar a diferenciação e se tornar ovulatório. Todavia, é necessário que a progesterona esteja em concentrações basais para que a maturação ocorra adequadamente e a ovulação aconteça (SUNDERLAND et al., 1994; GINTHER et al., 2016b). Portanto, o folículo ovulatório é sempre proveniente da última onda folicular em ciclos estrais naturais. Caso contrário, a alta concentração de progesterona causada por um corpo lúteo (CL) ativo no ambiente ovariano, faz *feedback* negativo com o LH, com a diminuição da amplitude e frequência dos pulsos dessa gonadotrofina hipofisária, a maturação final não ocorre e o folículo perde a dominância. Por conseguinte, a atresia acontece e uma nova onda folicular se inicia (SUNDERLAND et al., 1994; BARTLEWSKI et al., 1998; EVANS et al., 2000; GINTHER et al., 2016ab).

2.3 Esteroidogênese ovariana

A produção dos hormônios esteroides sexuais, estradiol e progesterona (P4), é chamada esteroidogênese ovariana (figura 1). Esses esteroides, contribuem para a regulação funcional do

ciclo reprodutivo via mecanismos de *feedback* com FSH e LH no eixo hipotalâmico-hipofisário-gonadal. Desta maneira, mecanismos endócrinos relacionados a secreção do GnRH e consequente liberação do FSH e LH induzem a ativação de mecanismos de ação local (parácrinos), desencadeando uma cascata de reações enzimáticas nas células da teca e da granulosa culminando na síntese dos esteroides (EVANS et al., 2000; LUO et al., 2011; SUMMERS et al., 2015; GINTHER et al., 2016ab; LARIMORE et al., 2016). O LH modula a atividade na célula da teca, enquanto o FSH age sobre as células da granulosa, a cooperação entre essas células é fundamental para a produção do estradiol conforme descrito pela teoria "duas células - duas gonadotrofinas" (HILLIER et al., 1994). O folículo antral maduro é o principal condutor da esteroidogênese, além do CL após a ovulação. Desta forma, antes do período peri-ovulatório, o FSH promove o aumento gradativo da síntese de estradiol, para promover o pico ovulatório do LH. À medida que a ovulação se aproxima, o folículo peri-ovulatório aumenta a produção de estradiol para induzir a ovulação e consequentemente formação do CL (SUNDERLAND et al., 1994; BARTLEWSKI et al., 1999, 2000). Tudo isso, ocorre através de ativação da via proteína kinase A (PKA) dependente de AMPc, que é a principal rota de modulação da esteroidogênese (DARBON et al., 1984; HILLIER et al., 1994).

A síntese dos esteroides sexuais nas células somáticas foliculares ocorre pela ação de atividades enzimáticas específicas. Assim, as células da teca no folículo antral inicialmente contêm apenas receptores de LH (LHr), e após a ligação ao receptor, o LH estimula a transcrição de genes expressos pela teca que codificam as enzimas necessárias para a conversão de colesterol em andrógenos (LUO et al., 2011). O colesterol pode ser transportado para o citoplasma da célula da teca via receptores de lipoproteína ou pode ser produzido pela via *síntese de novo* (revisado por MILLER e BOSE, 2011). O colesterol é então internalizado na mitocôndria através da proteína reguladora aguda esteroidogênica (STAR). Em seguida, o colesterol é convertido em pregnenolona nas mitocôndrias através da clivagem da cadeia lateral do colesterol pelo citocromo-P450 (CYP11A1). A pregnenolona então se difunde para fora das mitocôndrias e vai para o retículo endoplasmático liso onde é convertida em progesterona ou desidroepiandrosterona (DHEA) pela ação da enzima 3β-hidroxiesteroide desidrogenase (HSD3β) ou 17α-hidroxilase-17,20-desmolase (CYP17A1), respectivamente. A progesterona e DHEA por sua vez, são convertidas no andrógeno androstenediona novamente pela CYP17A1 ou HSD3β, respectivamente (revisado por MILLER e BOSE, 2011; SUMMERS et al., 2014).

Por outro lado, as células da granulosa do folículo antral inicialmente contêm apenas receptores de FSH (FSHr), e em resposta à ligação ao hormônio a cascata de transcrição das enzimas esteirodogênicas é ativada. Logo, a 17β-hidroxiesteroide desidrogenase (HSD17β) ou a aromatase (CYP19A1) convertem a androstenediona em testosterona ou estrona, respectivamente. A testosterona e a estrona são convertidas em estradiol-17β, através das mesmas enzimas CYP19A1 e HSD17β, respectivamente (LUO e WILTBANK, 2006; LARIMORE et al., 2016). Desta forma, a ação cooperativa entre as células somáticas foliculares se dá devido as células da teca não possuírem a enzima CYP19A1, em contraste a granulosa não possui CYP17A1, converte pregnenolona e progesterona em andrógenos (revisado por MILLER e BOSE, 2011).

Estudos demonstram que a administração de gonadotropinas afeta a esteroidogênese. A ação do FSH pode ser regulada pela ação de membros da superfamília dos fatores de transformação do crescimento beta (TGFβ) como GDF9 e sistemas BMPs. *In vivo,* o BMP15 é um inibidor potente do FSHr e da via PKA, bem como o GDF9 inibe a expressão do receptor LH dependente de FSH, a produção de AMPc e consequentemente a síntese de P4 e estradiol (revisado por SHIMASAKI et al., 2004). *In vitro,* o efeito do inibidor da luteinização destas BMPs é um mecanismo para manter a relação estradiol / P4, porque o aumento prematuro da concentração de P4 está correlacionado com a atresia folicular (ZHENG et al., 2008). As BMP suprimem a produção de P4 por redução de STAR e aumentam a produção de estradiol induzida por FSH pelo aumento da expressão de aromatase (CYP19A1). O estradiol melhora a ação de FSH por aumento progressivo da expressão de FSHr, LHr e seus próprios receptores como ERα, resultando em atenuação da apoptose e da atresia folicular (DARBONet al., 1984; revisado por COUSE e KORACH, 1999;YU et al., 2003; ZHENG et al., 2008).

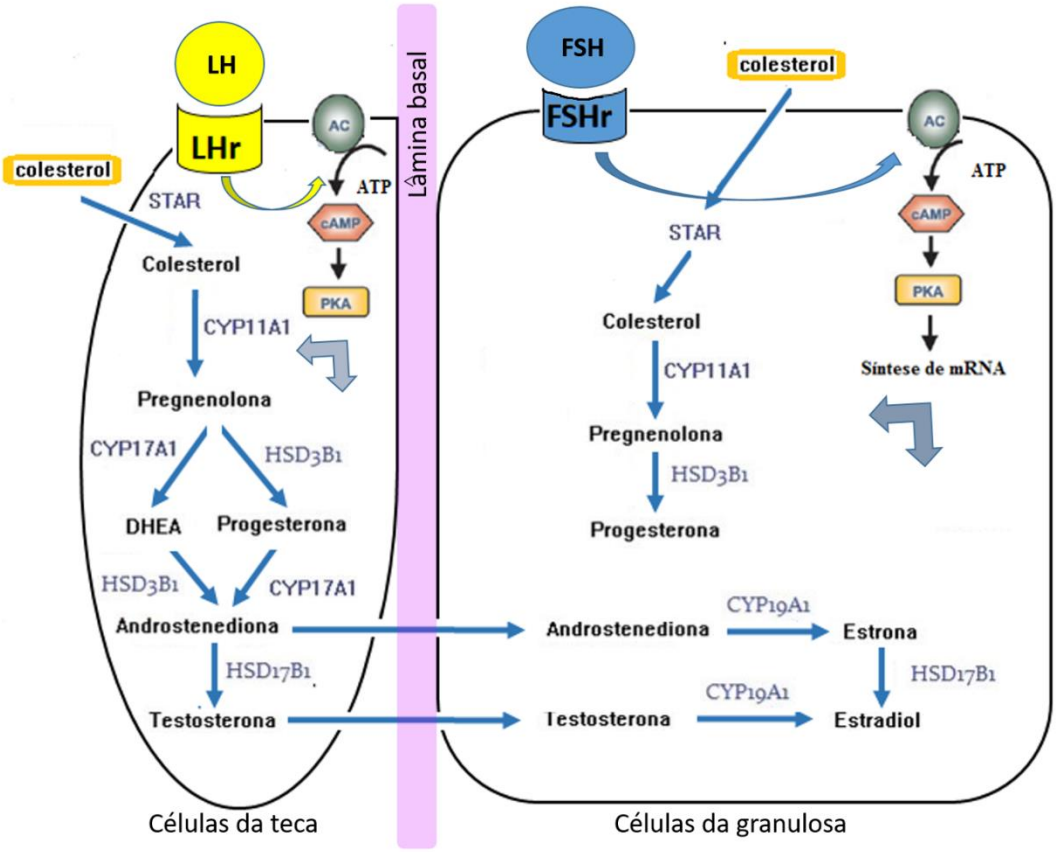

Figura 1. Resumo esquemático da esteroidogênese ovariana. Nas células da teca, o LH se liga ao receptor, isso ativa adenilciclase (AC) que gera adenosina trifosfato (ATP), ativando a síntese de AMPc e ativa a síntese de mRNA das enzimas esteroidogênicas na via PKA que ao final da cascata gera androstenediona e testosterona, essas passam pela lâmina basal e chegam a granulosa, ocorre então a aromatização que gera estradiol. O mesmo ocorre na granulosa quando o FSH liga aos receptores iniciando a cascata de eventos.

2.4 Estimulação ovariana em ovelhas doadoras de oócitos

Combinações hormonais variadas têm sido empregadas para aumentar a quantidade de folículos disponíveis para aspiração e elevar a taxa de recuperação oocitária via laparoscópica (*laparoscopic ovum pick up* - LOPU) em ovelhas (BALDASSARRE et al., 1996; BERLINGUER et al., 2004, 2007; MENCHACA et al., 2018).

Protocolos compostos por diferentes concentrações, origens (suínos e ovinos) e graus de pureza do FSH, em regime de múltiplas (três a seis) ou única administração, já foram testados

em pequenos ruminantes, com o intuito de promover o desenvolvimento folicular (BALDASSARRE et al., 1996; 2011; BERLINGUER et al., 2004; SOUSA et al., 2011; MENDES et al., 2017). Contudo, o FSH quando usado sozinho em regime de única aplicação é ineficaz (ARMSTRONG et al., 1994), devido sua curta meia-vida, que em ovelhas é de aproximadamente 268 ± 18 min na fase folicular e de 393 ± 15 min na fase luteal (LASTER, 1972). Por isso, o FSH em única aplicação é usado em associação com gonadotrofina coriônica equina (eCG), pois a meia-vida longa desse hormônio proporciona a continuação do estímulo iniciado pelo FSH (ARMSTRONG et al., 1994; BALDASSARRE et al., 1996). O FSH também pode ser combinado a substâncias que induzam sua liberação de forma lenta (ex. preparação de ácido hialurônico), tornando-o eficaz nesse regime (BALDASSARRE et al., 2011; MENCHACA et al., 2018).

O uso do FSH em única e múltipla administração foi avaliado em ovelhas, e não houve diferença entre os tratamentos com relação ao total de folículos aspirados, total de oócitos recuperados, taxa de recuperação, e nem no percentual de embriões clivados e blastocistos (BALDASSARE et al., 1996). Logo, muitos estudos optam pelo uso do tratamento simplificado em dose única, também chamado de *one-shot* (PIERSON et al., 2005; GIBBONS et al., 2007; TEIXEIRA et al., 2011). Em uma avaliação de dados experimentais, Baldassarre et al. (2012) relataram bons resultados usando 80 mg de FSH em três doses decrescentes para o regime de múltipla administração ou associado a 300 UI de eCG para *one-shot* em ovelhas e cabras. Outros estudos demonstraram que 120 mg de FSH também foi eficiente em regime de múltipla aplicação (SOUSA et al., 2011; MENDES et al., 2017).

De outro modo, os progestágenos são esteroides naturais ou sintéticos que exercem atividade semelhante à progesterona (P4). A P4 é o único progestágeno natural, sendo sua produção realizada principalmente pelo corpo lúteo após a ovulação. A P4 exógena, embora seja quimicamente produzida a partir de matéria-prima vegetal, é bioidêntica ao homologo endógeno. Enquanto que, outros progestágenos sintéticos, como o acetato de medroxiprogesterona (MAP), apesar de simularem os efeitos terapêuticos da P4, apresentam grande diferença na estrutura química, resultando em distintas ações a nível celular e efeitos adversos (revisado por LIEBERMAN e CURTIS, 2017, revisado por L'HERMITE, 2017). Em pequenos ruminantes, comumente são utilizados dispositivos intravaginais de liberação lenta de progesterona (P4) (TEIXEIRA et al., 2016), esponjas impregnadas com de acetato de medroxiprogesterona (MAP) (BRAGANÇA et al., 2018) ou fluorogesterona (FGA) (BERLINGER et al., 2007) para a sincronização do estro. Neste contexto de sincronização do

estro, o propósito de uso é inibir a secreção pulsátil do LH, permitindo a regressão do(s) folículo(s) dominante(s) pela atresia (revisado por SOUZA-FABJAN et al., 2014a).

Em protocolos pré-LOPU tradicionais (figura 2), os progestágenos são usados por 9-14 dias, geralmente combinados à administração de análogos de prostaglandina-F2α (PGF2α) para otimizar a sincronização das doadoras antes da estimulação com gonadotrofinas (BALDASSARRE et al., 1994, 1996, 2002; 2007; 2012). No entanto, o uso prolongado de progestágenos foi associado com menor taxa de produção de embriões obtidos pela a técnica de FIV (THAMMASIRI et al., 2016) e o uso de dispositivos de liberação de P4 (CIDR, *controlled internal drug release*) em protocolo curto apresentou maior taxa de gestação. Foi demonstrado também que tanto MAP quanto a P4 podem ser usadas com eficiência em protocolos curtos de seis dias em ovinos (UNGERFELD e RUBIANES, 1999). Estudos mais recentes têm aderido ao uso de progestágenos em tratamentos de curto prazo (*short-term*) visando melhores resultados na produção de embrião *in vivo* e *in vitro* (TEIXEIRA et al., 2011; CUADRO et al., 2018; MENCHACA et al., 2018).

Figura 2. Representação esquemática de protocolo de estimulação ovariana tradicional baseado no longo termo de uso de progestágenos 9-14 dias. Adaptado de Souza-Fabjan et al., 2014c.

A partir do benéfico recurso de tratamentos com progestágenos em curto prazo, o "protocolo Dia 0", foi proposto como metodologia para sincronizar o estro e induzir a ovulação, permitindo a utilização da primeira onda de crescimento folicular em programas de múltipla ovulação e transferência de embrião (MOTE, MENCHACA et al., 2009). Este protocolo

consiste na aplicação de um implante intravaginal contendo progestágeno associado à administração de um análogo da PGF2-α e gonadotrofina coriônica equina (eCG) 24 h antes ou na retirada do implante. Sequencialmente, às 36 h da remoção do dispositivo vaginal, é feita a indução da ovulação pela aplicação de GnRH, sendo o FSH iniciado logo após a ovulação, ou seja, na emergência da primeira onda folicular, momento que há ausência de folículos dominantes e o *pool* de folículos é mais homogêneo (MENCHACA et al., 2009; MENCHACA et al., 2010; BALARO et al., 2015). A presença de folículos dominantes no início do tratamento com FSH influencia negativamente a resposta à superovulação em ovinos (MENCHACA et al., 2010). Lançando-se mão dessa estratégia, o protocolo dia 0 foi empregado previamente à estimulação ovariana pré-LOPU. Neste caso um novo implante com progestágeno foi inserido no mesmo momento da aplicação do FSH com o objetivo de inibir a oscilação da secreção pulsátil do LH e, consequentemente, prevenir a maturação folicular precoce e evitar a ovulação, sendo o implante removido na LOPU (MENCHACA et al., 2018).

2.5 Obtenção de oócito por LOPU

Snyder e Dukelow (1974) foram pioneiros na realização da coleta de oócitos por LOPU em ovelhas. A partir deste estudo, a técnica foi sendo aprimorada ao longo dos anos (BALDASSARRE et al., 1994, 1996; TERVIT et al., 1996; STANGL et al., 1999), passando a ser uma útil ferramenta para a captura de oócitos em fêmeas vivas de pequenos ruminantes para uso em ARTs. Atualmente, a LOPU já está bem estabelecida como método de obtenção de oócitos visando a PIV em animais com alto valor genético (BALDASSARRE et al., 2007), em risco de extinção (SOUZA-FABJAN et al., 2013) ou para fins de pesquisa (SOUZA-FABJAN et al., 2014b; BRAGANÇA et al., 2018). Além disso, é útil para aplicação de outras biotecnologias que envolvem desenvolvimento *in vitro*, como a transgênese e clonagem por transferência nuclear de células somáticas (BALDASSARE et al., 2002; 2012).

A LOPU é uma técnica eficiente e menos invasiva que a laparotomia, onde os animais apresentam rápido restabelecimento pós-procedimento. Além disso, mesmo após repetidas coletas não incorre em aderências, fibrose ou lesões histológicas significativas nos ovários (TEIXEIRA et al., 2011), podendo ser observadas ocasionalmente pequenas aderências do omento para parede abdominal apenas no local de inserção dos trocaters, porém sem inviabilizar a fêmea para novas coletas (PIERSON et al., 2004). Adicionalmente, não há diferenças sobre o número de folículos visualizados, aspirados, oócitos recuperados, e nem na qualidade dos oócitos obtidos entre as repetidas sessões (PIERSON et al., 2004; GIBBONS et al., 2007;

TEIXEIRA et al. 2011). Dessa forma, coletas repetidas associadas a um protocolo de estimulação eficiente, podem proporcionar a otimização da obtenção de oócitos de boa qualidade e consequentemente incrementar a eficiência dos programas de PIVE (GIBBONS et al., 2007).

2.6 Qualidade de oócitos

Apesar dos grandes avanços nos programas de PIVE, um dos maiores desafios continua sendo a obtenção de oócitos mais competentes (revisado por PARAMIO e IZQUIERDO, 2016). A qualidade do oócito, está diretamente relacionada à capacidade de se desenvolver e gerar gestação, suportando todos os eventos necessários para isso, incluindo a aquisição das habilidades de: retomar a meiose, clivar após fertilização, desenvolver até o estágio de blastocisto, induzir gestação e levá-la a termo progredindo em boa saúde (revisado por SIRARD et al., 2006). Em vista disso, os oócitos sofrem um longo e complexo processo de desenvolvimento regulado por interações parácrinas e juncionais instrutivas com as células somáticas circundantes. Este diálogo entre oócito e células somáticas permite o intercâmbio de muitos sinais regulatórios que controlam o metabolismo do oócito, a remodelação do citoesqueleto, a progressão do ciclo celular e a fertilização, os quais são eventos chave para iniciar e sustentar a embriogênese precoce (revisado por LI e ALBERTINI, 2013). Todos esses eventos importantes para aquisição da competência ocorrem ao longo do crescimento folicular, principalmente na etapa final, de forma que o oócito com crescimento já finalizado passa a autorregular seu microambiente via fatores de secreção parácrinos que contribuem para plena competência (HUSSEIN et al., 2006).

Desse modo, a ação do FSH influencia a secreção de citocinas e fatores de crescimento pelo oócito, que estimulam a atividade mitótica nas células do cumulus e modulam a expressão de enzimas esteroidogênicas durante o desenvolvimento folicular (KHAN et al., 2015). A habilidade do oócito em retomar a meiose é adquirida após o surgimento da cavidade antral folicular, porém a plena competência é alcançada quando o folículo atinge seu tamanho completo (SIRARD et al., 1989; NIVET et al., 2012). Durante o estádio antral final, os picos pré-ovulatórios de LH quebram o sinal de inibição da meiose enviado pelas células somáticas foliculares, permitindo a progressão meiótica do núcleo oocitário até então estacionado em prófase I, iniciando o processo de maturação (SIRARD et al., 1989). Todavia, a maturação nuclear deve ocorrer alinhada com a citoplasmática e molecular, para que todos os eventos morfológicos e moleculares que dão suporte ao desenvolvimento aconteçam em sincronia,

garantindo assim a cascata de eventos cruciais que dão suporte ao desenvolvimento inicial até a ativação do genoma embrionário, tais como: a redistribuição das organelas citoplasmáticas; estoque de mRNA, energia e proteínas; capacitação do oócito para bloqueio à poliespermia e formação do pronúcleo após a fecundação (MEMILI et al., 1998; SIRARD et al., 1998; revisado por MERMILLOD et al., 2006; 2008).

O aumento da competência ao desenvolvimento do oócito durante o crescimento folicular está relacionado com o armazenamento de transcritos, que serão necessários para o sucesso do desenvolvimento embrionário inicial. Nos ruminantes, a atividade do genoma do embrião se inicia após o terceiro ciclo de divisão celular, quando este apresenta de 8-16 células (MEMILI et al., 1998). Assim, a atividade transcricional inferida pela análise de RNA ribossomal demonstra que o oócito é transcricionalmente inativo nos folículos primordiais. A síntese de RNA é retomada nos folículos secundários e o pico ocorre nos folículos terciários (HYTTEL et al., 2001). Isto parece coincidir com as fases de recrutamento e dominância folicular respectivamente. Em evidências reportadas em bovinos, o diâmetro do oócito aumentou proporcionalmente com o aumento do diâmetro folicular; oócitos obtidos de folículos >10 a 15 mm eram maiores que os obtidos de folículos de > 6 a 10 mm, que por sua vez, eram maiores os obtidos de folículos de 1 a 3 mm. Apesar dos oócitos provenientes dos folículos maiores apresentarem maturação similar aos oriundos de folículos menores, a formação de blastocistos a partir de oócitos recuperados de folículos maiores foi maior do que os oriundos de folículos menores (ARLOTTO et al., 1996). Contudo, mesmo os oócitos maiores apresentando maior potencial de desenvolvimento, isso não garante que oócitos totalmente crescidos sejam capazes de passar por todas as etapas do desenvolvimento embrionário a termo (revisado por SIRAD, et al., 2006; 2011). Por outro lado, os resultados de Yang et al. (2016), demonstraram que oócitos obtidos de pequenos folículos podem apresentar competência ao desenvolvimento similar àqueles provenientes de grandes folículos, apesar das diferenças na maturação meiótica e citoplasmática.

O folículo pré-ovulatório sofre um aumento súbito na síntese de RNA que precede a retomada da meiose (MEMILI et al., 1998). Isso parece ser funcionalmente importante para o sucesso do desenvolvimento embrionário, como evidenciado pela inibição da transcrição nesta fase por amanitina (inibidor de RNA-polimerase II). Logo, vários genes como *ZAR1 (namely zygote arrest* 1), *MATER* (*maternal antigen that embryo requires*), *GDF9* (*growth differentiation factor* 9) e *BMP15* (*bone morphogenetic protein* 15) foram identificados em

oócitos como necessários tanto durante a progressão meiótica, como para desenvolvimento inicial do embrião e a transição materno-zigótica. Dessa forma, tanto os genes de efeito materno, *ZAR1* e *MATER*, quanto os genes que codificam fatores de crescimento secretados pelo oócito, *GDF9* e *BMP15*, são expressos em todos os estágios de desenvolvimento, com maior expressão no oócito imaturo e decaindo em embriões de 8-16 células (em linhas gerais), apresentando um padrão ondulatório de expressão estádio-específico (BEBBERE et al., 2008).

Em sistemas de PIV, a suplementação do meio de maturação com *BMP15* e *GDF9* sozinhos ou em associação aumenta a taxa de produção de blastocistos (HUSSEIN et al., 2006). Um estudo em células da granulosa ovinas demonstrou que a expressão dos receptores de *BMP15* e *GDF9* é influenciada pelas concentrações de FSH e estradiol (CHEN et al., 2009). Também já foi demonstrado que membros da família *TGFβ1* (*transforming growth factor-beta1*), dos quais fazem parte GDF9 e *BMP15*, regulam a atividade do FSH na proliferação do cumulos e produção de esteroides desempenhando papel crucial no desenvolvimento folicular normal e incremento da competência oocitária (ZHENG et al., 2008). Por outro lado, *MATER* e *ZAR*1 são fundamentais para a progressão do desenvolvimento embrionário, sendo a expressão no oócito imaturo um preditor de competência (PENNETIER et al., 2006; UZBEKOVA et al., 2006; BEBBERE et al., 2008; URREGO et al., 2015).

Recentes estudos identificaram *RELN* (*reelin protein*) e seu ligante *LRP8* (*low-density lipoprotein receptor-related protein 8*) sendo ativamente expressos em folículos dominantes. Observou-se que RELN apresenta máxima expressão nas células tecais desses folículos, enquanto o mesmo ocorre com LRP8 nas células da granulosa, indicando a ativação de vias de sinalização por ação parácrina (FAYAD et al., 2007). RELN, que é um gene de matriz extracelular, foi associado com a regulação do estágio final de crescimento folicular e aquisição da competência (NIVET et al., 2013). Além disso, foi relacionado como participante da atividade das proteínas quinases, desempenhando um papel na rota progesterônica e possível participação do processo ovulatório (YANG et al., 2018). Evidências como estas sinalizam para descoberta de novos marcadores de competência, já que vários estudos utilizam a expressão gênica nas células da teca e ou granulosa como espelho da qualidade oocitária.

Estudos em bovinos demonstram que a competência ao desenvolvimento é adquirida entre a janela de surgimento do FSH e o pico de LH pré-ovulatório. Porém, para a máxima competência é necessária a queda das concentrações de FSH, que promovem a estabilização do processo de crescimento do oócito no período final da foliculogênese. Oócitos em crescimento

ativo apresentam reduzida capacidade de desenvolvimento até blastocisto (NIVET et al., 2012, 2013). Adicionalmente, quando o oócito é removido do ambiente folicular, a meiose é automaticamente retomada, fazendo com que os oócitos que ainda não completaram a formação da maquinaria citoplasmática, tornem-se incapazes de gerar embriões viáveis, dada a pobreza de subsídios de sustentação do desenvolvimento (revisado por SIRAD et al., 2006). Assim, a remoção prematura do oócito do ambiente folicular e as condições de cultivo *in vitro* talvez sejam os principais fatores responsáveis pela pobre qualidade dos oócitos maturados *in vitro* em comparação com aqueles maturados *in vivo* (RIZOS et al., 2002; TESFAYE et al., 2009). Nessas condições, a retomada da meiose prematuramente é causada pelo decrescimento das concentrações de cAMP (*cyclic adenosine monophosphate*) dentro do oócito, que causa uma falta de sincronia nos diferentes etapas de maturação (nuclear, citoplasmática e molecular). Dessa forma, a utilização de moduladores de cAMP tem sido uma alternativa em sistemas de MIV para melhorar a competência de desenvolvimento (revisado por LEAL et al., 2018). Contudo, em linhas gerais, os oócitos utilizados para MIV nos diferentes laboratórios ao redor do mundo, são obtidos a partir de folículos em diferentes estágios de desenvolvimento, que constituem uma população heterogênea, com diferentes níveis de competência ao desenvolvimento (revisado por PARAMIO e IZQUIERDO, 2016).

2.7 Seleção de oócitos para a PIVE

A qualidade intrínseca do oócito para desenvolvimento *in vitro* tem correlação com o tamanho do folículo de origem. Então são preferencialmente aspirados folículos médios a grandes (NIVET et al., 2012). Em ovelhas e cabras, são relatadas melhores taxas de produção de blastocistos utilizando-se oócitos oriundos de folículos entre 3-5 mm do que com aqueles provenientes de folículos <3 mm de diâmetro (BERLINGUER et al., 2004; GIBBONS et al., 2007; LAHOZ et al., 2013). Em vacas, folículos médios (7-10 mm) fornecem oócitos mais competentes (maior taxa de blastocisto) do que os pequenos (5-6 mm) e grandes (>10 mm) folículos. Nesta espécie, foi demonstrada a existência de uma correlação linear positiva entre o tamanho do folículo e a competência ao desenvolvimento em folículos até de 8 mm, porém por razões desconhecidas, essa correlação é negativa nos maiores folículos (NIVET et al., 2012).

A seleção de oócitos baseada na qualidade morfológica é amplamente utilizada nos protocolos de PIVE. Rotineiramente, se atribui uma classificação em graus conforme a uniformidade do citoplasma e o número de camadas do cumulos. Em pequenos ruminantes, comumente se usa a escala que vai de I (ótima) a IV (ruim): oócitos GI, apresentam citoplasma

homogêneo e múltiplas camadas compactas de células do cumulos; GII, citoplasma homogêneo e com 1-3 camadas compactas de células do cumulos; GIII, citoplasma heterogêneo e parcialmente ou completamente desnudo; GIV, disformes, citoplasma heterogêneo, cumulos expandido ou em degeneração (ALMEIDA et al., 2010; SOUZA-FABJAN et al., 2013). Geralmente, somente oócitos graus I e II, são utilizados para PIV. Entretanto, oócitos desnudos (classificados como GIII) obtidos por LOPU, quando apresentam citoplasma homogêneo, podem ser utilizados para PIVE com possibilidade de desenvolver até blastocisto (SOUZA-FABJAN et al., 2013, 2016).

Outra maneira de selecionar oócitos para PIVE é a aplicação do teste do ACB, que seleciona os oócitos com base na atividade da enzima glicose-6-fosfato desidrogenase (G6PDH). A G6PDH é componente da via das pentoses e fornece fosfato de ribose para a síntese de nucleotídeos e formação de ácidos graxos. No oócito em crescimento, a G6PDH está em plena atividade e, assim, quando este é submetido à coloração com ACB, o corante é consumido pela ação da enzima e retoma sua cor natural, sendo o oócito classificado como ACB negativo (ACB-). Enquanto que, no oócito com crescimento exponencial finalizado, a atividade desta enzima é baixa, assim o corante não é consumido, tornando o citoplasma do oócito azul, passando a ser classificado como ACB positivo (ACB+) (RODRIGUEZ-GONZALEZ et al., 2002; TORNER et al., 2008; CATALÁ et al., 2011). Visto que a plena aquisição da competência ao desenvolvimento ocorre no período final da foliculogênese, quando o folículo atinge o platô de crescimento (revisado por SIRARD et al., 2006; GIRARD et al., 2015), o método tem se demonstrado eficiente na seleção de oócitos mais competentes. Estudos apontam que o diâmetro do oócito e o percentual de ACB+ oriundos de folículos pequenos são menores que os provenientes de médios e grandes (SHABANKAREH et al., 2014). Contudo, oócitos ACB+ oriundos de pequenos e grandes folículos exibem similar competência, embora apresentem diferenças na maturação meiótica e citoplasmática (YANG et al., 2016). Logo, o teste do ACB promove a seleção de um *pool* de oócitos mais homogêneo e mais competente para uso em programas de PIVE (revisado por PARAMIO e IZQUIERDO, 2016).

3. REFERÊNCIAS BIBLIOGRÁFICAS

ARMSTRONG, D. T., IRVINE, B. J., EARL, C. R., MCLEAN, D. AND SEAMARK, R. F. Gonadotropin stimulation regimens for follicular aspiration and *in vitro* embryo production from calf oocytes. *Theriogenology*, v. 42, p. 1227-36, 1994.

ARLOTTO, T.; SCHWARTZ, J-L.; FIRST, N. L.; LEIBFRIED-RUTLEDGELB, M. L. Aspects of follicle and oocyte stage that affect *in vitro* maturation and development of bovine oocytes. *Theriogenology*, v. 45, p. 943-56, 1996.

BALDASSARRE, H.; PIERSON, J. T.; POULIN, S.; SNEEK, L.; ROGAN, D.; HOCKLEY, D. K. Preliminary report on the use of a slow release formulation for administration of FSH in three assisted reproduction applications in goats. *Reprod. Fertil. Dev*., v. 23, p. 255, 2011.

ALMEIDA, K.C., PEREIRA, A.F., ALCÂNTARA NETO, A.S., AVELAR, S.R., BERTOLINI, L.R., BERTOLINI, M., FREITAS, V.J., MELO, L.M. Real-time qRT-PCR analysis of EGF receptor in cumulus-oocyte complexes recovered by laparoscopy in hormonally treated goats. *Zygote*, v. 19, p. 127-136, 2010.

BENDSEN, E.; BYSKOV, A. G.; YDING, C.; WESTERGAARDL, L. G. Number of germ cells and somatic cells in human fetal ovaries during the first weeks after sex differentiation. *Hum. Reprod.,* v. 21, p. 30-35, 2006.

BALARO, M. F. A.; FONSECA, J. F.; OBAR, E.; CARDOSO, E. C.; BRANDÃO, F. Z. Is the Santa Inês sheep a typical non-seasonal breeder. *Trop. Anim. Health Prod.,* v.46, p.1533–1537, 2014.

BALARO, M. F. A.; BRANDÃO, F. Z.; PENEIRAS, A. B. V.; OBA, E.; FONSECA, J. F.; ALMOSNY, N. R. P.; CARDOSO, E. C. R. Productive performance, metabolic and hormonal profiles of Santa Inês ewes in winter and summer under tropical conditions. *Trop. Anim. Health Prod.,* v.47, p.627–631, 2015.

BALARO, M. F. A.; FONSECA, J. F.; BARBOSA, T. G. B.; SOUZA-FABJAN, J. M. G.; FIGUEIRA, L. M.; TEIXEIRA, T. A.; CARVALHEIRA, L. R.; BRANDÃO, F. Z. Potential role for GnRH in the synchronization of follicular emergence before the superovulatory Day 0 protocol. *Domest. Anim. Endocrinol.,* v. 54, p. 10-14, 2016.

BALDASSARRE, H.; FURNUS, C. C.; DE MATOS, D. G.; PESSI, H. *In vitro* production of sheep embryos using laparoscopic folliculocentesis: Alternative gonadotrophin treatments for stimulation of oocyte donors. *Theriogenology*, v.45, p.707-17, 1996.

BALDASSARRE, H.; WANG, B.; KAFIDI, N. et al. Advances in the production and propagation of transgenic goats using laparoscopic ovum pick-up and in vitro embryo production technologies. *Theriogenology*, v.57, p.275-284, 2002.

BALDASSARRE, H. Practical aspects for implementing *in vitro* embryo production and cloning programs in sheep and goats. *Anim. Reprod.*, v.9, p.188-94, 2012.

BARTLEWSKI, P. M.; BEARD, A. P.; COOK, S. J.; RAWLINGS, N. C. Ovarian follicular dynamics during anoestrus in ewes. *J. Reprod. Fertil.*, v.113, p.275-85, 1998.

BARTLEWSKI, P. M.; BEARD, A. P. Ovarian function in ewes at the onsent of the breeding season. *Anim. Reprod. Sci.*, v.57, p.67-88, 1999.

BARTLEWSKI, P. M.; VANDERPOL, J.; BEARD, A. P.; COOK, S. J.; RAWLINGS, N. C. Ovarian antral follicular dynamics and their associations with peripheral concentrations of gonadotropins and ovarian steroids in anoestrous Finnish Landrace ewes. *Anim. Reprod. Sci.*,v.58, p.273-91, 2000.

BEBBERE, D.; BOGLIOLO, L.; ARIU, F. ; et al. Expression pattern of zygote arrest 1 (ZAR1), maternal antigen that embryo requires (MATER), growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) genes in ovine oocytes and in vitro-produced preimplantation embryos. *Reprod. Fertil. Dev.*, v. 20, p.908-915, 2008.

BEG, M. A.; BERGFELT, D. R.; KOT, K.; WILTBANK, M. C. W.; GINTHER, O. J. Follicularfluid factors and granulosa-cell gene expression associated with follicle deviation in cattle. *Biol Reprod*., v.64, p.432–441, 2001.

BERLINGUER, F.; LEONI, G.; BOGLIOLO, L.; PINTUS, P.P.; ROSATI, I.; LEDDA, S.; NAITANA, S. FSH different regimes affect the developmental capacity and cryotolerance of embryos derived from oocytes collected by ovum pick-up in donor sheep. *Theriogenology*, v. 61, p. 1477-1486, 2004.

BERLINGUER, F.; GONZALEZ-BULNES, A.; SUCCU, S.; LEONI, G.; MOSSA, F.; BEBBERE, D.; ARIZNAVARRETA, C.; TRESGUERRES, J.A.F.; VEIGA-LOPEZ, A.;

NAITANA, S. Effects of progestagens on follicular growth and oocyte developmental competence in FSH-treated ewes. *Domest. Anim. Endocrinol.*, v.32, p.303–314, 2007.

CUADRO, F.; SANTOS-NETO, P. C.; PINCZAKA, A.; BARRERA, N.; CRISPO, M.; MENCHACA, A. Serum progesterone concentrations during FSH superstimulation of the first follicular wave affect embryo production in sheep. *Ani. Reprod. Sci.*, v.196, p.205-210, 2018.

CARABASTOS, M. Jo.; ELVIN, J.; MATZUK, M. M.; ALBERTINI, D. F. Caracterization of oocyte and follicle developmente in growth differenciation fator-9-decificient mice. *Dev. Biol.*, v.204.; p.373-84, 1998.

CATALÁ, MG.; IZQUIERDO, D.; UZBEKOVA, S.; MARATÓ, R.; ROURA, M.; ROMANGUERA, R.; PAPILLIER, P.; PARAMIO, T. Brilliant Cresyl Blue stain selects largest oocytes with highest mitochondrial activity, maturation-promoting factor activity and embryo developmental competence in prepubertal sheep. *Reproduction*, v.142, p.517–527, 2011.

CHEN, A. Q.; YU, S. D.; WANG, Z. G.; XU, Z. R.; YANG, Z. G. Stage-specific expression of bone morphogenetic protein type I and type II receptor genes: Effects of follicle-stimulating hormone on ovine antral follicles. *Anim. Reprod. Sci.*, v.111, p.391–399, 2009.

CHESSA, B.; PEREIRRA, F.; ARNOUD, F.; AMORIM, A.; GOYACHE, F.; et al. REVEALING THE HISTORY OF SHEEP DOMESTICATION USING RETROVIRUS INTEGRATIONS B. *Science*, n.24, v.324(5926): p.532–536, 2009.

COUSE, J. F.; KORACH, K. S. Estrogen Receptor Null Mice: What Have We Learned and Where Will They Lead Us? *Endocr. Rev.*, v.20, n.3, p.358–417, 1999.

DARBON, J-M.; KNECT, M.; RANTA, T.; DUFAU, M. L.; CATTG, K. Hormonal Regulation of Cyclic AMP-dependent Protein Kinase in Cultured Ovarian Granulosa Cells. *J. biol. Chem.*, v. 259, n. 23, p. 14778-14782, 1984.

DRIANCOURT, M. A. Regulation of ovarian follicular dynamics in farm animais. Implications for manipulation of reproduction. *Theriogenology*, v.55, p.1211-1239, 2001.

EVANS, A. C. O.; DUFFY, P.; HYNESS, N.; BOLAND, M. P. Waves of follicle development during the estrus cycle in sheep. *Theriogenology*, v.53, p.699-715, 2000.

FAO, STATISTICAL POCKETBOOK: WORLD FOOD AND AGRICULTURA, 2015. Disponível em: http://www.fao.org/3/a-i4691e.pdf acesso: 26 de junho de 2017.

FAYAD, T.; LEFEBVRE, R.; NIMPF, J.; SILVERSIDES, D. W.; LUSSIER, J. G. Low-density lipoprotein receptor-related protein 8 (LRP8) is upregulated in granulosa cells of bovine dominant follicle: molecular characterization and spatio-temporal expression studies. *Biol. Reprod.*, v.76, p.466–475, 2007.

GIBBONS, A.; PEREYRA BONNET, F.; CUETO, M.; CATALA, M.; SALAMONE, D.; GONZÁLEZ-BULNES, A. Procedure for maximizing oocyte harvest for *in vitro* embryo production in small ruminants. *Reproduction in Domestic Animals*, v. 42, p. 423-426, 2007.

GINTHER, O. J.; SIDDIQUI, M. A. R.; BALDRIGHI, J. M.; WOLF, C. A. Systemic effect of follicle-stimulating hormone and intraovarian effect of the corpus luteum on complete regression vs recovery of regressing wave-2 follicles in heifers. *Domest. Anim. Endocrinol.*, v.55, p.51–59, 2016a.

GINTHER, O. J.; BALDRIGHI, J. M.; SIDDIQUI, M. A. R.; ARAUJO, E. R. Complexities of follicle deviation during selection a dominant follicle in *Bos Taurus* heifers. *Theriogenology*, v.86, n.8, p.2012-19, 2016b.

GIRARD, A.; DUFORT, I.; DOUVILLE, G.; SIRARD, M. A. Global gene expression in granulosa cells of growing, plateau and atretic dominant follicles in cattle. *Reprod. Biol. Endocrinol.*, p.13-17, 2015.

HILLIER SG, WHITELAW PF, SMYTH CD. Follicular oestrogen synthesis: the 'two-cell, two-gonadotrophin's model revisited. *Mol. Cell. Endocrinol.*, v.100, p.51-54, 1994.

HIRSHFIELD, A. E. Development of follicles in the mammalian ovary. *Intern. Rev. Cytol.*, v.124, p.43-101, 1991.

HUSSEIN, T. S.; THOMPSON, J. G.; GILCHRIST, R. B. Oocyte-secreted factors enhance oocyte developmental competence. *Develop. Biol.*, v.296, p.514-21, 2006.

HYTTEL, P.; VIUFF, D.; FAIR, T.; et al. Ribosomal RNA gene expression and chromosome aberrations in bovine oocytes and preimplantation embryos. *Reproduction*, v. 122, p. 21-30, 2001.

IBGE, agropecuária, efetivos da agropecuária, 2015. Disponível em: http://brasilemsintese.ibge.gov.br/agropecuaria/efetivos-da-pecuaria.html acesso em: 26 de junho de 2017.

IETS, data retrieval committee reports. Newletter 2017. Disponível em: https://www.iets.org/pdf/comm_data/IETS_Data_Retrieval_Report_2016_v2.pdf acesso: 20 de dezembro de 2018.

JONES, T.W. Anatomy of the ovum in the ovary. *Br. Foreign Med. Rev.*, v.1, n.2, p.610-611, 1836.

JONES, T.W. On the ovum of man and the mammifera before and after fecundation. *Br. Foreign Med. Rev.*, v.16, n.32, p.513-557, 1843.

KEMLÄINEN, H.; ADAM, M.; MÄKI-JOUPPILA, J.; DAMDIMOPOULOU, P.; DAMDIMOPOULOU, A.; KERE, J.; HOVATTA, O.; LAAJALA, T. D.; AITTOKALLIO, T.; ADAMSKI, J.; RYBERG, H.; OHLSSON, C.; STRAUSS, J.; POUTANEN, M. The Hydroxysteroid (17β) Dehydrogenase Family Gene HSD17B12 Is Involved in the Prostaglandin Synthesis Pathway, the Ovarian Function, and Regulation of Fertility. *Endocinology*, v.157, n.10, p.3719-30, 2016.

KHAN, D. R., GUILLEMETTE, C., SIRARD, M. A. AND RICHARD, F. J. Caracterization of FSH signaling networks in bovine cumulus cells: a perspective on oocyte competence acquisition. *Mol. Hum. Reprod.*, v.21, p.688-701, 2015.

KULIK, L. J.; BERFELT, D. R.; KOT, K.; GINTHER, O. J. Follicle selection in catle: follicle deviation and codominance within sequential waves 1. *Biol. Reprod.*, v.65, p.839-846, 2001.

LAHOZ, B.; ALABART, J. L.; FOLCH, J.; SÀNCHEZ, P. S.; ECHEGOYEN, E.; COCERO, M. J. Influence of the FecXR Allele in Heterozygous Ewes on Follicular Population and Outcomes of IVP and ET using LOPU-Derived Oocytes. *Reprod. Dom. Anim.*, v.48, p.717–723, 2013.

LARIMORE, E. L.; AMUNDSON, O. L.; BRIDGES, G. A et al. Changes in ovarian function associated with circulating concentrations of estradiol before a GnRH-induced ovulation in beef cows. *Domest. Anim. Endocrinol.*, p. 2-9, 2016.

LASTER, D. B. Disappearance and uptake of [^{125}I] FSH in the rat, rabbit, ewe and cow. *J. Reprod. Fertil.*, v.30, p.407-15, 1972.

LEAL, G. R.; MONTEIRO, C. A. S.; SOUZA-FABJAN, J. M. G.; VASCONCELOS, C. O. P.; NOGUEIRA, L. A. G.; FERREIRA, A. M. R,; SERAPIÃO, R. V. Role of cAMP modulator supplementations during oocyte in vitro maturation in domestic animals. *Anim. Reprod. Sci.*, 2018, *in press*.

L'HERMITE, M. Bioidentical menopausal hormone therapy: registered hormones (non-oral estradiol ± progesterone) are optimal. *Climacteric*. *In press*. 2017.

LI, R.; ALBERTINI, D. F. The road to maturation: somatic cell interaction and self-organization of the mammalian oocyte. *Nat. Rev. Mol. Cell. Biol.*, v.14, p.141-153, 2013.

LIEBERMAN, A.; CURTIS, L. In Defense of Progesterone: A Review of the Literature. *Altern. Ther. Health Med*., v.23, n.6, p.24-32, 2017.

LUO, W.; WILTBANK, M. C. Distinct regulation by steroids of messenger RNAs for FSHR and CYP19A1 in bovine granulosa cells. *Biol. Reprod.,* v.75, p.217-25, 2006.

LUO, W.; GUMEN, A.; HAUGHIAN, J. M.; WILTBANK, M. C. The role of luteinizing hormone in regulating gene expression during selection of a dominant follicle in cattle. *Biol. Reprod.*, v.84, p.369-78, 2011.

MATOS, M. H. T.; LIMA-VERDE, I. B.; LUQUE, M. C.CA.; MAIA Jr., J. E.; SILVA, J. R. V.; CELESTINO, J. J. H.; MARTINS, F. S.; BÁO, S. N.; LUCCI, C. M.; FIGUEIREDO, J. R. Essential role of follicle stimulating hormone in the maintenance of caprine preantral follicle viability *in vitro. Zygote,* v.15, p.173-82, 2007.

MAGALHÃES, D. M.; FERNANDES, D. D.; ARAÚJO, V. R.; ALMEIDA, A. P.; MATOS, M. H. T.; FIGUEIREDO, J. R. Papel do Hormônio Folículo Estimulante na foliculogênese in vivo e in vitro Role of Follicle Stimulating Hormone in folliculogenesis in vivo and in vitro. *Rev. Bras. Reprod. Anim.*, v.33,p.171-182, 2009.

MEMILI, E.; DOMINKO, T.; FIRST, N.L. Onset of transcription in bovine oocytes and preimplantation embryos. *Mol. Reprod. Dev*., v. 51, p. 36-41, 1998.

MERMILLOD, P. ; LOCATELLI, Y. ; DALBIES-TRAN, R. ; et al. In vitro production of ruminant embryos: results, limits and perspectives. Symposium COA/ INRA Scientific Cooperation in Agriculture, 59–78, 2006.

MERMILLOD, P.; DALBIES-TRAN.; UZBEKOVA, S.; THÉLIE, A.; TRAVERSO, J-M.; PERREAU, C.; PAPILLIER, P. MARGOT, P. Factors Affecting Oocyte Quality: Who is Driving the Follicle? *Reprod. Dom. Anim.*, v.43, p.393-400, 2008.

MENCHACA, A.; VILARINO, M.; PINCZAK, A.; KMAID, S.; SALDAN, J. M. Progesterone treatment, FSH plus eCG, GnRH administration, and Day 0 Protocol for MOET programs in sheep. *Theriogenology*, v.72, p.477–483, 2009.

MENCHACA, A.; VILARIÑO, M.; CRISPO, M.; DE CASTRO, T.; RUBIANES, E. New approaches to superovulation and embryo transfer in small ruminants. *Reprod. Fertil. Devel*., v.22, p.113–18, 2010.

MENCHACA, A.; CUADRO, F.; DOS SANTOS-NETO, P.C.; BOSOLASCO, D.; BARRERA, N.; BRUN, V.; CRISPO, M. Oocyte developmental competence is improved by relatively greater circulating progesterone concentrations during preovulatory follicular growth. *Anim. Reprod. Sci.*, v.195, p.321-328, 2018.

MENDES, C. S.; BARBOSA, L. P.; ARAÚJO, M. L.; PINHEIRO, E. E. G.; LENTS, M. P.; AMORIM, L. S.; KIYA, C. K.; SANTANA, A. L. A. Follicular response and oocyte production following variations in ovarian stimulation in goats. *Theriogenology*, v.108, p.88-96, 2018.

MILLER, W.J.; BOSE, H.S. Early steps in steroidogenesis: intracellular cholesterol traffi cking. *J. Lipid Res.*, v.52, p.2116-2135, 2011.

NIVET, A. L.; BUNEL, A.; LABRECQUE, R.; BELANGER, J.; VIGNEAULT, C.; BLONDIN, P.; SIRARD, M. A. FSH withdrawal improves developmental competence of oocytes in the bovine model. *Reproduction* 143: 165–171, 2012.

NIVET, A. L.; VIGNEAULT, C.; BLONDIN, P.; SIRARD, M. A. Changes in granulosa cells' gene expression associated with increased oocyte competence in bovine. *Reproduction, v.*145, p.555–565, 2013.

OROZCO-LUCERO, E.; SIRARD, M. A.. Molecular markers of fertility in cattle oocytes and embryos: progress and challenges. *Anim. Reprod.*, v.11, p.183-94, 2014.

PENNETIER, S.; PERREAU, C.; UZBEKOVA, S.; THÉLIE, A.; DELALEU, B.; MERMILLOD, P.; DALBIÈS-TRAN. MATER protein expression and intracellular localization throughout folliculogenesis and preimplantation embryo development in the bovine. *BMC Develp. Biol.*, v.6, n.26, p.1-9, 2006.

PARAMIO, M.T.; IZQUIERDO, D. Recent advances in i*n vitro embryo* production in small ruminants. *Therionegology*, v. 86, n.1, p.152-159, 2016.

PEDROSA, S.; UZUN, M.; ARRANZ, JJ.; GUTIÉRREZ-GIL, B.; PRIMITIVO, F., S.; BAYÓN, Y. Evidence of three maternal lineages in near eastern sheep supporting multiple domestication events. *Proc. Biol. Sci*. v. 272, p. 2211–2217, 2005.

PIERSON, J., WANG, B., NEVEU, N., SNEEK, L., CÔTÉ, F., KARATZAS, N. C., BALDASSARRE, H. Effects of repetition, interval between treatments and season on the results from laparoscopic ovum pick up in goats. *Reprod. Fertil. Dev.*, v.16, n.8, p.795-9, 2004.

RIZOS, D.; WARD, F.; DUFFY, P.; BOLAND, M.; LONERGAN, P. Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: implications for blastocyst yield and blastocyst quality. *Mol. Reprod. Dev*., v. 61, p. 234–48, 2002.

RODRIGUEZ-GONZALEZ, E.; LOPEZ-BEJAR, M.; VELILLA, E.; PARAMIO, M. T. Selection of prepubertal goat oocytes using the Brilliant Cresyl Blue test. *Theriogenology*, v.57, p.1397–1409, 2002.

SALVIANO, M. B.; COLLARES, F. J. F.; BECKER, B. S.; RODRIGUES, B. A.; RODRIGUES, J. L. Bovine non-competent oocytes (BCB–) negatively impact the capacity of competent (BCB+) oocytes to undergo *in vitro* maturation, fertilisation and embryonic development. *Zygote*, v.24, p.245-251, 2015.

SANTOS, JULIANA D.R., BATISTA, RIBRIO I.T.P., MAGALHÃES, LIVIA C., JUNIOR, ALEXANDRE R. PAULA, SOUZA, SAMARA S., SALAMONE, DANIEL F.; et al. Overexpression of hyaluronan synthase 2 and gonadotropin receptors in cumulus cells of goats

subjected to one-shot eCG/FSH hormonal treatment for ovarian stimulation. *Animal Reproduction Science*, v.170, p.15-24, 2016.

SARAIVA, M. V. A.; CELESTINO, J. J. H.; CHAVES, R. N.; MARTINS, F. S.; BRUNO, J. B.; LIMA-VERDE, I. B.; MATOS, M. H. T.; SILVA, G. M.; PORFIRIO, E. P.; BÁO, S. N.; CAMPELLO, C. C.; SILVA, J. R. V.; FIGUEIREDO, J. R. Influence of different concentrations of LH and FSH on in vitro caprine primordial ovarian follicle development. *Small Rumin Res*, v.78, p.87-95, 2008.

SARAIVA, M.; MATOS, M.; FAUSTINO, L.; CELESTINO, J.; SILVA, J.; FIGUEIREDO, J. Hormônios hipofisários e seu papel na folículogênese. *Rev. Bras. Reprod. Anim*., v.34, n.4, p.206-221, 2010.

SARTORI, R.; FRICKE, P. M.; FERREIRA, J. C. P.; GINTHER, O. J.; WILTBANK, M. C. Follicular Deviation and Acquisition of Ovulatory Capacity in Bovine Follicles1. *Biol. Reprod.*, v.65, p.1403-1409, 2001.

SHABANKAREH, H. K.; AZIMI, G.; TORKI, M. Development competence of bovine oocytes selected based on follicle size and using the brilliant cresyl blue (BCB) test. *Iran. J. Reprod. Med.*, v.12, n.11, p.771-778, 2014.

SHIMASAKI, S., MOORE, R. K., OTSUKA, F., ERICKSON, G. F. The bone morphogenetic protein system in mammalian reproduction. *Endocrinol. Rev.*, v.25, p.72–101, 2004.

SIRARD, M. A.; FLORMAN, H. M.; LEIBFRIED-RUTLEDGE, M. L.; BARNES, F. L.; SIMS, M. L.; FIRST, N. L. Timing of nuclear progression and protein synthesis necessary for meiotic maturation of bovine oocytes. *Biol. Reprod.*, v.40, p.1257-63, 1989.

SIRARD, M-A.; RICHARD, F.; BLONDIN, P.; ROBERT, C. Contribution of the oocyte to embryo quality. *Theriogenology*, v. 65, p.126–36, 2006.

SIRARD, M-A. Follicle environment and quality of in vitro matured oocytes. *J. Assist. Reprod. Genet.*, v.28, p.483–488, 2011.

SKAPETAS, B.; KALAITZIDOU, M. Current status and prespectives of sheep sector in the world. *Livest. Res. Rural Dev*. v.29, article 21, 2017. http://www.lrrd29/2/skap29021.html acess: Jun 21, 2017.

SNYDER, D. A.; DUKELOW, W. R. Laparoscopic studies of ovulation, pregnancy diagnosis and follicle aspiration in sheep. *Theriogenology*, v.2, n.6, p.143-148, 1974.

SOUSA, F. C.; MELO, C. H. S.; TELES FILHO, A. C. A.; AVELAR, S. R. G.; MOURA, A. A. A.; MARTINS, J. A. M.; et al. Ovarian follicular response to different hormonal stimulation treatments in Canindé goats. *Anim. Reprod. Sci.*, v.125, p.88– 93, 2011.

SOUZA-FABJAN, J. M. G.; PEREIRA, A. F.; MELO, C. H. S.; SANCHEZ, D. J. D.; OBA, E., et al. Assessment of the reproductive parameters, laparoscopic oocyte recovery and the first embryos produced in vitro from endangered Canindé goats (Capra hircus). *Reprod. Biol.*, v.13, p.325-332, 2013.

SOUZA-FABJAN, J. M. G.; LOCATELLI, Y.; FREITAS, V. J. F. et al. Laparoscopic ovum pick up (LOPU) in goats: from hormonal treatment to oocyte possible destinations. *Rev. Bras. Ciên. Vet.*, v. 21, p.3-11, 2014a.

SOUZA-FABJAN, J. M. G.; LOCATELLI, Y.; DUFFARD, N. et al. In vitro embryo production in goats: Slaughterhouse and laparoscopic ovum pick up–derived oocytes have different kinetics and requirements regarding maturation media. *Theriogenology*, v. 81, p. 1021-1031, 2014b.

SOUZA-FABJAN, J. M. G.; PANNEAU, B.; DUFFARD, N.; LOCATELLI, Y.; VICENTE, J. F. F. MERMILLOD, P. In vitro production of small ruminant embryos: Late improvements and further research. *Theriogenology*, v.81, p.1149–1162, 2014c.

SOUZA-FABJAN, J. M. G; LOCATELLI, Y.; DUFFARD, N.; CORBIN, E.; BATISTA, R. I. T. B.; FREITAS, V. J. F.; BECKERS, J-F.; MERMILLOD, P. Intrinsic quality of goats oocytes already found denuded at collection for in vitro embryo production. *Theriogenology*, v.86, p. 1989-1998, 2016.

SOUZA-FABJAN, J. M. G.; ROSA, R. M.; BALARO, M. F. A.; PINTO, P. H. N.; SANTOS, G. B.; ARASHIRO, E. K. N. FONSECA, J. F.; UNGLERFELD, R.; BRANDÃO, F. Z. Effect of different hormonal combinations on follicular wave emergence and superovulatory response in sheep. *Theriogenology*, v.103, p.24-29, 2017.

STANGL, M.; KTIHHOLZER, B.; BESENFELDER, U.; BREM, G. Repeated endoscopic ovum pick-up in sheep. *Theriogenology.*, v.52, p.709-716, 1999.

SUMMERS, A. F.; POHLMEIER, W. E.; SARGENT, K. M.; COLE, B. D., et al. Altered Theca and Cumulus Oocyte Complex Gene Expression, Follicular Arrest and Reduced Fertility in Cows with Dominant Follicle Follicular Fluid Androgen Excess. *Plos One*, v.9, Issue 10, e110683, 2014.

SUNDERLAND, S. J.; CROWE, M. A.; BOLAND, M. P.; ROCHE, J. F.; IRELAND, J. J. Selection, dominance and atresia of follicles during the oestrous cycle of heifers. *J. Reprod. Fertil.*, v.101, p.547-55, 1994.

TEIXEIRA, P. P. M.; PADILHA, L. C.; OLIVEIRA, M. E. F.; MOTHEO, T. F.; SILVA, A. S. L.; BARROS, F. F. P. C.; COUTINHO, L. N.; FLORES, F. N.; LOPES, M. C. S.; et al. Laparoscopic ovum collection in sheep: Gross and microscopic evaluation of the ovary and influence on oocyte. *Anim. Reprod. Sci.*, v. 127, p. 169-175, 2011.

TEIXEIRA, T. A.; DA FONSECA, J. F.; DE SOUZA-FABJAN, J. M.; DE REZENDE, C. L.; DE MOURA, F. D. A.; BRANDÃO, F. Z. Efficiency of different hormonal treatments for estrus synchronization in tropical Santa Inês sheep. *Trop. Anim. Health Prod.*, v. 48, p. 545-551, 2016.

TESFAYE, D.; GHANEM, N.; CARTER, F.; FAIR, T.; SIRARD, M.; HOELKER, M.; et al. Gene expression profile of cumulus cells derived from cumulusoocyte complexes matured either in vivo or in vitro. *Reprod. Fertil. Dev.*, v. 21, p. 451–61, 2009.

THAMMASIRI, J.; NAVANUCRAW, C.; URIYAPONGSON, S.; KHANTHUSAENG, V.; KAMOLLIRT, C. Expression of cumulus-oocyte complex genes and embryonic development in goats subjected to progestogen-based estrus synchronization. *Theriogenology*, v.86, p.612-18, 2016.

TERVIT, H. R.. Laparoscopy/laparotomy oocytes recovery and juvenile breeding. *Anim. Reprod. Sci.*, v.42, p.227-238, 1992.

TISDALL, D. J., WATANABE, K., HUDSON, N. L., SMITH, P. AND MCNATTY, K. P. FSH receptor gene expression during ovarian follicle development in sheep. *J. Mol. Endocrinol.* v.15, n.3, p.273-81, 1995.

TORNER, H.; GHANEM, N.; AMBROS, C.; HÖLKER, M.; TOMEK, W.; PHATSARA, C.; ALM, H.; SIRARD, M-A.; KANIT, W.; SCHELLANDER, K.; TESFAYE, D. Focus on Mammalian Embryogenomics Molecular and subcellular characterisation of oocytes screened

for their developmental competence based on glucose-6-phosphate dehydrogenase activity. *Reproduction*, v.135, p.197–212, 2008.

UNGERFELD, R.; RUBIANES, E. Effectiveness of short-term progestogen primings for the induction of fertile oestrus with eCG in ewes during late seasonal anoestrus. *Anim. Sci.*, v.68, p.349-352, 1999.

URREGO R; HERRERA-PUERTA E; CHAVARRIA N.A; CAMARGO O; WRENZYCKI C; RODRIGUEZ-OSORIO N. Follicular progesterone concentrations and messenger RNA expression of MATER and OCT-4 in immature bovine oocytes as predictors of developmental competence. *Theriogenology*, v.83, p.1179–1187, 2015.

VAN DEN HURK, R.; ZHAO, J. Formation of mammalian oocytes and their growth, differentiation and maturation within ovarian follicles. *Theriogenology*, v.63, p.1717-1751, 2005.

YANG, M.; HALL, J.; FAN, Z.; REGOUSKI, M.; MENG, Q.; RUTIGLIANO, H. M.; et al. Oocytes from small and large follicles exhibit similar development competence following goat cloning despite their differences in meiotic and cytoplasmic. *Theriogenology*, v.86, p.2302-2311, 2016.

YANG, F.; WANG, M.; ZHANG, B.; XIANG, W.; ZHANG, K.; CHU, M.; WANG, P. Identification of new progestogenassociated networks in mammalian ovulation using bioinformatics. *BMC Syst. Biol.*, v.12, p.36, 2018.

YU, Y.; LI, W.; HAN, Z.; LUO, M.; CHANG, Z.; TAN, J. The effect of follicle-stimulating hormone on follicular development, granulosa cell apoptosis and steirodogenesis and its mediation by insulin-like growth factor-I in the goat ovary. *Theriogenology*, v.60, n.9, p.1691-704, 2003.

ZHENG, X.; PRINCE, C. A.; TREMBLAY, Y.; LUSSIER, J. G.; CARRIÈRE, P. D. Role of transforming growth factor-β1 in gene expression and activity of estradiol and progesterone-generating enzymes in FSH-stimulated bovine granulosa cell. *Reproduction*, v.136, p.447-457, 2008.

UZBEKOVA, S.; ROY-SABAU, M.; DALBIÈS-TRAN, R.; PERREAU, C.; PAPILLIER, P.; MOMPART, F.; THELIE, A.; PENNETIER, S.; COGNIE, J.; CADORET, V.; ROYERE, D.;

MONGET, P.; MERMILLOD, P. Zygote arrest 1 gene in pig, cattle and human: evidence of different transcript variants in male and female germ cells. *Reprod. Biol. Endocrinol*., v.4, p.12, 2006.

4. OBJETIVOS

4.1 Objetivo geral

Avaliar o efeito de diferentes protocolos hormonais para estimulação ovariana, com base na dose e regime de administração do FSH e fonte de progestágeno utilizada durante a estimulação, considerando seus efeitos sobre a resposta ovariana e modulação da expressão gênica que impactam a qualidade de complexos cumulos oócitos (CCOs) imaturos em ovelhas da raça Santa Inês.

4.2 Objetivos específicos

- Avaliar o efeito do FSH (80 ou 120 mg, em dose única ou múltipla) e da fonte de progestágeno (progesterona e acetato de medroxiprogesterona) na:
  - População folicular e a obtenção de CCOs;
  - Competência do oócito ao desenvolvimento;
  - Expressão de genes relacionados a qualidade, esteroidogênese e apoptose em CCOs.

Capítulo 2

FSH dose and strategy of administration for ovarian stimulation affect gene expression in sheep cumulus-oocyte complexes

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FSH dose and strategy of administration for ovarian stimulation affect gene expression in sheep cumulus-oocyte complexes

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Running head: FSH protocol affects gene expression in sheep COCs

Abstract. The present study evaluated the effect of four ovarian stimulation protocols on the follicular population and molecular status of cumulus–oocyte complexes (COCs). Twelve Santa Inês ewes (in a cross-over design) received 80 or 120 mg FSH alone in a multiple-dose (MD80 and MD120) regimen or in combination with 300 IU equine chorionic gonadotrophin (eCG) in a one-shot (OS80 and OS120) protocol. The follicular population, COC recovery rate, mean COCs per ewe and the rate of brilliant Cresyl blue-positive (BCB^+) COCs were similar among treatments ($P > 0.05$). The expression of markers of oocyte competence (*ZAR1*, zygote arrest 1; *MATER*, maternal antigen that embryo requires; *GDF9*, growth differentiation factor 9; *BMP15*, bone morphogenetic protein 15; Bcl-2, B-cell lymphoma 2; BAX, Bcl-2 associated X protein) and the steroidogenic pathway (*ER*α, oestrogen receptor a; *LHr*, LH receptor; *FSHr*, FSH receptor; *STAR*, steroidogenic acute regulatory protein) was affected by stimulation. Specifically, the expression of markers of the steroidogenic pathway was reduced with increasing FSH dose in the OS protocol. FSH at a dose of 80 mg reduced the expression of FSH*r* and *ER*a in the OS versus MD protocol. Conversely, in MD protocol, only *LHr* was affected by increasing FSH dose. In conclusion, 80 mg FSH in the MD or OS protocol was sufficient to promote the development of multiple follicles and obtain fully grown (BCBþ) oocytes. The MD protocol may be more appropriate for the production of better quality oocytes.
Additional keywords: superstimulation, oocyte, brilliant cresyl blue, molecular biology, ewe.

Introduction

Assisted reproductive technologies (ARTs) have enabled the rapid propagation of embryos with genetically desirable characteristics for the animal breeding industry and the supply of biological material for basic research (Orozco-Lucero and Sirard 2014). Most ARTs, such as *in vitro* embryo production, transgenesis or cloning, require the recovery of a great number of

good quality oocytes (Souza-Fabjan *et al*. 2014b). In live females of monovular species, hormonal ovarian stimulation is performed as the first step in the application of these biotechnologies, as this stimulation increases the number of follicles available and thus the number of oocytes recovered (Gibbons *et al*. 2007; reviewed by Baldassarre 2012; Souza-Fabjan *et al*. 2013; Dias *at al*. 2013). However, oocyte quality is essential for the success of any ART technique (Dias *et al.* 2014).

Several ovarian stimulation protocols have been proposed for small ruminants. Different concentrations, origins (porcine or ovine) and degrees of purity of follicle-stimulating hormone (FSH), applied in either multiple doses (three to six) or as a "one-shot" regime, have been reported (Baldassarre *et al*. 1996, 2002; Berlinguer *et al*. 2004; Sousa *et al*. 2011). In general, although the short half-life of FSH (Laster 1972) makes it ineffective when administered alone in a single dose (Armstrong *et al*. 1994; Baldassarre *et al*. 1996), the use of either 80 or 120 mg of FSH in multiple doses is efficient to promote follicle development (Sousa *et al*. 2011; reviewed by Baldassarre 2012; Santos *et al*. 2016). Indeed, the use of 80 mg of FSH applied in single dose with 300 IU of equine chorionic gonadotropin (eCG), the so called "one-shot" scheme, is efficient and often used in small ruminants, due to be a practical single handling (Baldassarre *et al*. 2002, 2003, 2012; Pierson *et al*. 2004; Teixeira *et al*. 2011). However, little is known about the molecular status of oocytes generated under different stimulatory treatments in small ruminants.

Exogenous support of gonadotropin led to physiological alterations and affected intrafollicular regulatory mechanisms. Subordinate follicles can be rescued from atresia conditions, and multiple follicles can reach dominance and become able to ovulate, maintaining a high FSH plasma concentration (Adams *et al*. 1992; Yu *et al*. 2003; Pinto *et al.* 2017). Recent studies in cows using microarray analysis have shown that global gene expression in cumulus cells is altered by the use of stimulatory treatment (Dias *et al*. 2013) and can affect the oocyte

development competence (Blondin *et al*. 2002; Nivet *et al.* 2012; Labrecque *et al*. 2013). The quality of the female gamete depends on the specific gene products (transcripts and proteins) stored in the cytoplasm during oocyte growth to support early development and during transcriptionally inactive periods, from maturation to activation of the embryonic genome (Krisher 2004; Sirard 2010).

The action of FSH in cumulus cells is primarily mediated by the cAMP-dependent protein kinase A (PKA) pathway (Hillier *et al*. 1994). Briefly, when FSH binds to its receptor (FSHr), adenyl cyclase is activated, enabling cAMP production. The increased cAMP concentration activates PKA. Subsequently, the expression and/or activity of components of the steroidogenic machinery, such as the steroidogenic acute regulatory protein (STAR), serves as the starting point of the cascade of cholesterol transformation (Wood and Straus III 2002; reviewed by Shimasaki *et al*. 2004). At the end of the cascade, 17β-estradiol (E_2) production enhances FSH action through a gradual increase in the expression of FSHr, LHr and its own receptors, including ERα (reviewed by Couse and Korach 1999). Additionally, growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) produced by oocytes regulate FSH activity in the cumulus through the modulation of steroid production (Otsuka *et al*. 2005; Lahoz *et al*. 2013; Kona *et al*. 2016). Thus, transcripts of genes, such as GDF9, BMP15, maternal antigen that embryo requires (MATER) and zygote arrest 1 (ZAR1), serve as potential markers of oocyte development competence (reviewed by Orozco-Lucero and Sirard 2014; Bebbere *et al*. 2008), as do intracellular proteins that regulate the apoptotic process by the activation of caspases, such as BAX (pro-apoptotic) and Bcl-2 (anti-apoptotic).

In ART, immature oocytes are often selected according to their morphological features (Almeida *et al*. 2010; Avelar *et al*. 2012; Souza-Fabjan *et al*. 2016). The brilliant cresyl blue (BCB) test can also be applied to determine oocyte growth (Turner *et al*. 2008; Catalá *et al*.

2011; Shabankareh *et al*. 2014). The germinal vesicle (GV)-stage oocyte is a relatively stable model to observe differences in accumulated mRNA levels in regard of its competence to form a viable embryo (Lambrecque *et al*. 2013). Based on the findings described here, we tested two hypotheses: one, the stimulatory treatment regardless of the protocol affects the steroidogenic pathway and oocyte quality; two, given an equivalent growth phase, the expression of gene transcripts related to steroidogenic machinery will increase in the cumulus-oocyte complex (COC) with increasing FSH dose in different regimes; and three, oocyte quality molecular markers may be favorably affected by the multiple-dose regime, since it may be more physiological. Thus, the aim of the present study was to evaluate the effect of four stimulation protocols on the follicular population and molecular status of the COC, as evidenced by the gene expression of markers of oocyte quality (GDF9, BMP15, MATER, ZAR1, Bcl-2 and BAX) and transcripts involved in steroidogenesis (STAR, FSHr, LHr and ERα) in Santa Inês ewes.

Materials and Methods

Experimental Design

Twelve Santa Ines ewes underwent ovarian stimulation using four different protocols (Sousa *et al*. 2011; Baldassare 2012) in a crossover design (four treatments, three ewes per treatment, four replicates). There was a 21-day interval between replicates. The one-shot with 80 mg of FSH plus 300 IU of eCG was considered the standard treatment (Baldassarre *et al*. 2002, 2003, 2012). Ovarian dynamics was monitored by ultrasonography from sponge removal (estrus synchronization) to the laparoscopic ovum pick up (LOPU) procedure. A total of four LOPU sessions was performed, and the recovered COCs were morphologically graded and tested using BCB. BCB^+ COCs were used for gene expression analysis. The estradiol (E_2) concentration was

evaluated in the follicular fluid. Additionally, two ewes that had not received any treatment underwent LOPU in the last two sessions (n=4). COC recovery was graded and tested using BCB. The BCB^{+} COCs from the non-treated ewes were used as a control calibrator in the gene expression analysis.

Location, Conditions and Animal Care

The study was performed from September to December, 2016 at the Unidade de Pesquisa Experimental em Caprinos e Ovinos (UNIPECO) da Universidade Federal Fluminenese, in Cachoeiras de Macacu, Rio de Janeiro, Brazil (latitude 22°27′45″ S). All procedures were approved by the local Ethical Committee for Animal Use of the Universidade Federal Fluminense (protocol #721/2015) and conducted under the ethical principles of the Sociedade Brasileira de Experimentação Animal. Fourteen clinically healthy, adult, multiparous Santa Inês ewes (mean body weight of 53 ± 4.9 kg and body condition score of 3.7 ± 0.3) were housed and received chopped elephant grass (*Pennisetum purpureum*) and 200 g/animal of concentrate (12% crude protein) twice daily, and water and mineralized salt were provided *ad libitum*.

Hormonal Stimulation

Estrus synchronization was performed with intravaginal sponges containing 60 mg of medroxyprogesterone acetate (Progespon®; Schering Plough Animal Health, SP, Brazil) used for 6 days. One day prior to sponge removal, 300 IU of eCG (Novormon 5000®; MSD Animal Health, SP, Brazil) and 0.12 mg of cloprostenol sodium (Estron®, Tecnopec, São Paulo, Brazil) were administered intramuscularly (i.m.), as well as 0.025 mg of lecirelin (Gestran Plus®; Tecnopec, SP, Brazil) at 36 h after progestagen withdrawal (Balaro *et al*. 2016). Every 12 h from sponge removal, the ewes were submitted to estrus detection with teaser rams for sexual

behavior evaluation. Stimulation was initiated (considered day zero – D0) at 80 h after sponge removal and 36 h prior to LOPU. Moreover, a new sponge was placed (removed at LOPU). Stimulation protocols were performed by using the following the schemes: multiple doses (MD), 80 or 120 mg of FSH (Folltropin-V®, Bioniche Animal Health, Ontario, Canadá) applied in three decreasing doses (50, 30 and 20%) every 12 h (MD_{80} and MD_{120}); or one-shot (OS), 80 (standard protocol) or 120 mg of FSH plus 300 IU of eCG (OS_{80} and OS_{120}). Figure 1 illustrates the experimental treatments and procedures adopted in the present study.

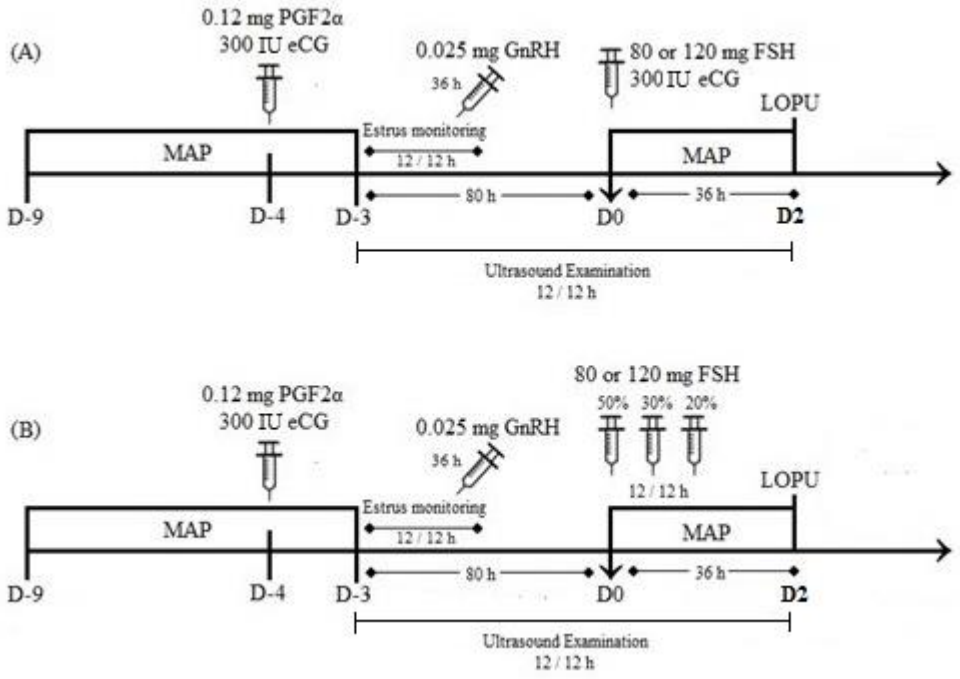

Fig. 1. Representative scheme of the experimental design. Previous estrus synchronization treatment was applied in all groups to reach ovulation on Day 0. At Day 0, a new progesterone sponge was inserted, and ovarian stimulation was performed with (A) only one dose of FSH (either 80 or 120 mg) and 300 IU eCG (one-shot, OS_{80} and OS_{120}) or (B) multiple decreasing doses of FSH (totaling 80 or 120 mg) (multiple doses, MD_{80} or MD_{120}).

Ovarian Assessment

Transrectal ultrasonography was performed every 12 h from the day of the first sponge removal to the day of LOPU. The ovulatory parameters (ovulation and size of largest follicle) and the number, position, and diameter of the ovarian follicles were recorded. Follicles were classified according to their size as small (<3.0 mm), medium (3–5.0 mm) or large (>5.0 mm). The follicular population was observed immediately prior to the start of ovarian stimulation (Day 0) and immediately before LOPU (Day 2). Evaluations were performed using an ultrasound (SonoScape, Shenzhen, China) equipped with a 7.5-MHz linear transducer in B-mode. Color Doppler mode was used to assess the corpora lutea (CL) status (regression or formation post-ovulation at D0).

COC recovery

Donor ewes were deprived of food for 36 h and water for 24 h prior to laparoscopic procedures. Preanesthetic medication was achieved by the administration of 0.2 mg/kg diazepam (Diazepam, Teuto®, Goiania, Brazil), 0.1 mg/kg acepromazine i.v. (Acepran®, Vetnil, São Paulo, Brazil), and 0.4 mg/kg morphine i.m. (Dolo Moff®, São Paulo, Brazil). Anesthesia was induced with 6 mg/kg ketamine (Dopalen®, Vetbrands, São Paulo, Brazil) and 10 mg 1% propofol (Profolen®, Balusiegel, São Paulo, Brazil) i.v. and maintained with isoflurane (Forane®, Abbott Laboratórios, Rio de Janeiro, Brazil) via endotracheal intubation. For local anesthesia, 2% lidocaine (Lidovet®, Bravet, Rio de Janeiro, Brazil) was injected. LOPU was performed according to Baldassare *et al*. (2003). Briefly, visible follicles (2-8 mm) were aspirated using a 20 G needle with a short bevel connected to a 50 cm cannula, which was connected to a silicone cork (Handle Cook®, São Paulo, Brazil) and a Falcon tube (50 mL). The aspiration pressure was regulated at 36 mmHg produced by a vacuum pump (WTA, São Paulo,

Brazil). The aspiration medium was TCM199 (M2520, Sigma-Aldrich, St. Louis, USA) supplemented with 20 IU/mL heparin (Calbiochem 375095; Merck Chemicals, Nottingham, UK), 0.2 mM pyruvate (P4562, Sigma), 100 IU penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin (A5955, Sigma) and 10% BSA (A9647, Sigma). Initially, 4 mL of the aspiration medium was added into the collection tube, and another 4 mL was later added to wash the aspiration system, for a final volume of 8 mL.

COC grading and BCB test

COCs were isolated under a stereomicroscope and graded based on visual assessment of morphology (cellular layers and cytoplasmic uniformity) considering the quality as Grade I/II (good), III (acceptable) and IV (poor) (Souza-Fabjan *et al*. 2016). For inference of the developmental competence, viable COCs (GI, GII and GIII) from each treatment were washed once and exposed to 26 µM BCB (B5388, Sigma) diluted in DMPBS supplemented with 10% BSA (A9647, Sigma) and 0.2 mM pyruvate (P4562, Sigma) for 60 min on a hot plate at 37 ºC and ambient atmosphere. Then, COCs were washed two times, placed in polyvinyl alcohol (PVA, P8136, Sigma) for 5 min and classified according to oocyte cytoplasm staining as BCB+ (blue cytoplasm) or BCB- (colorless cytoplasm). Pools of 10 BCB+ COCs from the same treatment were recovered (with a minimal amount of medium) at once with a pipette calibrated at 2 µL, transferred to DNAse/RNAse-free cryotubes, snap frozen and stored in a liquid nitrogen canister for later gene expression analysis.

Estradiol concentration in follicular fluid

Following the LOPU procedure, the collection tube containing 8 mL of follicular content was left to rest for decantation. After 5 min, 300 µL of supernatant was aliquoted in triplicate and

stored at -20 °C. The samples from each ewe were separately aliquoted. The measurement was made by radioimmunoassay (RIA) using a commercial kit (17b-Estradiol - ImmuChem™ Double Antibody - MP Biomedicals, LLC, Diagnostics Division - Orangeburg, NY 10962) according to the manufacturer's instructions. The samples were analyzed in a single assay, with a standard dilution of 1:2 and an intra-assay coefficient of variation of 7%. After the determination of the E_2 concentration, the mathematical correction was made by multiplying the observed concentration by the dilution factor.

Gene expression analysis

To assess the abundance of mRNA encoding protein markers of oocyte competence (MATER, ZAR1, BMP15, GDF9, BAX, Bcl-2) and the steroidogenic pathway (FSHr, LHr, STAR and ERα), a control (non-stimulated ewe) was used as a calibrator to compare parameters of physiological molecular status vs. each treatment.

The samples were analyzed by quantitative real-time polymerase chain reaction (qRT-PCR) after reverse transcription according to Batista *et al*. (2014). Total RNA was extracted from 3 pools of 10 BCB+ COCs/group using an RNAeasy Micro kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and reverse transcribed using the Superscript III kit (Invitrogen, Carlsbad, CA, USA). The total RNA from each pool for treatment group was quantified using 1 μL of sample and a ND-100 spectrophotometer (NanoDrop Products, Wilmington, DE, USA). Relative quantification by qRT-PCR was performed in duplicate using a commercial SYBR Green kit (Power SYBR Green, Applied Biosystem), 400 ng of cDNA, nuclease-free water and specific primers (Table 1) for every reaction. Template cDNA was denatured at 95 °C for 15 min, followed by 45 cycles of denaturation at 94 °C for 15 s, gene-specific primer annealing temperature for 30 s and elongation at 60 °C for 30 s. After each PCR run, a melting curve analysis was performed to confirm that a single specific product was

generated. No template controls (NTCs), comprising the PCR reaction mix without DNA template, were also run with each primer to confirm the absence of contamination.

Table 1. Sequences of the specific primers used in the analysis of gene expression in immature sheep cumulus oocyte complexes (BCB+) recovered by laparoscopy after different ovarian stimulation treatments.

Gene	Sequence of primers (5'- 3')	Annealing temperature (°C)	Size (bp)	Reference
GAPDH	GGGAAA TCG TGC GTG ACA TTA AG TGTGTT GGCGTAAGGTCTTTG	60	273	Bebbere *et al.* 2008
ZAR1	CAC TGC AAG GAC TGC AAT ATC CAG GTG ATA TCC TCC ACT C	60	137	Bebbere *et al.* 2008
MATER	CAG CCT CCA GGA GTT CTT TG GAC AGC CTA GGA GGG TTT CC	59	212	Bebbere *et al.* 2008
GDF9	CAG ACG CCA CCT CTA CAA CA CAG GAA AGG GAA AAG AAA TGG	58	198	Bebbere *et al.* 2008
BMP15	GGG TTC TAC GAC TCC GCT TC GGT TAC TTT CAG GCC CAT CAT	59	273	Bebbere *et al.* 2008
BAX	CCT GGG ATC TTG AAA CTC TCC TT CTG AGC CAG GCT GAA ATC AAA A	60	566	Chakravarthi *et al.* 2017
Bcl-2	GCC GAG TGA GCA GGA AGA C GTT AGC CAG TGC TTG CTG AGA	60	214	Chakravarthi *et al.* 2017
ERα	GAA TCT GCC AAG GAG ACT CG CCT GAC AGC TCT TCC TCC TG	60	187	Hogg *et al.* 2011
LHr	TCC GAA AGC TTC CAG ATG TT GAA ATC AGC GTT GTC CCA TT	60	199	Hogg *et al.* 2011
FSHr	TAA GCA CTT GCC AGC TGT TC CTC ATC GAG TTG GGT TCC AT	60	196	Hogg *et al.* 2011
STAR	GCA TCC TCA AAG ACC AGG AG CTT GAC ACT GGG GTT CCA CT	60	194	Hogg *et al.* 2011

Statistical analysis

Outcome variables related to sexual behavior, ovulatory parameters, follicular population, recovery rate, total number of COCs, morphological quality and BCB staining of the COCs per ewe and E_2 concentration were evaluated for normality using the Lilliefors test. The main effect of FSH dose and administration regime and the interaction between these parameters were assessed by analysis of variance (ANOVA), and differences between means were compared by Tukey's test. Data without a normal distribution (follicular population) were submitted to square root transformation prior to ANOVA. For gene expression analysis, primer efficiency was tested using the LinRegPCR software (Ramakers *et al*. 2003) for each reaction. Relative quantification was performed by REST software (Pfaffl *et al*. 2002) based on comparative Ct quantification. The values are shown as differences relative to the calibrator. Statistical analyses were performed by using a Statistical Analysis System program (SAEG® 9.0, Federal University of Viçosa, Viçosa, Brazil). Differences were considered significant when $P < 0.05$. Results are shown as the mean ± SD.

Results

Estrus synchronization and follicular wave

After estrus synchronization with the first progestogen sponge (from Day -3 to Day 0 in Figure 1), sexual behavior and ovulatory parameters were evaluated. Overall, the estrous response rate was 85% (41/48), the interval from sponge removal to estrus was 27.5 ± 10.8 h, the interval from estrus to ovulation was 26.4 ± 11.4 h, the duration of estrus was 32.0 ± 13.9 h, the ovulation rate was 98% (47/48), the number of ovulations was 1.2 ± 0.4, and the largest follicle diameter (mm) was 6.4 ± 0.9. These parameters did not differ among ewes from all four experimental groups.

Ovarian response to the stimulation treatments

The data on follicular population are shown in Table 2. At Day 0, no differences were found among treatments in relation to the number of follicles in each category. In OS_{80}, OS_{120} and $MD_{120,}$ the number of small follicles was greater than that of medium follicles, which was greater than the number of large follicles. In the MD_{80} group, the number of small follicles was also greater than that of medium and large follicles, which did not differ from each other. At Day 2, there was no effect of FSH dose and administration regime, as well as no interaction of these factors on the number of follicles in any category. In OS_{120}, MD_{80} and $MD_{120,}$ the number of medium follicles was greater than the number of small follicles, which in turn was larger than that of large follicles. Even in the OS_{80} group, the number of medium follicles was greater than that of small and large follicles, which did not differ from each other.

Table 2. Ovarian follicular population immediately before (Day 0) and after (Day 2) hormonal stimulation treatments in Santa Inês ewes (mean ± SD).

	Day 0			Day 2		
Treatment	Small (<3 mm)	Medium (3-5 mm)	Large (>5 mm)	Small (<3 mm)	Medium (3-5 mm)	Large (>5 mm)
OS_{80}**	8.5 ± 3.6 [A]	4.3 ± 2.0 [B]	0.1 ± 0.3 [C]	3.5 ± 3.5 [A]	5.7 ± 2.4 [B]	1.3 ± 1.2 [A]
OS_{120}**	9.2 ± 3.2 [A]	2.8 ± 1.2 [B]	0.1 ± 0.3 [C]	3.4 ± 2.4 [A]	7.9 ± 2.3 [B]	0.9 ± 1.4 [C]
MD_{80}**	9.4 ± 3.3 [A]	2.4 ± 2.4 [B]	0.3 ± 0.5 [B]	3.8 ± 3.4 [A]	8.1 ± 2.2 [B]	0.5 ± 0.9 [C]
MD_{120}**	8.3 ± 5.1 [A]	3.3 ± 1.8 [B]	0.1 ± 0.3 [C]	3.3 ± 2.0 [A]	7.8 ± 3.6 [B]	0.8 ± 1.0 [C]

n= 12 ewes/treatment

* Within a row, A,B differ ($P < 0.05$) among the follicular category, within the same day and treatment.

** OS_{80}: 80 mg of FSH plus 300 IU of eCG applied in one-shot;

**OS_{120}: 120 mg of FSH plus 300 IU of eCG, applied in one-shot;

**MD_{80}: 80 mg of FSH applied in multiple decreasing doses;

**MD_{120}: 120 mg of FSH applied in multiple decreasing doses.

COC recovery and BCB test

Quantitative and qualitative aspects of COCs produced from the four hormonal treatments are shown in Table 3. There were no differences among treatments in the parameters evaluated. However, there was an effect of the administration regime on COC morphology, where MD resulted in a greater number of GII COCs than OS. There was no difference in the amount of the good morphology quality COCs (GI + GII) in the following treatment groups: OS_{80} (56%,

39/70), OS_{120} (66%, 43/65), MD_{80} (74%, 61/82) and MD_{120} (70%, 64/91). Regardless of the regimen applied, i.e., considering only the dose (80 mg or 120 mg of FSH), the number of COCs per ewe was as follows: total, 6.3 ± 0.9 or 6.5 ± 1.8; viable, 6.0 ± 1.0 or 6.3 ± 1.6; and BCB+, 4.6 ± 1.5 or 5.1± 1.1, respectively.

Table 3. Effect of different ovarian stimulation treatments on the quantity and quality (morphology and BCB test) of immature cumulus oocyte complexes (COCs) recovered by laparoscopy in Santa Inês ewes (mean ± SD).

Treatment	Recovery (%)	Total COC	Viable* COC	Good quality* COC	BCB+ (%)
OS_{80}^{**}	65 (70/107)	5.8 ± 0.6	5.4 ± 0.9	3.3 ± 0.7^{b}	72
OS_{120}^{**}	59 (65/110)	5.4 ± 1.0	5.3 ± 0.8	3.6 ± 0.6^{b}	88
MD_{80}^{**}	68 (82/120)	6.8 ± 0.9	6.6 ± 0.9	5.1 ± 0.4^{a}	80
MD_{120}^{**}	70 (91/130)	7.6 ± 1.9	7.3 ± 1.3	5.3 ± 2.3^{a}	76

n= 12 ewes/treatment

* Within a column, a,b differ ($P < 0.05$) among the treatments.

*Viable: GI + GII + GIII; *Good quality: GI + GII;

**OS_{80}: 80 mg of FSH plus 300 IU of eCG applied in one-shot;

**OS_{120}: 120 mg of FSH plus 300 IU of eCG, applied in one-shot;

**MD_{80}: 80 mg of FSH applied in multiple decreasing doses;

**MD_{120}: 120 mg of FSH applied in multiple decreasing doses.

Gene expression

Regardless of the dose and strategy of administration, ovarian stimulation protocols altered the gene expression pattern of the steroidogenic pathway and oocyte quality markers in COCs with complete exponential growth (BCB^+) compared with the control. The steroidogenic pathway genes were down-regulated, except for FSHr in OS_{80}, which was not differentially expressed. Oocyte quality markers were up-regulated in OS_{80} (ZAR1, BMP15, GDF9, BAX and Bcl-2), OS_{120} (GDF9, BAX and Bcl-2), MD_{80} (MATER, ZAR1, BMP15, GDF9, BAX and Bcl-2) and MD_{120} (ZAR1, GDF9, BAX and Bcl-2) compared to control.

Different doses (80 vs. 120 mg) of FSH were compared within the same application regime, and the results are shown in Figure 2. Steroidogenic pathway transcripts (FSHr, ERα and STAR) and BMP15 were down-regulated in OS_{120} (Figure 2A). However, only LHr was down-regulated in MD_{120} (Figure 2B).

Different application regimes (OS vs. MD) using the same FSH dose were compared, and the results are shown in Figure 3. The steroidogenic pathway (FSHr and ERα) was down-regulated in OS_{80}. While, LHr was down-regulated in MD_{120}.

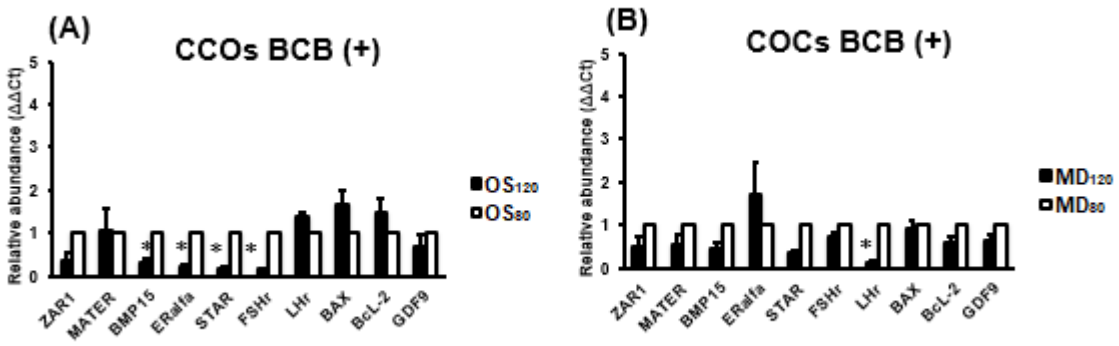

Fig. 2. Effect of FSH dose on the gene expression profile in cumulus oocytes complexes (COC). Relative gene expression is shown in COCs derived from different hormonal protocols for ovarian stimulation (OS_{80}: 80 mg of FSH plus 300 IU of eCG applied in one-shot; OS_{120}: 120 mg of FSH plus 300 IU of eCG applied in one-shot; MD_{80}: 80 mg of FSH applied in multiple decreasing doses; MD_{120}: 120 mg of FSH applied in multiple decreasing doses). Columns with an asterisk (*) within each gene differ ($P < 0.05$) among groups. The data for the OS_{80} (A) and MD_{80} (B) groups were used as a calibrator (=1). The results are shown as the fold change (mean ± SD) relative to the calibrator.

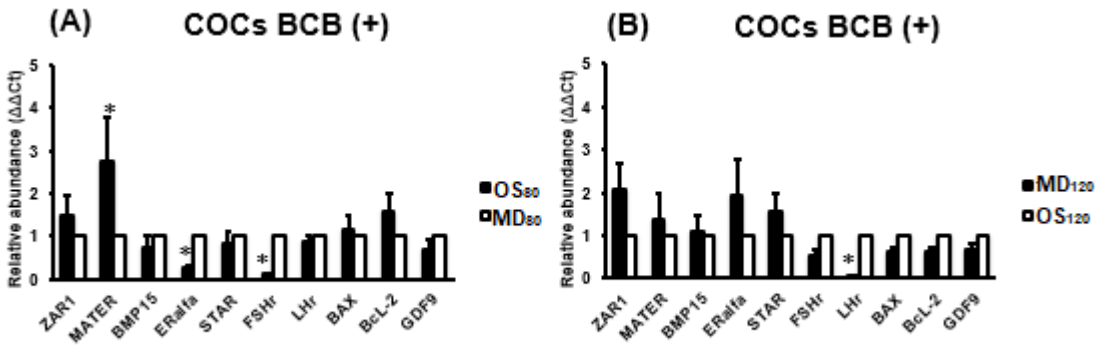

Fig. 3. Effect of FSH administration strategy on the profile of gene expression in cumulus-oocyte complexes (COC). Relative gene expression is shown in COCs derived from different hormonal protocols for ovarian stimulation (OS_{80}: 80 mg of FSH plus 300 IU of eCG applied in one-shot; OS_{120}: 120 mg of FSH plus 300 IU of eCG applied in one-shot; MD_{80}: 80 mg of FSH applied in multiple decreasing doses; MD_{120}: 120 mg of FSH applied in multiple decreasing doses). Columns with an asterisk (*) within each gene differ ($P < 0.05$) among groups. The data for the MD_{80} (A) and OS_{120} (B) groups were used as a calibrator (=1). The results are shown as the fold change (mean ± SD) relative to the calibrator.

Estradiol concentration in the follicular fluid

The 17β-estradiol concentration in the aspirated fluid was not different ($P > 0.05$) among all four treatment groups. The mean, minimum and maximum for each treatment were as follows: OS_{80} (11.511 ± 11.991, 1.561 and 43.680 ng/dL in 98 follicles), OS_{120} (5.235 ± 2.406, 817 and 9162 ng/dL in 110 follicles), MD_{80} (5.217 ± 4.783, 1.439 and 18.862 ng/dL in 120 follicles) and MD_{120} (7.544 ± 6.893, 585 and 25.405 ng/dL in 108 follicles). All data were within the maximum and minimum points of the curve.

Discussion

The effect of four stimulation protocols on the follicular population and molecular status of the COCs were evaluated in Santa Ines ewes. As expected after synchronization, sexual behavior and ovulatory parameters were not different among groups, since the same protocol has been used until recently. A higher estrus response rate (85%) and ovulation rate (98%) were observed as well reported by Balaro *et al*. (2016) that obtained 78% estrus response and 89% ovulation rates using the same synchronization protocol. The results demonstrated that follicular population at Day 0 (immediately before the start of stimulation) was similar in all groups, and the number of small follicles was significantly greater than that of medium and large follicles. At Day 2 (day of LOPU), there was neither an effect of the FSH dose nor the administration regime on the follicular population, as reported by Baldassarre *et al*. (2002) and in contrast with what was reported by Sousa *et al*. (2011). Regardless of the strategy of stimulation, FSH promoted a significant increase in the number of medium follicles and a decrease in the number of small follicles (Table 2), consistent with the findings of Yu *et al*. (2003), Berlinguer *et al.* (2004) and Gibbons *et al.* (2007). In small ruminants, the COCs from medium follicles (3-5 mm) are positively correlated with better maturation, cleavage, and blastocyst rates and are of the preferential diameter for aspiration to IVP (Lahoz *et al*. 2013; Souza-Fabjan *et al*. 2014a). A study in cows demonstrated that COCs from medium follicles (7-10 mm) presented greater developmental competence than those from small (5-6 mm) and large (>10 mm) follicles. Additionally, the existence of a positive correlation between follicle size (until 8 mm) and developmental competence was observed, and for unknown reasons, the correlation was negative in large follicles (Nivet *et al*. 2012).

An effect of the administration regimen on COC morphological quality was observed in the present study, where MD treatments produced a greater amount of GII COCs than did OS treatments. Curiously, a similar effect was reported by Santos *et al.* (2016). The rate of

BCB+ COCs was not different among the groups. The relationship between viable COCs (GI, GII and GIII) and the BCB+ rate demonstrated that GIII (denuded but with homogeneous cytoplasm) may have satisfactory competence, as proposed by Souza-Fabjan *et al*. (2016). The BCB test in IVP programs has been used to select oocytes that have completed the exponential growth phase and thus have theoretically acquired the capability to develop *in vitro*, promoting the selection of a more homogeneous oocyte pool (Catalá *et al*. 2011; reviewed by Paramio and Izquierdo 2016). In a previous study, the percentage of immature BCB+ COCs from small follicles was lower than that from medium or large follicles (Shabankareh *et al*. 2014). In another study, BCB+ COCs from small and large follicles exhibited similar developmental competence, despite their differences in meiotic and cytoplasmic maturation (Yang *et al.* 2016).

The results of the present study provide new information on the dose effect and strategy of FSH administration on the relative expression patterns of the genes associated with steroidogenesis (ERα, STAR, FSHr and LHr) and markers of quality (ZAR1, MATER, and BMP15 and GDF9, BAX and Bcl-2) in cumulus-oocyte complexes. Regardless of the dose of FSH and strategy of administration, the genes involved in steroidogenesis were down-regulated, and markers of quality were up-regulated compared to the non-stimulated control calibrator. These findings suggest that the increase in circulating FSH concentration induces a reduction in the expression of FSHr and consequently other receptors and enzymes of the PKA pathway as a mechanism for maintaining healthy intrafollicular estrogen levels. Consistent with this hypothesis, the intrafollicular estrogen concentration was similar among the different treatment groups. According to Dias *et al*. (2013), multiple pre-ovulatory follicles that develop after stimulation are not typical dominant follicles. A total of 470 genes are differentially expressed in granulosa cells (GCs) from stimulated compared to non-stimulated follicles. Stimulation primarily affects cellular growth and development functions, delaying GC differentiation due the up-regulation of genes associated with cell growth and proliferation, which causes a blunted

response to LH signaling in GCs from stimulated follicles compared to that in GCs from non-stimulated dominant follicles, where these genes are down-regulated. Nevertheless, not all functions are equally affected, e.g., steroidogenic function, as the intrafollicular E_2 concentration and E_2:progesterone ratio were similar between stimulated and non-stimulated follicles.

The improvement of the gene expression of quality markers, such as GDF9 (in all treatment groups) and BMP15 in OS_{80} and MD_{80}, justifies the reduction in the steroidogenic pathway. GDF9 and BMP15 synergistically regulate FSH activity in the proliferation and steroid production of GCs, playing a crucial role in normal follicular development and following a stage-specific pattern of expression *in vivo* (Otsuka *et al*. 2005; Bebbere *et al*. 2008; Lahoz *et al*. 2013; Kona *et al*. 2016). The transforming growth factor-β (TGFβ) superfamily members exert luteinization inhibitor activity in GCs (reviewed by Shimasaki *et al*. 2004). *In vitro*, GDF9 alone stimulates basal steroidogenesis in preovulatory GCs, but in co-culture with FSH, GDF9 induces the dose-dependent suppression of cAMP production, and consequently, progesterone (P4) and E_2 synthesis attenuate FSH-dependent LHr expression (Vitt *et al*. 2000). BMP15 inhibits FSHr expression and cAMP signaling, thereby reducing the expression of PKA pathway components, including STAR protein and LHr (Otsuka *et al*. 2001). The expression of GDF9 and BMP15 is FSH independent (reviewed by Shimasaki *et al*. 2004), but in the present study, GDF9 was influenced by exogenous FSH, and BMP15 was affected by FSH dose (80 mg). FSH may play a role in the modulation of the cumulus response to oocyte-secreted factors such as GDF9 and BMP15 (Khan *et al*. 2015).

Other markers of oocyte quality that were up-regulated in stimulated BCB^+ COCS included MATER (MD_{80}) and ZAR1 (MD_{80}, OS_{80} and MD_{120}). MATER and ZAR1 are required for early development prior to zygotic genome activation, and these proteins have similar expression patterns. The abundance of mRNA transcripts of MATER and ZAR1 is a good

predictor of the development competence in immature GV oocytes (Bebbere *et al*. 2008; Urrego *et al*. 2015), indicating that stimulation protocols can enhance COC quality depending on the dose and administration strategy, e.g., 80 mg of FSH showed better modulation of both maternal effect genes in MD_{80} and of ZAR1 in $OS_{80.}$ However, 120 mg of FSH administered in an MD regime also increased ZAR1 expression. This fact confirmed our hypothesis that MD is more suitable for producing better quality COCs. In addition, we considered apoptosis proteins, including BAX (pro-apoptotic molecules) and Bcl-2 (antiapoptotic molecules), as markers of COC quality. In all stimulation protocols evaluated in the present study, BAX and Bcl-2 were abundantly expressed. However, the relative balance between the different pro- and anti-apoptotic proteins, reflecting the formation of homodimers and heterodimers (neutralization), defines the route of programmed cell death (reviewed by Zörnig *et al*. 2001). These data suggested the neutralization of the molecular mechanisms of apoptosis. Apoptosis is the mechanism responsible for promoting atresia. A study using microarray analysis of stimulated follicles demonstrated that despite stimulation rescue of follicles from atresia, some molecules involved with that pathway continue to be triggered. In contrast, other known anti-atresia markers were up-regulated after stimulation (Dias *et al*. 2013).

Comparing different doses of FSH (80 vs. 120 mg) within the same administration regime (Figure 2) revealed that the steroidogenic pathway (FSHr, ERα and STAR) and BMP15 (quality marker) were reduced by increased FSH dose in OS. Comparing different regimes (OS vs. MD) using the same FSH dose (Figure 3) revealed that the steroidogenic pathway (FSH and ERα) was reduced in $OS_{80.}$ Conversely, in MD, only LHr was reduced in both comparisons. These findings indicated that a lower FSH concentration (80 mg) causes a better response to steroidogenic pathway signaling, and the MD regime promoted less suppression of the expression of these genes. The eCG synergism did not contribute to the effect on FSHr expression in OS, as demonstrated by the comparison between different regimes at the same

FSH dose. eCG has similar pituitary gonadotropin activity and even has high affinity for FSHr and LHr (Wei *et al*. 2016). The long half-life of eCG enabled the continued development of the follicles whose growth had been initiated by the bolus injection of FSH (Armistrong *et al*. 1994; Baldassarre *et al*. 1996), as used in the OS regime. FSHr is essential for FSH action and is primarily expressed by cumulus and mural granulosa cells in sheep (Tisdall *et al*. 1995). FSHr expression in the present study was reduced with increasing FSH dose, consistent with the findings of Santos *et al*. (2016). The reduced expression of LHr at MD_{120} compared to that at OS_{120} may indicate a better quality of COCs in MD. The abundance of LHr expression in GCs from immature COCs was associated with the poor quality of the oocytes (Vigone *et al*. 2015; Santos *et al.* 2016), as LHr expression has been reported as a parameter to differentiate immature oocytes as competent or non-competent to *in vitro* development (Vigone *et al*. 2015). The suppressed expression of STAR in OS_{120} compared to that in $OS_{80,}$ likely indicates a mechanism in which GDF9 and/or BMP15 inhibit early luteinization. TGFβ members act through this mechanism to maintain the E_2/P_4 ratio, as a premature increase in P_4 levels is correlated with follicular atresia (Zheng *et al*. 2008). ERα was reduced in OS_{120} (compared to OS_{80}) and OS_{80} (compared to MD_{80}). This reduction may be a consequence of the reduced FSHr and PKA pathways. Increased expression of E_2 receptors has been associated with the attenuation of apoptosis and follicular atresia (reviewed by Couse and Korach 1999). Perhaps MD_{80} produced better quality COCs than the other treatments tested, despite the similar E_2 concentrations in all treatments.

In conclusion, 80 mg of FSH in MD or OS regimens was sufficient to promote the development of multiple follicles available for COC recovery by LOPU, generating good morphology quality and fully grown (BCB+) COCs that are predicted as more competent for *in vitro* development. The MD regime may be more appropriate for producing better quality oocytes.

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Conflict of interest

All authors declare that they do not have any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations.

References

Adams, G. P., Matteri, R. L., Kastelic, J. P., Ko, J. C. and Ginther, O. J. (1992). Association between surges of follicle-stimulating hormone and the emergence of follicular waves in heifers. *J. Reprod. Fert.* **94**, 177–88. doi:10.1530/jrf.0.0940177

Almeida, K. C., Pereira, A. F., Alcântara Neto, A. S., Avelar, S. R. G., Bertolini, L. R., Bertolini, M., Freitas, V. J. F. and Melo, L. M. (2010). Real-time qRT-PCR analysis of EGF receptor in cumulus-oocyte complexes recovered by laparoscopy in hormonally treated goats. *Zygote* **19**, 127-36. doi:10.1017/S0967199410000225

Armstrong, D. T., Irvine, B. J., Earl, C. R., McLean, D. and Seamark, R. F. (1994). Gonadotropin stimulation regimens for follicular aspiration and *in vitro* embryo production from calf oocytes. *Theriogenology* **42**, 1227-36. doi: 10.1016/0093-691X(94)90871-0

Avelar, S. R. G., Moura, R. R., Sousa, F. C., Pereira, A. F., Almeida, K. C., Melo, C. H. S., Teles-Filho, A. C. A., Baril, g., Melo, L. M., Teixeira, D. I. A. and Freitas, V. J. F. (2012). Oocyte production and *in vitro* maturation in Canindé goats following hormonal ovarian

stimulation. *Anim. Reprod.* **9(1)**, 27-32.

Balaro, M. F. A., Fonseca, J. F., Barbosa, T. G. B., Souza-Fabjan, J. M. G., Figueira, L. M., Teixeira, T. A., Carvalheira, L. R. and Brandão, F. Z. (2016). Potential role for GnRH in the synchronization of follicular emergence before the superovulatory Day 0 protocol. *Domest. Anim. Endocrinol.* **54**, 10-4. doi: 10.1016/j.domaniend.2015.07.002

Baldassarre, H., Furnus, C. C., de Matos, D. G. and Pessi, H. (1996). *In vitro* production of sheep embryos using laparoscopic folliculocentesis: Alternative gonadotrophin treatments for stimulation of oocyte donors. *Theriogenology* **45**, 707-17.

Baldassarre, H., Wang, B., Kafidi, N., Keefer, C., Lazaris, A. and Karatzas, C. N. (2002) Advances in the production and propagation of transgenic goats using laparoscopic ovum pick-up and *in vitro* embryo production technologies. *Theriogenology* **57**, 275-284.

Baldassarre, H., Keefer, C., Wang, B., Lazaris, A. and Kartazas, C. N. (2003). Nuclear Transfer in Goats Using *In Vitro* Matured Oocytes Recovered by Laparoscopic Ovum Pick-Up. *Cloning and steam cells* **5**, 279-285. doi: 10.1089/153623003772032781

Baldassarre, H. (2012). Practical aspects for implementing *in vitro* embryo production and cloning programs in sheep and goats. *Anim. Reprod.* **9**, 188-94.

Batista, R. I., Raposo, N. R., Campos-Junior, P. H., Pereira, M. M., Camargo, L. S., Carvalho, B. C., Gama, M. A. and Viana, J. H. (2014). Trans-10, cis-12 conjugated linoleic acid reduces neutral lipid content and may affect cryotolerance of in vitro-produced crossbred bovine embryos. *J Anim. Sci. Biotechnol.* **5**, 33. doi: 10.1186/2049-1891-5-33

Bebbere D, Bogliolo L, Ariu F, Fois S, Leoni GG, Tore S, Succu S, Berlinguer F, Naitana S, Ledda S. Expression pattern of zygote arrest 1 (ZAR1), maternal antigen that embryo requires (MATER), growth differentiation factor 9 (GDF9) and bone morphogenetic protein

15 (BMP15) genes in ovine oocytes and in vitro-produced preimplantation embryos. *Reprod. Fertil. Dev.* **20(8),** 908-9015, 2008. doi.org/10.1071/RD08095.

Berlinguer, F., Leoni, G., Bogliolo, L., Pintus, P. P., Rosati, I., Ledda, S. and Naitana, S. (2004). FSH different regimes affect the developmental capacity and cryotolerance of embryos derived from oocytes collected by ovum pick-up in donor sheep. *Theriogenology* **61**, 1477-86. doi:10.1016/j.theriogenology.2003.08.005

Blondin, P., Bousquet, D., Herménégilde, T., Barnes, F., Sirard, M. A. (2002). Manipulation of Follicular Development to produce developmentally competent bovine oocytes. *Biol. Reprod.* **66(1)**, 38-43. doi: 10.1095/biolreprod66.1.38

Catalá, M. G., Izquierdo, D., Uzbekova, S., Marató, R., Roura, M., Romanguera, R., Papillier, P. and Paramio, T. (2011). Brilliant Cresyl Blue stain selects largest oocytes with highest mitochondrial activity, maturation-promoting factor activity and embryo developmental competence in prepubertal sheep. *Reproduction* **142**, 517–27. doi: 10.1530/REP-10-0528

Chakravarthi, V.P., Kona, S.S., Siva Kumar, A.V., Bhaskar, M., Rao, V.H. Quantitative expression of antiapoptotic and proapoptotic genes in sheep ovarian follicles grown in vivo or cultured in vitro. *Theriogenology*. 2015 1;83(4):590-5. doi: 10.1016/j.theriogenology.2014.10.024.

Couse, J. F. and Korach, K. S. (1999). Estrogen Receptor Null Mice: What Have We Learned and Where Will They Lead Us? *Endocr. Rev.* **20**, 358–417. doi:10.1210/edrv.20.3.0370

Dias, F. C. F., Khan, M. I. R., Sirard, M. A., Adams, G. P. and Singh, J. (2013). Differential gene expression of granulosa cells after ovarian superstimulation in beef cattle. *Reproduction* **146(2)**, 181-91. doi: 10.1530/REP-13-0114

Dias, F. C. F., Khan, M. I. R., Sirard, M. A., Adams, G. P. and Singh, J. (2014). Granulosa cell function and oocyte competence: Super-follicles, supper-moms and super-stimulation in cattle. *Anim. Reprod. Sci.* **149(1-2)**, 80-9. doi: 10.1016/j.anireprosci.2014.07.016

Gibbons, A., Pereyra Bonnet, F., Cueto, M., Catalá, M., Salamone, D. and González-Bulnes, A. (2007). Procedure for maximizing oocyte harvest for *in vitro* embryo production in small ruminants. *Reprod. Domest. Anim.* **42**, 423-426. doi: 10.1111/j.1439-0531.2006.00802.x

Hillier, S. G., Whitelaw, P. F. and Smyth, C. D. (1994). Follicular oestrogen synthesis: the 'two-cell, two-gonadotrophin' model revisited. *Mol. Cell. Endocrinol.* **100**, 51-54.

Hogg K, McNeilly AS, Duncan WC. Prenatal androgen exposure leads to alterations in gene and protein expression in the ovine fetal ovary. *Endocrinology*, **152(5),** 2048-59, 2011. doi: 10.1210/en.2010-1219.

Khan, D. R., Guillemette, C., Sirard, M. A. and Richard, F. J. (2015). Caracterization of FSH signaling networks in bovine cumulus cells: a perspective on oocyte competence acquisition. *Mol. Hum. Reprod.* **21**, 688-701. doi: 10.1093/molehr/gav032

Kona, S. S. R., Chakravarthi, V. P., Kumar, A. V. N. S., Srividya, D., Padmaja, K. and Rao, V. H. **(**2016). Quantitative expression patterns of GDF9 and BMP15 genes in sheep ovarian follicles grown in vivo or cultured in vitro. *Theriogenology* **85**, 315–22. doi: 10.1016/j.theriogenology.2015.09.022

Krisher, R. L. (2004). The effect of oocyte quality on development. *J. Anim. Sci*. **82**, 14-23.

Lahoz, B., Alabart, J. L., Folch, J., Sànchez, P. S., Echegoyen, E. and Cocero, M. J. (2013). Influence of the FecXR Allele in Heterozygous Ewes on Follicular Population and Outcomes of IVP and ET using LOPU-Derived Oocytes. *Reprod. Domest. Anim.* **48**, 717–723. doi: 10.1111/rda.12150

Laster, D. B. (1972). Disappearance and uptake of [^{125}I]FSH in the rat, rabbit, ewe and cow. *J Reprod Fertil* **30**, 407-15. doi: 10.1530/jrf.0.0300407

Lambrecque, R., Vigneault, C., Blondin, P. and Sirard, M. A. (2013) Gene expression analysis of bovine oocytes with high developmental competence obtainedfrom FSH-stimulated animals. *Mol. Reprod. Dev.* **80**, 428–40. doi: 10.1002/mrd.22177

Nivet, A. L., Bunel, A., Labrecque, R., Belanger, J., Vigneault, C., Blondin, P. and Sirard, M. A. (2012). FSH withdrawal improves developmental competence of oocytes in the bovine model. *Reproduction* **143**, 165–71. doi: 10.1530/REP-11-0391

Orozco-Lucero, E. and Sirard, M. A. (2014). Molecular markers of fertility in cattle oocytes and embryos: progress and challenges. *Anim. Reprod.* **11**, 183-94.

Otsuka, F., Yamamoto, S., Erickson, G. F. and Shimasaki, S. (2001). Bone morphogenetic protein-15 inhibits follicle-stimulating hormone (FSH) action by suppressing FSH. *J. Biol. Cham.* **276(14)**, 11387-92. doi: 10.1074/jbc.M010043200

Otsuka, F., Moore, R. K., Wang, X., Sharma, S., Miyoski, T. and Shimasaki, S. (2005). Essential Role of the Oocyte in Estrogen Amplification of Follicle-Stimulating Hormone Signaling in Granulosa Cells. *Endocrinology* **146**, 3362–7. doi: 10.1210/en.2005-0349

Paramio, M. T. and Izquierdo, D. (2016). Recent advances in *in vitro* embryo production in small ruminants. *Theriogenology* **86(1)**, 152-9. doi: 10.1016/j.theriogenology.2016.04.027

Pfaffl, M. W., Horgan, G. M. and Dempfle, L. (2002). Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic. Acids. Res.* **30**, 36.

Pierson, J., Wang, B., Neveu, N., Sneek, L., Côté, F., Karatzas, N. C., Baldassarre, H. (2004). Effects of repetition, interval between treatments and season on the results from laparoscopic ovum pick up in goats. *Reprod. Fertil. Dev.* **16(8)**, 795-9. doi: 10.1071/RD04066

Pinto, P. H. N., Bragança, G. M., Balaro, M. F. A., Arashiro, E. K. N., Santos, G. B., Souza, G. N., Souza-Fabjan, J. M. G., Fonseca, J. F. and Brandão, F. Z. (2017). Colour-Doppler ultrasound imaging lutea in superovulated sheep. *Reprod. Domest. Anim.* doi: 10.1111/rda.13089

Ramakers, C., Ruijter, J. M., Deprez, R. H. L. and Moorman, A. F. M. (2003). Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci. Let*. **339**, 62–6.

Santos, J. D. R., Batista, R. I. T. P., Magalhães, L. C., Paula, A. R. Jr., Souza, S. S., Salamone, D. F., Bhat, M. H., Teixeira, D. I. A., Freitas, V. J. F. and Melo, L. M. (2016). Overexpression of hyaluronan synthase 2 and gonadotropin receptors in cumulus cells of goats subjected to one-shot eCG/FSH hormonal treatment for ovarian stimulation. *Anim. Reprod. Sci.* doi: 10.1016/j.anireprosci.2016.03.008

Shabankareh, H. K., Azimi, G. and Torki, M. (2014). Development competence of bovine oocytes selected based on follicle size and using the brilliant cresyl blue (BCB) test. *Iran. J. Reprod. Med.* **12**, 771-778.

Shimasaki, S., Moore, R. K., Otsuka, F. and Erickson, G. F. (2004). The bone morphogenetic protein system in mammalian reproduction. *Endocr. Rev*. **25**, 72–101. doi: 10.1210/er.2003-0007

Sirard, M. A. (2010). Activation of the embryonic genome. *Soc. Reprod. Fertil. Suppl.* **67**, 145-58.

Sousa, F. C., Melo, C. H. S., Teles Filho, A. C. A., Avelar, S. R. G., Moura, A. A. A., Martins, J. A. M., Freitas, V. J. F. and Teixeira, D. I. A. (2011). Ovarian follicular response to different hormonal stimulation treatments in Canindé goats. *Anim. Reprod. Sci.* **125**, 88– 93. doi: 10.1016/j.anireprosci.2011.02.015

Souza-Fabjan, J. M. G., Pereira, A. F., Melo, C. H. S., Sanchez, J. D., Oba, E., Mermillod, P., Melo, L. M., Teixeira, D. I. A. and Freitas, V. J. F. (2013). Assessment of the reproductive parameters, laparoscopic oocyte recovery and the first embryos produced in vitro from endangered Canindé goats (Capra hircus). *Reprod. Biol.* **13 (4)**, 325-32. doi: 10.1016/j.repbio.2013.09.005

Souza-Fabjan, J. M. G., Locatelli, Y., Duffard, N., Corbin, E., Touzé, J. L., Perreau, C., Beckers, J. F., Freitas, V. J. and Mermillod, P. (2014a). *In vitro* embryo production in goats: Slaughterhouse and laparoscopic ovum pick up–derived oocytes have different kinetics and requirements regarding maturation media. *Theriogenology* **81**, 1021-31. doi: 10.1016/j.theriogenology.2014.01.023

Souza-Fabjan, J. M. G., Locatelli, Y., Freitas, V. J. F. and Mermillod, P. (2014b). Laparoscopic ovum pick up (LOPU) in goats: from hormonal treatment to oocyte possible destinations. *R. Bras. Ci. Vet.* **21**, 3-11. doi: 10.4322/rbcv.2014.021

Souza-Fabjan, J. M. G., Locatelli, Y., Duffard, N., Corbin, E., Batista, R. I. T. B., Freitas, V. J. F., Beckers, J. F., Mermillod, P. (2016). Intrinsic quality of goats oocytes already found denuded at collection for *in vitro* embryo production. *Theriogenology* **86**, 1989-98. doi: 10.1016/j.theriogenology.2016.06.021

Souza-Fabjan, J. M. G., Rosa, R. M., Balaro, M. F. A., Pinto, P. H. N., Santos, G. B., Arashiro, E. K. N., Fonseca, J. F., Ungerfeld, R., Brandão, F. Z. (2017). Effect of different hormonal

combinations on follicular wave emergence and superovulatory response in sheep. *Theriogenology* in press. doi: 10.1016/j.theriogenology.2017.07.036

Teixeira, P. P. M., Padilha, L. C., Oliveira, M. E. F., Motheo, T. F., Silva, A. S. L., Barros, F. F. P. C., Coutinho, L. M., Flôre, N. F., Lopes, M. C. S., Bandarra, M. B., Silva, M. A. M., Vasconcelos, R. O., Rodrigues, L. F. S. and Vicente, W. R. R. (2011). Laparoscopic ovum collection in sheep: Gross and microscopic evaluation of the ovary and influence on oocyte production. *Anim. Reprod. Sci.* **127**, 169– 75. doi: 10.1016/j.anireprosci.2011.08.001

Tisdall, D. J., Watanabe, K., Hudson, N. L., Smith, P. and McNatty, K. P. (1995). FSH receptor gene expression during ovarian follicle development in sheep. *J. Mol. Endocrinol.* **15(3)**, 273-81. doi: 10.1677/jme.0.0150273

Turner, H., Ghanem, N., Ambros, C., Hölker, M., Tomek, W., Phatsara, C., ALM, H., Sirard, M. A., Kanit, W., Schellander, K. and Tesfaye, D. (2008). Focus on mammalian embryogenomics molecular and subcellular characterisation of oocytes screened for their developmental competence based on glucose-6-phosphate dehydrogenase activity. *Reproduction* **135**, 197–212. doi: 10.1530/REP-07-0348

Urrego, R., Herrera-Puerta, E., Chavarria, N. A, Camargo, O., Wrenzycki, C. and Rodriguez-Osorio, N. (2015). Follicular progesterone concentrations and messenger RNA expression of MATER and OCT-4 in immature bovine oocytes as predictors of developmental competence. *Theriogenology* **83**, 1179–87. doi: 10.1016/j.theriogenology.2014.12.024

Vigone, G., Merico, V., Redi, C.A., Mazzini, G., Garagna, S. and Zuccotti, M. (2015). FSH and LH receptors are differentially expressed in cumulus cells surrounding developmentally competent and incompetent mouse fully grown antral oocytes. *Reprod. Fertil. Dev.* **27**, 497-503. doi: 10.1071/RD13251

Vitt, U. A., Hayashi, M., Klein, C. and Hsueh, A. J. W. (2000). Growth differentiation factor-9 stimulates proliferation but suppress the follicle-stimulating hormone-induced differentiation of cultured granulosa cell from small antral and preovulatory rat follicles. *Biol. Reprod.* **62(2)**, 370-7. doi: 10.1095/biolreprod62.2.370

Wei, S. C., Gong, Z. D., Zhao, H. W., Liang, H. Q., Lai, L. J. and Deng, Y. Y. (2016). Equine chorionic gonadotropin influence on sheep oocyte *in vitro* maturation, apoptosis, and follicle-stimulating hormone receptor and luteinizing hormone receptor expression. *Genet. Mol. Res.* **15**, 1-13. doi: 10.4238/gmr15049162

Wood, J. R. and Strauss III, J. F. (2002). Multiple signal transduction pathway regulate ovarian steroidogenesis. *Rev. Endocr. Metab. Disord.* **3**, 33-46.

Yang, M., Hall, J., Fan, Z., Regouski, M., Meng, Q., Rutigliano, H. M., Stott, R., Rood, K. A., Panter, K. E. and Polejaeva, I. A. (2016). Oocytes from small and large follicles exhibit similar development competence following goat cloning despite their differences in meiotic and cytoplasmic. *Theriogenology* **86**, 2302-11. doi: 10.1016/j.theriogenology.2016.07.026

Yu, Y., Li, W., Han, Z., Luo, M., Chang, Z. and Tan, J. (2003). The effect of follicle-stimulating hormone on follicular development, granulosa cell apoptosis and steirodogenesis and its mediation by insulin-like growth factor-I in the goat ovary. *Theriogenology* **60**, 1691-704. doi: 10.1016/j.theriogenology.2003.08.001

Zheng, X., Prince, C. A., Tremblay, Y., Lussier, J., G. and Carrière, P. D. (2008). Role of transforming growth factor-β1 in gene expression and activity of estradiol and progesterone-generating enzymes in FSH-stimulated bovine granulosa cell. *Reproduction* **136**, 447-57. doi: 10.1530/REP-07-0316

Zörnig, M., Hueber, A., Baum, W. and Evan, G. (2001). Apoptosis regulators and their role in tumorigenesis. *Biochim. Biophys. Acta.***1551**, 1-37. doi: 10.1016/S0304-419X(01)00031-2

Capítulo 3

Exogenous progestogen does not affect first-wave follicle populations and oocyte quality during ovarian stimulation with FSH in sheep

Em tramitação – aceito com correções
Domestic Animal Endocrinology

Exogenous progestogen does not affect first-wave follicle populations and oocyte quality during ovarian stimulation with FSH in sheep

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Running title: The use of progestin devices does not affect oocyte quality

ABSTRACT

The effect of short-term administration of medroxyprogesterone acetate (MPA) or natural progesterone (P_4) during ovarian stimulation with FSH on oocyte recovery was investigated in Santa Inês ewes. Ewes were treated with intravaginal sponge containing MPA for 6 days; GnRH was applied 36 h after sponge removal and FSH in three injections at 80 h after (D0, shortly after ovulation). At the first FSH dose, the ewes received either a new MPA sponge (n=10) or a CIDR impregnated with P_4 (n=10) or did not receive any device (n=10). Ovarian dynamics were assessed every 12 h by transrectal ultrasonography from D-3 to D2. Oocytes were recovered by laparoscopic ovum pick up (LOPU) on D2 and graded by morphological quality. The number of small, medium and large follicles at D0 and D2 (ultrasound exams); number of both follicles aspirated and oocytes recovered at LOPU, recovery rate, and oocyte grade did not differ ($P>0.05$) among treatments. Thus, the short-term use of MPA or P_4 during ovarian stimulation did not affect the first-wave follicle population or morphological quality of oocytes. We would suggest that, in this protocol, the use of exogenous progestin is unnecessary.

Keywords: progestin, Day 0 protocol, oocyte, laparoscopy

1. Introduction

Progesterone (P_4) modulates various reproductive functions such as follicular growth, initial nutritional support of the embryo, and it blocks estrus expression and ovulation by actions in the hypothalamus. Because of this latter function, P_4 (natural progestogen) and its analogue (synthetics progestogen) have been commonly used in estrus synchronization protocols and to blockade the LH surge during ovarian stimulation to allow oocyte recovery in donor ewes [1,2]. However, recent evidence has suggested that exogenous progestogen may have a deleterious effect on embryo quality, mainly when used in long-term treatment [1,2].

The "Day 0 protocol" was proposed to synchronize ovulation to embryo transfer programs [3,4]. It involves the application of a progestin-releasing device for six days prior to the initiation of superovulation by FSH soon after normal ovulation; i.e., during emergence of the first follicular wave [5,6]. Recent research demonstrates that the application of a controlled device for internal release (CIDR) that contained progesterone during FSH stimulation of the first wave of estrous cycle was able to improve embryo yield [7]. In addition, it was reported that under high progesterone concentrations, FSH stimulation prior to LOPU enhanced quality of cumulus-oocyte complexes (COCs) and oocyte fertilization rate for *in vitro* embryo production (IVP) in sheep [8]. However, a comparative effect of exogenous progestogen (natural and synthetics) used during ovarian stimulation with FSH on populations of follicles and morphological quality of oocytes produced has not yet been demonstrated. Therefore, we developed the following hypotheses: 1) The use of exogenous progestogen during ovarian stimulation would not affect the population of follicles but would affect the quality of COCs, and 2) After estrus synchronization by day zero protocol, when ovulation is confirmed, exogenous progestogen support may not be essential, during ovarian stimulation for COC collection. Thus, this study aimed to investigate the effect of the synthetic (MPA) and natural (P_4) progestogen, on first-wave populations of follicles and oocyte quality during ovarian stimulation of Santa Inês ewes.

2. Materials and Methods

This study was approved by the Ethical Committee for Animal Use of the Universidade Federal Fluminense (protocol #721/2015).

2.1. Experiment location, animals and design

This study was performed at Unipeco, Brazil (22°27' S, 43°39' W) in October of 2017 (non-breeding season). Thirty multiparous and clinically healthy Santa Inês ewes (mean ± SD: 3.9 ±

1.0 years old, 51 ± 5.9 kg of body weight and 3.2 ± 0.6 of body condition score/scale 0-5) were kept housed and received chopped elephant grass (*Pennisetum purpureum*) plus 200 g/animal of concentrate (12% crude protein) twice daily, water and mineralized salt *ad libitum*. All ewes had their estrus synchronized followed by implementation of the Day 0 protocol [5]. Our previous study [4] defined the best stimulatory protocol used at present study, D0 was designed as first application of FSH. Ovarian assessment was performed as described below by ultrasonography on D-3 to D2. Experimental design is illustrated in the Fig. 1.

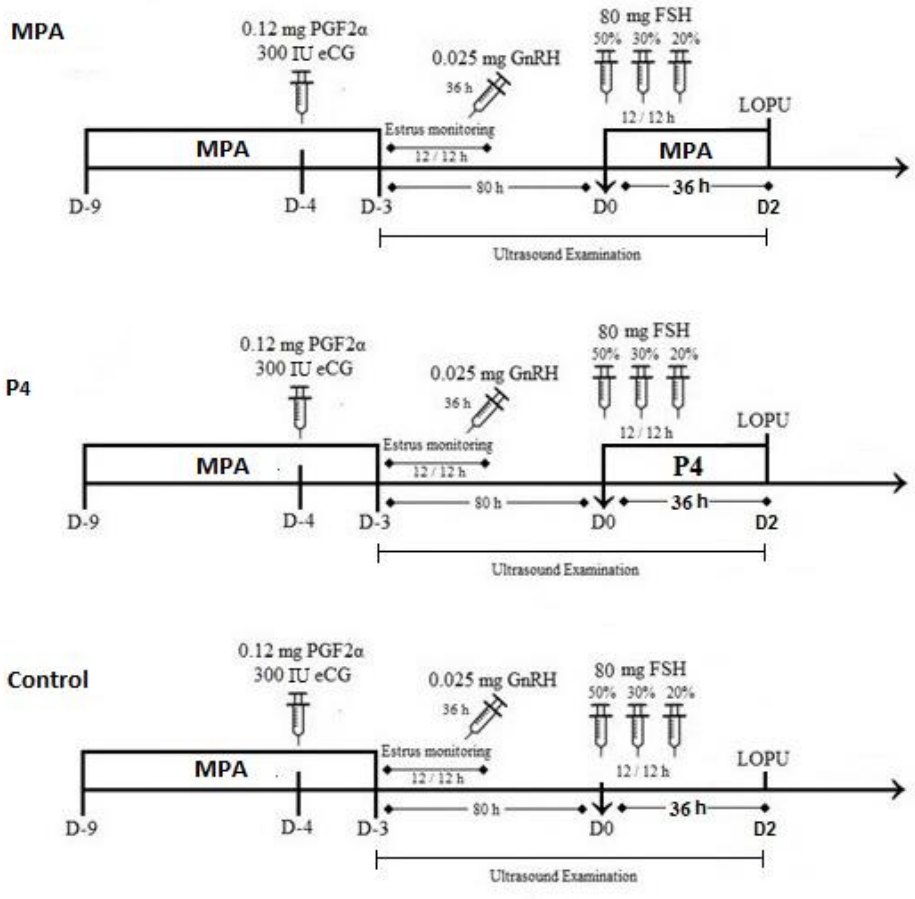

Fig. 1. Scheme of the experimental design. An estrus synchronization treatment was applied to all groups to reach ovulation on Day 0, when ovarian stimulation started, and a new progestin device was inserted that contained either: (1) Medroxyprogesterone acetate – MPA, (2) Natural progesterone – P_4 or (3) no device - control.

2.2. Estrus synchronization and ovarian stimulation

All ewes had their estrus synchronized by the use of intravaginal sponges containing 60 mg of MPA (Progespon®; Schering Plough Animal Health, SP, Brazil) for six days. One day before sponge removal, 300 IU eCG (Novormon 5000®; MSD Animal Health, SP, Brazil) and 0.12 mg cloprostenol sodium (Estron®, Tecnopec, São Paulo, Brazil) were administered intramuscularly (i.m.), as well as 0.025 mg lecirelin (Synthetic GnRH, Gestran Plus®; Tecnopec, SP, Brazil) 36 h after sponge removal [5]. Ovarian stimulation started on D0, at 44 h after GnRH, i.e., 80 h after sponge removal. Ewes received 80 mg of FSH (Folltropin-V, Bioniche Animal Health, Ontario, Canada) via three administrations (40 mg, 24 mg, and 16 mg, respectively) every 12 h.

2.3. Treatments

On D0, ewes in the progestogen-treated groups (n=10 per group) received either intravaginal 60 mg of MPA (MPA; Progespon, Zoetis, São Paulo, Brazil), or a CIDR impregnated with 0.33 mg P_4 (P_4, Eazi-Breed CIDR, Zoetis). Control ewes (n=10) did not receive any device (only luteal P_4 was thus present). The devices were removed immediately after LOPU (Fig. 1).

2.4. Estrus behavior, ovarian assessment, COC recovery and grade

Estrus behavior was assessed using teaser rams and the female acceptance of mounting was considered decisive to confirm estrus, the first and last mounting defined the duration of estrus. B-mode transrectal ultrasonography (SonoScape, Shenzhen, China, 7.5-MHz linear transducer) was performed every 12 h from D-3 to D2. The ewes were restrained in standing position for examination and the procedure was carried out as described by [9]. Ovulation was characterized when the largest follicle visualized on the previous day was no longer observed, and was also

confirmed later by corpora lutea (CL) presence and their vascularization (functionality) by color Doppler mode. The population of follicles at Day 0 and Day 2 were used to compare the ovary status before and after treatments, respectively. Follicle diameters were classified as small (<3 mm), medium (3-5 mm) or large (>5 mm). COCs were recovered by LOPU and graded according to the morphological features as: G1 (multilayers of compacted cumulus and homogeneous ooplasm), G2 (one to three layers and homogeneous ooplasm), G3 (one incomplete layer or denuded but homogeneous ooplasm) and G4 (shapeless, expanded cumulus and/or degenerated). G1 and G2 are considered to have good quality, G3 acceptable and G4 poor quality [4].

2.5. Statistical analyses

Variables related to estrus behavior, ovulatory parameters, population of follicles, recovery rate, total number of COCs and morphological quality were tested for normality using the Lilliefors test. Parametric data were analyzed by ANOVA and Tukey test. Population of follicles were analyzed, independently, for each day (D0 and D2) making comparisons of the follicular categories within each treatment and among treatments. Non-parametric as estrus response data were assessed by chi-square test. The SAEG 9.0 statistical analysis system program was used. Differences were considered significant when $P<0.05$.

3. Results

3.1. Estrus synchronization and follicular wave

Overall, there were no differences among treatments in terms of estrus behavior and ovulatory parameters: estrous response rate [80% (range: 70-90%), 24/30], initial sponge removal to onset of estrus (25.3 ± 2.4 h), initial sponge removal to ovulation (52.0 ± 2.4 h), estrus onset to ovulation (28.5 ± 2.6 h), duration of estrus (35.3 ± 3.0 h). The ovulation rate [97% (29/30)],

ovulations per ewe (1.3 ± 0.1), and the largest follicle diameter (6.7 ± 0.2 mm) also did not differ among treatment groups.

3.2. Populations of follicles and COC quality

Data on population of follicles are shown in Table 1. On Day 0, no difference was observed among treatments in relation to the number of follicles in each category. In all groups, the number of small follicles was greater than that of medium follicles, which was greater than the number of large follicles. On Day 2, there was no effect of exogenous progestin on the number of follicles in any category. Regardless of the progestin source, the number of small and medium follicles was similar, and in turn, larger than the number of large follicles in all treatments. The variables obtained by LOPU procedure (follicles visualized and aspirated, number of COC recovered and recovery rate) and COC quality, as judged by morphological appearance, are shown in Table 2. There was no difference among treatments for such variables.

Table 1. Populations of follicles on Day 0 (beginning of ovarian stimulation) and Day 2 (day of LOPU) after ovarian stimulation and insertion of the progestin-releasing devices in hair ewes (mean ± SEM)

Groups	Day 0			Day 2		
	Small (<3 mm)	Medium (3-5 mm)	Large (>5 mm)	Small (<3 mm)	Medium (3-5 mm)	Large (>5 mm)
MPA	8.6 ± 1.1 ^{A}	2.7 ± 0.8 ^{B}	0.1 ± 0.1 ^{C}	5.8 ± 0.5 ^{A}	5.4 ± 0.7 ^{A}	0.1 ± 0.1 ^{B}
P_4	8.7 ± 0.8 ^{A}	2.0 ± 0.5 ^{B}	0.1 ± 0.1 ^{C}	4.8 ± 1.0 ^{A}	5.2 ± 0.7 ^{A}	0.6 ± 0.3 ^{B}
Control	8.8 ± 0.8 ^{A}	2.8 ± 0.8 ^{B}	0.2 ± 0.1 ^{C}	5.3 ± 0.6 ^{A}	5.3 ± 0.6 ^{A}	0.5 ± 0.3 ^{B}

Within a row, A,B differ ($P < 0.05$) among the follicular category, within the same day and treatment.

MPA: Ewes received sponge containing 60 mg of medroxyprogesterone acetate during stimulation.

P4: Ewes received CIDR containing 0.33 mg of progesterone (P_4) during stimulation

Control: Ewes did not receive any progestin device during the stimulation.

Table 2. Total number of follicles visualized and aspirated, number of COC recovered and recovery rate, quality of COCs using exogenous progestin during ovarian stimulation in hair ewes (mean ± SEM)

Groups	Follicles		COC	Recovery	Good	Acceptable	Poor
	Visualized	Aspirated	Recovered	%	G1 + G2	G3	G4
MPA	10.1 ± 1.3	9.7 ± 1.3	5.9 ± 1.0	61	4.3 ± 0.9	1.4 ± 0.5	0.2 ± 0.1
P_4	10.3 ± 0.8	10.3 ±0.8	8.2 ± 0.7	80	5.3 ± 0.8	2.3 ± 0.8	0.5 ± 0.2
Control	9.9 ± 1.1	9.3 ± 1.1	6.2 ± 1.3	67	3.7 ± 0.7	2.0 ± 0.6	0.5 ± 0.2

(P>0.05)

*COC grading according with the cellular layers of cumulus and cytoplasmic uniformity: G1 (multilayered compacted cumulus and homogeneous ooplasm); G2 (one to three layers and homogeneous ooplasm); G3 (one incomplete layer or denuded but homogeneous ooplasm); G4 (shapeless, expanded cumulus and degenerated) [4].

MPA: Ewes received sponge containing 60 mg of medroxyprogesterone acetate during stimulation.

P4: Ewes received CIDR containing 0.33 mg of progesterone (P_4) during stimulation

Control: Ewes did not receive any progestin device during the stimulation.

4. Discussion

This study investigated for the first time the effect of two exogenous (natural and synthetic progestogen) vs endogenous P_4 (from CL; control) during ovarian stimulation with FSH on ovarian dynamics and oocyte production. The results demonstrated that exogenous progestogen does not affect the first-wave follicle population and oocyte morphological quality in sheep.

Recently, it was reported that exogenous P_4 improved the number of grade 1 oocytes, despite the total number of COCs/ewe and populations of follicles were not different between treated and non-treated ewes [8]. Evidences based in our previous study [4] suggest that this difference between our results and Menchaca et al. [8] is not because different FSH dose or administration regime. Perhaps it is related to the time of the last FSH administration (FSH withdrawal - coasting time). In cows, it was demonstrated that coasting time influences the development competence, but the COC morphological grade was not mentioned [10]. Interestingly, different FSH-metabolic clearance rates were found during superovulation protocol between ewes treated with exogenous P_4 or MPA implant, although the number of ovulations and embryo recovery rate were not affected [11]. These observations open a new perspective for future research in hormonal protocols for oocyte production in sheep.

As expected, no differences in the estrus behavior or ovulatory parameters among experimental groups were observed after estrus synchronization, since the same protocol was used to treat all ewes. These results corroborate those of our previous studies [4,5] demonstrating that the Day 0 protocol effectively synchronizes ovulation and the emergence of the first follicular wave, as previously reported [6]. The time chosen to initiate the stimulatory treatment was deemed appropriate based on the large number of small follicles and absence of large follicles [5].

On Day 0, the numbers of small, medium and large follicles were similar across groups. The number of small follicles was greater than the number of medium follicles, and 85% of the ewes did not have large follicles, typically characteristic of the occurrence of a new wave of follicles post-ovulation. This finding is consistent with that of previous reports [5,6]. On Day 2, the population of follicles was not different among treatments. The number of small follicles decreased, and the number of medium follicles increased within each group compared to D0, an effect that is expected following ovarian stimulation [4].

In conclusion, short-term use of an exogenous progestogen during the ovarian stimulation protocol does not affect the population of first-wave follicles or morphological quality of the COCs, thus rejecting our first hypothesis. In the other hand, it supports the hypothesis that when the occurrence of ovulation is confirmed, additional supply of progestogens by the use of exogenous devices is not required during the FSH treatment. We believe that luteal concentrations of P_4 are sufficient to blockade the surge of the LH and avoid oocyte maturation *in vivo* during the ovarian stimulation protocol.

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Conflict of interest

None of the authors have any conflict of interest to declare.

References

[1] Thammasiri, J., Navanucraw, C., Uriyapongson, S., Khanthusaeng, V., Kamollirt, C., 2016. Expression of cumulus-oocyte complex genes and embryonic development in goats

subjected to progestogen-based estrus synchronization. Theriogenology 86, 612-18.

[2] Mendes, C.S., Barbosa, L. P., Araújo, M.L., Pinheiro, E.E.G., Lents, M.P., Amorim, L.S., Kiya, C.K., Santana, A.L.A., 2017. Follicular response and oocyte production following variations in ovarian stimulation in goats. Theriogenology, *In press*. https://doi.org/10.1016/j.theriogenology.2017.11.016.

[3] Menchaca, A., Vilariño, M., Crispo, M., de Castro, T., Rubianes, E., 2010. New approaches to superovulation and embryo transfer in small ruminants. Reprod. Fertil. Devel. 22, 113–18.

[4] Bragança, G.M., Batista, R.I.T., Souza-Fabjan, J.M.G, Alfradique, V.A.P., Arashiro, E.K.N., Cosentino, I.O., Pinto, P.H.N., Camargo, L.S.A., da Fonseca, J.F., Brandão, F.Z., 2018. FSH dose and strategy of administration for ovarian stimulation affect gene expression in sheep cumulus-oocyte complexes. Reprod. Fertil. Dev. 30, 1234-44. https://doi.org/10.1071/RD17337

[5] Balaro, M.F., Fonseca, J.F., Barbosa, T.G., Souza-Fabjan, J.M.G., Figueira, L.M., Teixeira, T.A., Carvalheira, L.R., Brandão F.Z., 2016. Potential role for GnRH in the synchronization of follicular emergence before the superovulatory Day 0 protocol. Domest. Anim. Endocrinol. 54, 10-4.

[6] Menchaca, A., Vilarino M., Crispo, M., Pinczak, A., Rubianes, E., 2007. Day 0 Protocol: Superstimulatory treatment initiated in the absence of a large follicle improves ovarian response and embryo yield in goats. Theriogenology, 68, 1111–1117.

[7] Cuadro, F., dos Santos-Neto, P.C., A. Pinczak N., Barrera, N., Crispo, M., Menchaca, A., 2018. Serum progesterone concentrations during FSH superstimulation of the first follicular wave affect embryo production in sheep. Anim. Reprod. Sci. 196, 205-210.

[8] Menchaca, A., Cuadro, F., dos Santos-Neto, P.C., Bosolasco, D., Barrera, N., Brun, V.,

Crispo, M., 2018. Oocyte developmental competence is improved by relatively greater circulating progesterone concentrations during preovulatory follicular growth. Anim. Reprod. Sci. 195, 321-328.

[9] Figueira, L.M., Fonseca, J.F., Arashiro, E., Souza-Fabjan, J., Ribeiro, A., Oba, E., Viana, J., Brandão, F.Z., 2015. Colour Doppler Ultrasonography as a Tool to Assess Luteal Function in Santa Inês Ewes. Reprod. Domest. Anim. 50(4), 643-50.

[10] Nivet, A.L., Bunel, A., Labrecque, R., Belanger, J., Vigneault, C., Blondin, P., and Sirard, M. A., 2012. FSH withdrawal improves developmental competence of oocytes in the bovine model. Reproduction **143.**

[11] Bartlewski, P. M., Seaton, P., Szpila, P., Oliveira, M. E. F., Murawski, M., Schwarz, T., Kridli, R. T., Zieba, D. A., 2015. Comparison of the effects of pretreatment with Veramix sponge (medroxyprogesterone acetate) or CIDR (natural progesterone) in combination with an injection of estradiol-17β on ovarian activity, endocrine profiles, and embryo yields in cyclic ewes superovulated in the multiple-dose Folltropin-V (porcine FSH) regimen. Theriogenology 84, 1225-37

Capítulo 4

Exogenous progestogens: impacts on molecular status of cumulus-oocyte complexes and its implication in the oocyte quality in sheep

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Exogenous progestogens: impact on molecular status of immature cumulus-oocyte complexes in sheep

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Abstract. This study evaluated the role of progesterone (P_4) and medroxyprogesterone acetate (MAP) on molecular status of immature cumulus-oocyte complexes (COCs) and its implication on oocyte quality in sheep. The number of viable COC per ewe and the rate of COC screened for developmental competence by brilliant cresyl blue (BCB^+) were similar ($P > 0.05$) among treatments, respectively (P_4: 7.7 ± 0.7, 58%; MAP: 5.7 ± 1.0, 61% and control: 5.7 ± 1.1, 65%). The COCs gene expression was altered by exogenous progestogens compared to the control group: markers of steroidogenic pathway (*FSHr*, FSH receptor; *LHr*, LH receptor; *Erα*, estradiol receptor α) and of quality (*ZAR1*, zygote arrest 1; *GDF9* and *Bcl-2*, B-cell lymphoma 2) were in abundance in P_4 ($P < 0.05$). Indeed, *RELN* (reelin protein) was downregulated and *Bcl-2* was upregulated in MAP ($P < 0.05$). In the P_4 *vs.* MAP comparison, *FSHr*, *LHr* and *RELN* genes were upregulated ($P < 0.05$) in the P_4 group. In conclusion, P_4 and MAP promoted dissimilar effects on trascriptome profiling of immature BCB^+ COCs, possibly due to the differences in progestogens chemical structure and serum progesterone concentrations; exogenous P_4 impacted positively on the profile of genes related to oocyte quality.

Additional keywords: progestin, oocyte competence, gene expression

Introduction

Hormonal manipulation of ovarian function using the association of gonadotropins and progestogens are widely applied in assisted reproductive technologies to improve oocyte recovery in live female of monovular species (Berlinger *et al.* 2007; Kuang *et al*. 2015; Thammisiri *et al*. 2016). Stimulatory protocols with follicle stimulating hormone (FSH) increase follicular population (Mendes *et al*. 2017; Bragança *et al.* 2019) and alter global gene expression in oocyte and cumulus cells (Dias *et al.* 2013; Nivet *et al.* 2013; Bragança *et al.* 2018). FSH enhances the profile of genes associated with oocyte developmental competence

depending of the dose and administration regimen (Bragança *et al*. 2018), or coasting time (Nivet *et al.* 2012). Progestogen support during gonadotropin treatments is used to inhibit LH pulse frequency, allowing regression of dominant follicle(s) by atresia, and thus, avoiding ovulation (Greyling and van der Nest 2000; Kuang *et al.* 2015). Progestogens are natural or synthetic steroids that exert progesterone-like activity. Progesterone (P_4) is the only natural progestogen and it is produced primarily by corpus luteum after ovulation. Progesterone analogues, as medroxyprogesterone acetate (MAP), simulate the therapeutic effects of P_4, despite some differences in chemical structure. These differences result in different actions at the cellular level (for review, see Lieberman and Curtis 2017; L'Hermite 2017). Progesterone has important role in mammalian oocyte maturation, but its impact on oocyte quality is not yet well clarified (for review, see Lonergan 2011). In addition, no information is available about the effect of P_4 *vs.* MPA during antral follicular growth on gene expression of immature COCs.

Recent evidence using intravaginal P_4 administration during FSH stimulation of the first wave of the estrous cycle, showed that embryo yield is improved as *in vivo* (Cuadro *et al.* 2018) as *in vitro* (Menchaca *et al.* 2018) sheep embryo production systems. The authors demonstrated that this improvement was associated to a positive effect of P4 on oocyte developmental competence (Menchaca *et al.* 2018). Bartlewski *et al.* (2015) evaluated the use of P_4 *vs* MAP during a conventional superovulation protocol and no difference was observed in terms of number of ovulation and embryo recovered. Conversely, some studies reported deleterious effect of exogenous progestogens on *in vitro* embryo production (Berlinger *et al.* 2007), mainly in long-term based treatments (Thammisiri *et al.* 2016).

There is a gap in the literature about the impact of exogenous progestogen in the molecular level and its consequence to oocyte competence. The current study evaluates how P_4 and MAP modulate the expression of genes related to acquisition of the oocyte competence, such as growth factors (i.e. *BMP15*, bone morphogenetic protein 15; *GDF9*, growth

differentiation factor 9), maternal effect genes (i.e. *MATER*, maternal antigen that embryo requires; *ZAR1*, zygote arrest 1), steroidogenic pathway (i.e. *FSHr*, FSH receptor; *LHr*, luteinizing hormone receptor; *STAR*, acute regulatory protein; *ERα*, estradiol receptor alpha), apoptosis regulatory proteins (i.e. *Bcl-2*, B-cell lymphoma 2; *BAX*, Bcl-2 associated X protein) (for review, see Hillier *et al.* 1994; Zöorning *et al.* 2001; Bebbere *et al.* 2008), and extracellular matrix and ligand (i.e. *RELN*, reeling protein; *LRP8*, low-density lipoprotein receptor-related protein 8) recently related with regulation of final follicle growth (Fayad *et al.* 2007; Nivet *et al.* 2012; Yang *et al.* 2018).

The oocyte quality is related to their developmental competence, sustaining profound changes in structural, molecular and biochemical levels, including maternal zygotic transition. This capability is acquired during the follicular growth, mainly at final phase. However, an oocyte fully grown does not ensure that is capable of undergoing all the steps of embryonic development to term (for a review, see Sirard *et al.* 2006; and Sirard 2011). Nevertheless, studies demonstrated that oocytes which have already finished the exponential growth phase, screened by brilliant cresyl blue (BCB), are more competent and present greater blastocyst rate (Torner *et al.* 2008; Mohammadi-Sangcheshmeh *et al.* 2014). Thus, we tested the hypothesis that P_4 and MAP promote dissimilar effects on molecular status of COCs impacting the oocyte quality. The aim of present study was to evaluate the effect of P_4 and MAP on molecular status of immature cumulus-oocyte complex (COCs) and its implication for oocyte quality in sheep.

Materials and Methods

Experimental design

Thirty multiparous ewes were allocated into three experimental groups (n=10 ewes per group) to receive two different intravaginal devices containing P4 or MAP, while other group remained

as control (no device). The females were treated during FSH ovarian stimulation performed at the first follicular phase, soon after ovulation (Day 0), that was induced by an estrous synchronization treatment (Figure 1). Follicular aspiration was performed on Day 2 by laparoscopic ovum pick up (LOPU) and recovered COCs were morphologically graded and tested to BCB. BCB^{+} COCs were used for gene expression analysis. Blood samples were collected from the jugular vein using vacutainer tubes to determine serum P_4 concentrations from Day -1 to Day 2.

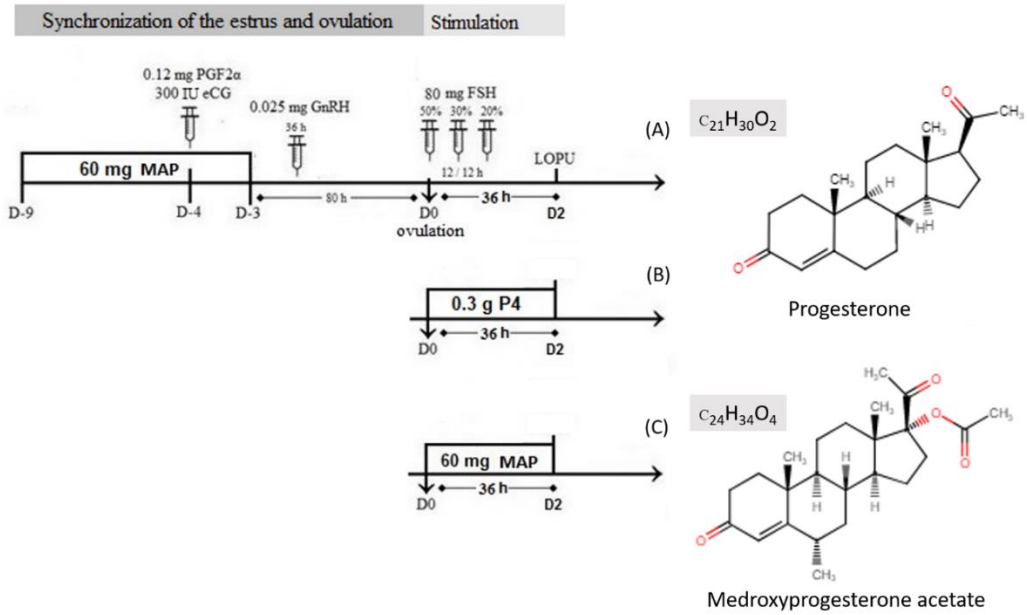

Fig. 1. Representative scheme of the experimental design. At 80 h after sponge removal of the estrous synchronization treatment (Day 0), ovarian stimulation was performed with 80 mg of FSH in three decreasing doses every 12 h. At first FSH dose the females were allocated to one of three experimental groups: (A) No progestogen support was applied in the control group (exposed to endogenous progesterone); (B) progesterone (P_4) was administrated by intravaginal devices (CIDR-G); and (C) medroxyprogesterone acetate (MAP) was administrated by intravaginal devices (sponges). (A) and (B) were exposed to endogenous and endogenous plus exogenous P_4 (bio-identical, same chemical structure), respectively, while (C) was exposed to MAP synthetic progestogen analogue (with different chemical structure). Source for molecule structure (Marvin 2018).

Local conditions and animal care

The study was performed at *Unidade de Pesquisa Experimental em Caprinos e Ovinos (UNIPECO) da Universidade Federal Fluminense*, in Cachoeiras de Macacu, Rio de Janeiro, Brazil (22° S, month october). All procedures were approved by local Ethical Committee for Animal Use of the Universidade Federal Fluminense (protocol #721/2015) and were conducted under the ethical principles of the Brazilian Society of Animal Experimentation. Thirty multiparous Santa Inês ewes were used, with 51 ± 5.9 kg (Mean ± SD) of body weigth and 3.2 ± 0.6 (0 to 5 scale) body condition score, clinically healthy, feeding on chopped elephant grass (*Pennisetum purpureum*) and 200 g/animal of concentrate (12% crude protein) twice daily, and receiving water and mineralized salt *ad libitum*.

Estrous synchronization

All ewes received an estrous synchronization treatment using a short-term protocol described by Balaro *et al*. (2016). Briefly, intravaginal sponges containing 60 mg MAP (Progespon®; Schering Plough Animal Health, SP, Brazil) were applied for six days. One day before sponge removal, 300 IU eCG (Novormon 5000®; MSD Animal Health, SP, Brazil) and 0.12 mg cloprostenol sodium (Estron®, Tecnopec, São Paulo, Brazil) were administered intramuscularly (i.m.), as well as 0.025 mg lecirelin (Gestran Plus®; Tecnopec, SP, Brazil) was given 36 h after sponge removal.

Progestogen sources during ovarian stimulation

From 80 h after sponge withdrawal (defined as Day 0, *i.e.*, soon after ovulation) 80 mg of FSH (Folltropin-V®, Vetoquinol, Paris, France) were administered in three injections (50, 30 and 20%) every 12 h for stimulation of the first follicular wave of the estrous cycle. At the first FSH

dose, ewes received either an intravaginal device containing 0.33 mg of progesterone (CIDR, Eazi-Breed, Zoetis) (P_4 group) or an intravaginal sponge containing 60 mg MAP, while the control group did not receive any progestogen device and remained untreated during the early luteal phase (*i.e.*, endogenous P_4).

Serum P_4 determinations

Blood samles were collected and centrifuged (2600 x *g*) and serum was stored at -20 °C until progesterone determinations, which was performed by a solid phase radioimmunoassay by using a commercial kit (ImmuChem, MP Biomedicals, Santa Ana, CA, USA). The sensitivity and intra-assay coefficient were 0.05 ng/mL and 11%, respectively. All data were within the maximum and minimum point of the curve.

COCs selection and BCB test

COCs were recovered by laparoscopic ovum pick up (LOPU) as previously described (Bragança *et al*. 2018). COCs were isolated under stereomicroscope and graded based on visual assessment of morphology (number of cellular layers and cytoplasmic uniformity) considering the quality as Grade I⁄II (good), III (acceptable) and IV (poor) (Souza-Fabjan *et al*. 2013). For inference of the developmental competence, viable COCs (GI, GII and GIII) from each treatment were washed once and exposed to 26 μM BCB (B5388, Sigma) diluted in DMPBS supplemented with 10% BSA (A9647, Sigma) and 0.2 mM pyruvate (P4562, Sigma), for 60 min on a hot plate at 37 ºC and ambient atmosphere. Then, COCs were washed two times, placed in polyvinyl alcohol (PVA, P8136, Sigma) for 5 min and classified according to oocyte cytoplasm staining as: BCB^+ (blue cytoplasm) and BCB^- (colorless cytoplasm). Pools of 5 BCB^+ COC from the same treatment were recovered (with minimal amount of medium a**s** possible) at once

with a pipette calibrated at 2 μL, transferred to DNAse/RNAse free cryotubes, subjected to snap frozen and stored in liquid nitrogen canister for later gene expression analysis.

Gene expression analysis

The abundance of mRNA encoding proteins that plays a role in oocyte quality, such as growth factors (*i.e.*, *BMP15* and *GDF9*), maternal effect genes (*i.e.*, *MATER* and *ZAR1*), steroidogenic pathway (*i.e.*, *FSHr, LHr, STAR* and *ERα*), extracellular matrix and ligand (*RELN* and *LRP8*) and apoptosis (*i.e.*, *BAX* and *Bcl-2*) was evaluated in immature BCB^+ COCs from P_4 and MAP treated ewes. A control group (non-progestogen treated ewes) was used as calibrator to comparative parameters of physiological molecular status. Samples were analyzed by reverse transcription quantitative real time polymerase chain reaction (RT-qPCR), according to Batista *et al*. (2014). Total RNA extraction was performed from three pools of 5 COCs BCB+ per group using RNAeasy Micro kit (Qiagen, Hilden, Germany) according to manufacturer's instructions, as well as reverse transcription using the Superscript III kit (Invitrogen, Carlsbad, CA, USA). Total RNA quantification from each pool was performed using 1 μL of sample and a ND-100 spectrophotometer (NanoDrop Products, Wilmington, DE, USA). Relative quantification by RT-qPCR was performed in duplicates using the commercial kit SYBR Green (Power Sybr Green, Applied Biosystem), 400 ng of cDNA, nuclease free water and specific primers (Table 1) for every reaction. Template cDNA was denatured at 95 °C for 15 min, followed by 45 cycles of 94 °C for 15 s; gene-specific primer annealing temperature for 30 s and elongation at 60 °C for 30 s. After each PCR run, a melting curve analysis was performed to confirm that a single specific product was generated. No template controls (NTC), comprised of the PCR reaction mix without DNA template were also run with each primer to confirm the absence of contaminations.

Table 1. Sequences of the specific primers used in the analysis of gene expression in imature brilliant cresyl blue positive cumulus-oocyte complexes (BCB^+) recovered by laparoscopy after different ovarian stimulation treatments in Santa Inês ewes

Gene	Sequence of primers (5'- 3')	Annealing temperature ($^{\circ}C$)	Size (bp)	Reference
GAPDH	GGGAAA TCG TGC GTG ACA TTA AG TGTGTT GGCGTAAGGTCTTTG	60	273	Bebbere *et al*. 2008
ZAR1	CAC TGC AAG GAC TGC AAT ATC CAG GTG ATA TCC TCC ACT C	60	137	Bebbere *et al*. 2008
MATER	CAG CCT CCA GGA GTT CTT TG GAC AGC CTA GGA GGG TTT CC	59	212	Bebbere *et al.* 2008
GDF9	CAG ACG CCA CCT CTA CAA CA CAG GAA AGG GAA AAG AAA TGG	58	198	Bebbere *et al.* 2008
BMP15	GGG TTC TAC GAC TCC GCT TC GGT TAC TTT CAG GCC CAT CAT	59	273	Bebbere *et al.* 2008
BAX	CCT GGG ATC TTG AAA CTC TCC TT CTG AGC CAG GCT GAA ATC AAA A	60	566	Chakravarthi *et al.* 2017
Bcl-2	GCC GAG TGA GCA GGA AGA C GTT AGC CAG TGC TTG CTG AGA	60	214	Chakravarthi *et al.* 2017
ERα	GAA TCT GCC AAG GAG ACT CG CCT GAC AGC TCT TCC TCC TG	60	187	Hogg *et al.* 2011
LHr	TCC GAA AGC TTC CAG ATG TT GAA ATC AGC GTT GTC CCA TT	60	199	Hogg *et al.* 2011
FSHr	TAA GCA CTT GCC AGC TGT TC CTC ATC GAG TTG GGT TCC AT	60	196	Hogg *et al.* 2011
STAR	GCA TCC TCA AAG ACC AGG AG CTT GAC ACT GGG GTT CCA CT	60	194	Hogg *et al.* 2011
RELN	CAGCCAAAGGACTTCACCCA CGAGTGCTTACTAGCACAACC	60	161	NM_001306121.1
LRP8	ACACCTGTGGCTGTTGTTCA CCAGAGGCAGGAAAGACCTG	60	161	NM_001097565.1

Statistical analysis

Variables with normal distribution were analyzed by analysis of variance (ANOVA), and the significance of differences between mean values was determined using Tukey's test. Data

presented as a frequency or percentage (i.e. proportion of BCB^+ COCs) were analyzed by chi-square test. For gene expression analysis, primer efficiency was tested using LinRegPCR software (Qiagen; Ramakers *et al.* 2003) for each reaction. Relative quantification was performed using REST software (Qiagen; Pfaffl *et al.* 2002) based on comparative Ct quantification. The values are given as differences relative to the calibrator. Statistical analyses were performed using a statistical analysis system program (SAEG 9.0; Universidade Federal de Viçosa). Differences were considered significant when $P < 0.05$. Data are given as the mean ± S.E.M.

Results

COC recovery and gene expression in immature BCB^+COCs

There was no effect of progestogen source on total number of viable or BCB+ COCs, as shown in Table 2. Gene expression was altered by exogenous progestogens compared to the control (Figure 2ab): Steroidogenic pathway receptors (*FSHr, LHr, Erα*), as well as markers of oocyte quality (*ZAR1*, *GDF9* and *Bcl-2*) genes were in abundance in P_4 ($P < 0.05$). Conversely, *RELN* and *Bcl-2* genes were downregulated in MAP ($P < 0.05$). In the comparison between the different devices, *FSHr*, *LHr* and *RELN* genes were upregulated ($P < 0.05$) in P_4 compared to MAP group.

Table 2. Effect of different progestogen treatments during stimulation of first follicular wave with FSH on the quality (morphology and BCB test) of immature cumulus oocyte complexes (COCs) recovered by laparoscopy in Santa Inês ewes (mean ± SE).

Groups	COCs selection		
	Viable COCs*/ewe	% BCB^{+***}	% BCB^{-***}
P4**	7.7 ± 0.7	58	42
MPA**	5.7 ± 1.0	61	39
Control**	5.7 ± 1.1	65	35

n = 10 ewes per treatment

*Viable COCs: GI + GII + GIII COCs;

**P4: Ewes received CIDR containing 0.33 mg of progesterone (P4) during stimulation of the first follicular wave;

**MPA: Ewes received sponge containing 60 mg of medroxyprogesterone acetate during stimulation of the first follicular wave;

**Control: Ewes did not receive any progestogen device during the stimulation of first follicular wave, and thus were under endogenous P4 produced in the early luteal phase.

***BCB^{+}: Competent fully grown COCs.

***BCB^{-}: Noncompetent growing COCs.

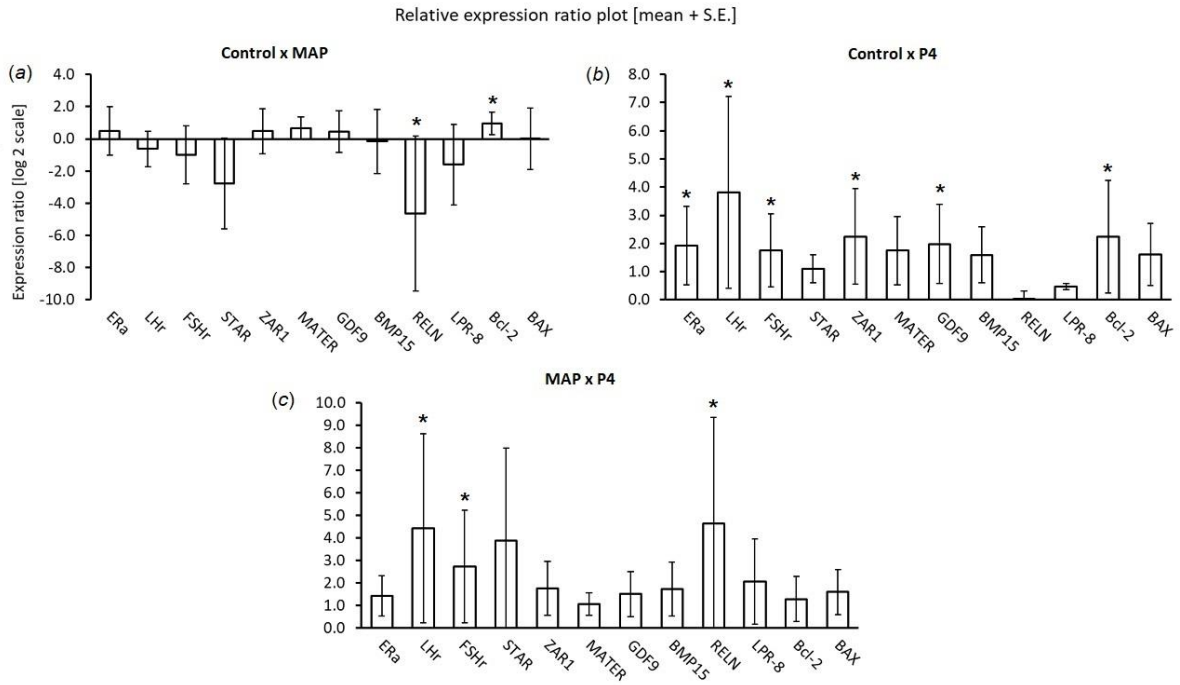

Fig. 2. Relative abundance of mRNA in immature BCB^{+}COCs obtained from (A) MAP or (B) P_4 treatment compared to the control (calibrator). (C) Comparison between the two progestogen tested, MAP (calibrator) vs P_4.

Serum progesterone concentrations

All ewes from the three experimental groups had non detectable serum progesterone concentrations one day prior (Day -1) and on the day that treatments began (on Day 0, at first FSH admnistration). Progesterone concentrations were affected by day and type of device ($P < 0.05$; Fig. 3), which was greater on Day 1 and Day 2 in P_4 group ($P < 0.05$), compared to MAP and control groups. The mean concentrations on Day 2 were 1.0 ± 1.0, 0.6 ± 0.3 and 3.7 ± 0.7 ng/mL ($P < 0.05$) in the control, MAP and P_4 group, respectively.

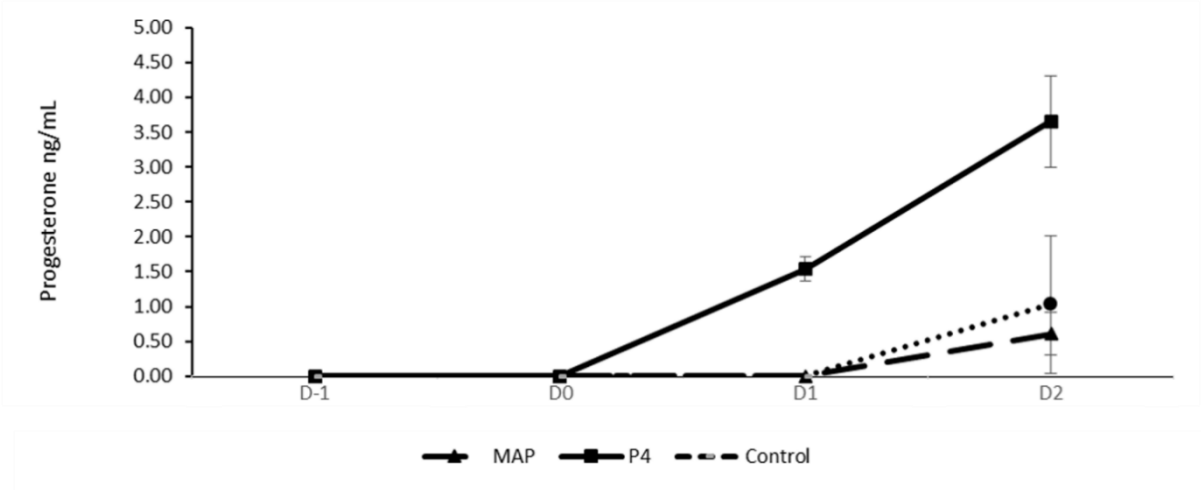

Fig. 3. Serum progesterone (P4) concenrations in Santa Inês ewes treated with progesterone (P4 group), medroxyprogesterone acetate (MAP group), and non-treated ews (control group) during the early luteal phase. Day 0 was defined at 80 h after sponge removal in an estrous synchronization treatment.

Discussion

This study provides new information about the impact of exogenous progestogens on the molecular status of immature cumulus-oocyte complexes (COCs) in the sheep model. The results demonstrate that P_4 and MAP cause dissimilar effects on gene expression in fully grown COCs (BCB+). This effect probably occurred due to the variations in chemical structure of these progestogens, resulting in different activity and inducing different transcriptome profiling.

Regarding our criterium for COC selection, there was no difference in number of COCs screened for their development competence by BCB test among the P_4, MAP and control groups. In addition, our recent data (Bragança *et al*. 2019, accepted for publication) showed no differences in follicular population and COCs morphological features (GI, GII, GIII and GIV) among the three treatments. The higher developmental competence of the BCB^+ COC

compared to the BCB^- COC may be related to the favorable conditions of the follicular development during the final phases of folliculogenesis, and has already been demonstrated in sheep (Mohammadi-Sangcheshmeh *et al.* 2012; 2014) and cows (Alm *et al.* 2005, Torner *et al.* 2008). Therefore, only BCB^+ COCs were used in the present study, resulting in most homogeneous samples which enable to attribute the differences in gene expression induced by treatments. Although no effect on ovarian dynamics (Bragança *et al*. 2019), P_4 and MAP promoted different effects on molecular status of fully grown COCs, confirming our hypothesis.

As expected, P_4 treatment promoted greater progesterone serum concentration in comparison with MAP and control. Low serum progesterone in MAP and control was a consequence of recent formation of the corpus luteum. This data is in agreement with Cuadro *et al.* (2018) that has reported higher progesterone serum concentration in ewes that received a P_4 implant instead ewes in early luteal phase (control) during the FSH stimulation of the first follicular wave. Similarly, Bartlewski *et al.* (2015) observed greater progesterone concentration in exogenous P4-treated than in MAP-treated ewes during superovulation protocol (Bartlewski *et al.* 2015). It is well known that MAP serum concentration is not detected by the RIA-kit used for progesterone measure. For MAP detection, it is necessary to use specific antiserum (Ortiz *et al.* 1977; Shrimanker *et al.* 1978). We speculate that this possibly occurs because of the divergence in the chemical structure between MAP and P_4. The MAP molecule presents a methyl (CH_3^-) radical at C6 position and a methyl acetate ($C_2H_3O_2^-$) radical at C17 position (to see Fig. 1), while in P_4 molecule this radical is absent. The exogenous P_4 is bio-identical to the endogenous (luteal) hormone (L'Hermite 2017).

The abundance of mRNA encoding proteins expressed in the oocyte (i.e. *MATER, ZAR1, BMP15* and *GDF9*), cumulus (i.e. *RELN, LRP8, FSHr, LHr, ERα* and *STAR*) or both (i.e. *BAX* and *Bcl-2*) were evaluated in BCB^+ COC. In P_4 group, six genes were upregulated (*ZAR1, GDF9, Bcl2, FSHr, LHr* and *ERα*) compared to the control (calibrator) and in MAP two

genes were affected (*RELN* was downregulated and *Bcl2* upregulated) compared to the control (calibrator). However, comparing P_4 *vs.* MAP (calibrator), three genes were upregulated *(RELN*, *FSHr* and *LHr*) in P_4 group.

The difference in *RELN* abundance (downregulated in MAP compared to control and upregulated in P_4 compared to MAP) was not related to serum progesterone concentration, since *RELN* expression was not different between P_4 and control. The contrast in *RELN* mRNA expression perhaps is due the different chemical structure of the progestogens. We presume that P_4 and MAP pharmacokinetics activate distinct pathways, despite the similar therapeutics effect (pharmacodynamics). *RELN* expression is maximal in theca cells of dominant follicles and binding *LRP8* in granulosa cells by paracrine action, activating the downstream signaling pathways (Fayad *et al*. 2007) which regulate the final follicle growth stage (Nivet *et al*. 2013). Yang *et al*. (2018) reported *RELN* involvement with protein kinases activity and suggested a role in progestogenic pathway. Conversely, Fayad *et al.* (2007) has demonstrated that *RELN* and its ligand LRP8 are predominantly expressed in the dominant follicles and similarly downregulated during ovulatory process and luteinization. Thus, we considered the *RELN* abundance as an important marker of oocyte competence acquisition.

We associate the gene expression profile observed in P_4 group to the rise of serum progesterone concentrations. Recently, Menchaca *et al.* (2018) demonstrated that high serum progesterone induced by P_4 intravaginal device enhanced oocyte developmental competence by increase *in vitro* cleavage rate and embryo development in sheep (compared to the non-treated ewes). Cuadro *et al.* (2018) has reported similar effects in *in vivo* embryo production system in sheep that received or not exogenous progesterone during the growing phase of the follicular wave. The authors found an improvement in the percentage of fertilized oocyes after insemination, greater number of transferable embryos per donor, and better quality of the collected embryos. Otherwise, when P_4 priming was compared with MAP treatment, Bartlewski

et al. (2015) reported no differences in terms of number of corpora lutea and embryo recovered in superovulated ewes.

In support of the upregulation of *ZAR1* in P_4-treated in relation to control group, we considered as a good indicative of quality improvement and possibily it corroborates with the findings of Menchaca *et al.* (2018) and Cuadro *et al*. (2018) already mentioned. *ZAR1* is well known as a good predictor of developmental competence mainly in immature oocytes. *ZAR1* mRNA plays a role in early embryo development until the zygotic genome activation (Uzbekova *et al.* 2006; Bebere *et al*. 2008).

FSH levels directly influence *FSHr, LHr* and *ERα* (Wei *et al.* 2017; Bragança *et al*. 2018) by downstream activation of *FSHr*/adenylciclase/cAMP/PKA cascade to trigger estradiol (E_2) production (Hillier *et al.* 1994; Silva *et al*. 2006; Ulloa-Aguirre *et al.* 2018). An interaction between progesterone and FSH was already evidenced (Baby *et al.* 2011; Menchaca *et al.* 2018), and we suggest that this interaction could be in function of the P_4 molecule structure, maybe the methyl acetate radical on MAP molecule prevent this interaction. Bartlewski *et al.* (2015) observed a longer FSH-metabolic clearance rates in P_4 than in the MPA-treated ewes. Based on this finding, and those reported by Knecht *et al.* (1984) and Wei *et al.* (2017), we speculate that the longer FSH bioavailability in P_4-treated ewes possibly maintained the stimulus of downstream pathways FSH-induced, enabling the expression of these receptors, including LHr through raising of cAMP, despite progesterone inhibition of LH release pulsatile frequency by feedback mechanism. The upregulation of these receptors (*FSHr, LHr* and *ERα*) may promote better condition to oocyte answer to the *in vitro* maturation condition regarding the role of hormonal regulation during the maturation process.

The greater serum progesterone also affected *GDF9* expression, probably by extended FSH bioavailability in the P_4 group. Exogenous FSH affects *GDF9*, as we previously demonstrated (Bragança *et al.* 2018). *GDF9* stimulates granulosa cells proliferation and acts in

the regulatory process during final follicle growth prior to ovulation; before LH surge, cumulus cells require *GDF9* to support the metabolic cascades (for review, see Shimasaki *et al.* 2004; Otsuka *et al.* 2011). Hence, P_4-treated group seems to promote more appropriate COCs gene modulation.

Apoptosis proteins regulate cell death and are good markers of cell viability. The anti-apoptotic *Bcl2* gene was upregulated in both P_4 and MAP group at present study. Similar results were obtained by Thammisiri *et al.* (2016) in a short-term MAP-treated protocol. These evidences indicate the positive effect of exogenous progestogens applied during ovarian stimulation on apoptosis modulation cascade.

Nilsen and Brinton (2003) demonstrated the divergent impact of P_4 and MAP on mitogen-activated protein kinase (MAPK) pathway for mechanism of neuroprotection in neuron culture, which reinforces our hypothesis about both progestogens activating different downstream pathways, despite the similar therapeutic effects. Exogenous support of P_4 seems to exert a beneficial effect to oocyte quality based in the profile of gene expression (compared to the MAP and control). In the other hand, MAP had a negative effect (compared to the control), mainly because the downregulation of *RELN* in this group, since this extracellular matrix glycoprotein is involved in 47 distinct cell functions, including MAPK pathway (Yang *et al*. 2018), which is necessary for germinal vesicle breaking down (GVBD) and oocyte maturation (for review, see Silvestre *et al.* 2011). However, to confirm this deleterious effect of MAP future research is necessary to evaluate the *in vitro* development, molecular features of the embryos, polyspermy regulation, cryopreservation and/or implantation.

In conclusion, P_4 and MAP promote dissimilar effects on molecular status of immature COC fully grown (BCB^+) in sheep. Exogenous P_4 impacts positively on the gene expression, probably due to their chemical structure and greater serum progesterone concentration that induce this difference on its biological activity.

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Conflict of interest

All authors declare that they do not have any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations.

References

Alm, H., Torner, H., Lohrke, B., Viergutz, T., Ghoneim, I. M. and Kanitz, W. (2005). Bovine blastocyst development rate in vitro is influenced by selection of oocytes by brilliant cresyl blue staining before IVM as indicator for glucose-6-phosphate dehydrogenase activity. *Theriogenology* **63**, 2194–2205. doi:10.1016/j.theriogenology.2004.09.050.

Baby, T. E. and Bartlewski, P. M. (2011). Progesterone as the driving regulatory force behind serum FSH concentrations and antral follicular development in cycling ewes. *Reprod. Fertil. Develop.* **23**, 303–310. doi: 10.1071/RD10121

Balaro, M. F. A., Fonseca, J. F., Barbosa, T. G. B., Souza-Fabjan, J. M. G., Figueira, L. M., Teixeira, T. A., Carvalheira, L. R. and Brandão, F. Z. (2016). Potential role for GnRH in the synchronization of follicular emergence before the superovulatory Day 0 protocol. *Domest.*

Anim. Endocrinol. **54**, 10-4. doi: 10.1016/j.domaniend.2015.07.002

Batista, R. I., Raposo, N. R., Campos-Junior, P. H., Pereira, M. M., Camargo, L. S., Carvalho, B. C., Gama, M. A. and Viana, J. H. (2014). Trans-10, cis-12 conjugated linoleic acid reduces neutral lipid content and may affect cryotolerance of in vitro-produced crossbred bovine embryos. *J. Anim. Sci. Biotechnol.* **5**, 33. doi: 10.1186/2049-1891-5-33

Bartlewski, P. M., Seaton, P., Szpila, P., Oliveira, M. E. F., Murawski, M., Schwarz, T., Kridli, R. T. and Zieba, D. A. (2015). Comparison of the effects of pretreatment with Veramix sponge (medroxyprogesterone acetate) or CIDR (natural progesterone) in combination with an injection of estradiol-17β on ovarian activity, endocrine profiles, and embryo yields in cyclic ewes superovulated in the multiple-dose Folltropin-V (porcine FSH) regimen. *Theriogenology* **84**, 1225-1237. doi: 10.1016/j.theriogenology.2015.07.002

Bebbere, D., Bogliolo, L., Ariu, F., Fois, S., Leoni, G. G., Tore, S., Succu, S., Berlinguer, F., Naitana, S. and Ledda, S. (2008). Expression pattern of zygote arrest 1 (ZAR1), maternal antigen that embryo requires (MATER), growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) genes in ovine oocytes and in vitro-produced preimplantation embryos. *Reprod. Fertil. Dev.* **20**, 908-915. doi: 10.1071/RD08095.

Berlinguer, F., Gonzalez-Bulnes, A., Succu, S., Leoni, G., Mossa, F., Bebbere, D., Ariznavarreta, C., Tresguerres, J. A. F., Veiga-Lopes, A. and Naitana, S. (2007). Effects of progestagens on follicular growth and oocyte developmental competence in FSH-treated ewes. *Domest. Anim. Endocrinol.* **32**, 303-14. doi:10.1016/j.domaniend.2006.04.007.

Bragança, G. M., Batista, R. I. T., Souza-Fabjan, J. M. G, Alfradique, V. A. P., Arashiro, E. K. N., Cosentino, I. O., Pinto, P. H. N., Camargo, L. S. A., da Fonseca, J. F. and Brandão, F. Z. (2018). FSH dose and strategy of administration for ovarian stimulation affect gene expression in sheep cumulus-oocyte complexes. *Reprod. Fertil. Dev.* **30**, 1234-1244. doi:

doi.org/10.1071/RD17337

Bragança, M. G., Souza-Fabja, J. M. G., Ribeiro, L. S., Brair, V. L., Côrtes, L. R., Souza, C. V., Batista, R. I. T. P., Fonseca, J. F., Menchaca, A. and Brandão, F. Z. (2019). Exogenous progestogen does not affect first-wave follicle populations and oocyte quality during ovarian stimulation with FSH in sheep. *Domest. Anim. Endocrinol. Acepted for publication.*

Cuadro, F., Santos-Neto, P. C., Pinczaka, A., Barrera, N., Crispo, M. and Menchaca, A. (2018). Serum progesterone concentrations during FSH superstimulation of the first follicular wave affect embryo production in sheep. *Anim. Reprod. Sci.* **196**, 205-210. doi: 10.1016/j.anireprosci.2018.08.011

Dias, F. C. F., Khan, M. I. R., Sirard, M. A., Adams, G. P. and Singh, J. (2013). Differential gene expression of granulosa cells after ovarian superstimulation in beef cattle. *Reproduction* **146**, 181-191. doi: 10.1530/REP-13-0114

Fayad, T., Lefebvre, R., Nimpf, J., Silversides, D. W. and Lussier, J. G. (2007). Low-density lipoprotein receptor-related protein 8 (LRP8) is upregulated in granulosa cells of bovine dominant follicle: molecular characterization and spatio-temporal expression studies. *Biol. Reprod.* **76**, 466–475. doi:10.1095/biolreprod.106.057216

Greyling, J. P. C. and van der Nest, M. (2000). Synchronization of oestrus in goats: dose effect of progestagen. *Small Rumin. Res.* **36**, 201-207.

Hillier, S. G., Whitelaw, P. F. and Smyth, C. D. (1994). Follicular oestrogen synthesis: the 'two-cell, two-gonadotrophin' model revisited. *Mol. Cell. Endocrinol.* **100**, 51-54.

Knecht, M., Darbon, J.M., Ranta, T., Baukal, A.J. and Catt, K.J. (1984). Estrogens enhance the adenosine 3′,5′-monophosphate-mediated induction of follicle-stimulating hormone and luteinizing hormone receptors in rat granulosa cells. *Endocrinology* **115**, 41-49. doi: 10.1210/endo-115-1-41

Kuang Y., Chen Q., Fu Y., Wang Y., Hong Q., Lyu, Q., Ai Ai and Shoham Z. (2015). Medroxyprogesterone acetate is an effective oral alternative for preventing premature luteinizing hormone surges in women undergoing controlled ovarian hyperstimulation for in vitro fertilization. *Fertil. Steril.* **104**, 62-70. doi: 10.1016/j.fertnstert.2015.0

L'Hermite, M. (2017). Bioidentical menopausal hormone therapy: registered hormones (non-oral estradiol ± progesterone) are optimal. Climacteric. *In press*. doi: 10.1080/13697137.2017.1291607

Lieberman, A. and Curtis, L. (2017). In Defense of Progesterone: A Review of the Literature. *Altern. Ther. Health Med*. **23**(6), 24-32.

Lonergan, P. (2011). Influence of progesterone on oocyte quality and embryo development in cows. *Theriogenology* **76**, 1594–1601. doi: 10.1016/j.anireprosci.2018.06.010

Marvin JS chemical editor program (2018). Available in: https://www.drugbank.ca/ access: January, 24th of 2019.

Menchaca, A., Cuadro, F., dos Santos-Neto, P.C., Bosolasco, D., Barrera, N., Brun, V. and Crispo, M., (2018). Oocyte developmental competence is improved by relatively greater circulating progesterone concentrations during preovulatory follicular growth. *Anim. Reprod. Sci.* **195**, 321-328. doi: 10.1016/j.anireprosci.2018.06.010

Mendes, C.S., Barbosa, L. P., Araújo, M.L., Pinheiro, E.E.G., Lents, M.P., Amorim, L.S., Kiya, C.K., Santana, A.L.A., 2017. Follicular response and oocyte production following variations in ovarian stimulation in goats. *Theriogenology, In press*. doi: 10.1016/j.theriogenology.2017.11.016.

Mohammadi-Sangcheshmeh, A., Soleimani, M., Deldar, H., Salehi, M., Soudi, S., Hashemi, S. M., Schellander, K. and Hoelker, M. (2012). Prediction of oocyte developmental competence in ovine using glucose-6-phosphate dehydrogenase (G6PDH) activity

determined at retrieval time. *J. Assist. Reprod. Genet.* **29**, 153–158. doi:10.1007/s10815-011-9625-6.

Mohammadi-Sangcheshmeh, A., Veshkini, A., Hajarizadeh, A., Jamshidi-Adegani, F., Zhandi, M., Abazari-kia, A. H., Cinar, M. U., Soleimani, M. and Gastal, E. L. (2014). Association of glucose-6-phosphate dehydrogenase activity with oocyte cytoplasmic lipid content, developmental competence, and expression of candidate genes in a sheep model. *J. Assist. Reprod. Genet.* **31**, 1089-1098. doi: 10.1007/s10815-014-0264-6

Nivet, A. L., Bunel, A., Labrecque, R., Belanger, J., Vigneault, C., Blondin, P. and Sirard, M. A. (2012). FSH withdrawal improves developmental competence of oocytes in the bovine model. *Reproduction* **143**, 165–71. doi: 10.1530/REP-11-0391.

Nivet, A. L., Vigneault, C., Blondin, P. and Sirard, M. A. (2013). Changes in granulosa cells' gene expression associated with increased oocyte competence in bovine. *Reproduction* **145**, 555-565. doi: 10.1530/REP-13-0032

Ortiz, A., Hirol, M., Stanczyk, F. Z., Goebelsmann, U. and Mishell, D. R. (1977). Serum medroxyprogesterone acetate (MPA) concentrations and ovarian function following intramuscular injection of depo-MPA. *J. Clin. Endocrinol. Metab.* **44**, 32-38. doi:10.1210/jcem-44-1-32

Otsuka, F., Tavish, M. K. and Shimasaki, S. (2011). Integral Role of GDF-9 and BMP-15 in Ovarian Function. *Mol. Reprod. Dev.* **78**, 9–21. doi:10.1002/mrd.21265

Pfaffl, M. W., Horgan, G. M. and Dempfle, L. (2002). Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic. Acids. Res.* **30**, 36.

Ramakers, C., Ruijter, J. M., Deprez, R. H. L. and Moorman, A. F. M. (2003). Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci. Let*. **339**, 62–6.

Silva, J. M., Hamel, M., Sahmi, M. and Prince, C. A. (2006). Control of oestradiol secretion and of cytochrome P450 aromatase messenger ribonucleic acid accumulation by FSH involves different intracellular pathways in oestrogenic bovine granulosa cells in vitro. *Reproduction* **132**, 909–917. doi: 10.1530/REP-06-0058

Silvestre F, Boni R, Fissore RA, Tosti E. (2011). Ca2+ signaling during maturation of cumulus-oocyte complex in mammals. *Mol. Reprod. Dev.* **78**, 744-56. doi: 10.1002/mrd.21332

Sirard, A. M., Richard, F., Blondin, P. and Robert, C. (2006). Contribution of the oocyte to embryo quality. *Theriogenology* **65**, 126-136. doi:10.1016/j.theriogenology.2005.09.020

Sirard, M. A. (2011). Follicle environment and quality of in vitro matured oocytes. *J. Assist. Reprod. Genet.* **28**, 483–488. doi: 10.1007/s10815-011-9554-4

Shimasaki, S., Moore, R. K., Otsuka, F. and Erickson, G. F. (2004). The bone morphogenetic protein system in mammalian reproduction. *Endocr. Rev*. **25**, 72–101. doi: 10.1210/er.2003-0007

Shrimanker, K., Saxena, B. N., Fotherby, K. (1978). A radioimmunoassay for serum medroxyprogesterone acetate. *J. Steroid. Biochem.* **9**, 359-63. doi: 10.1016/0022-4731(78)90631-3

Souza-Fabjan, J. M. G., Pereira, A. F., Melo, C. H. S., Sanchez, J. D., Oba, E., Mermillod, P., Melo, L. M., Teixeira, D. I. A. and Freitas, V. J. F. (2013). Assessment of the reproductive parameters, laparoscopic oocyte recovery and the first embryos produced in vitro from endangered Canindé goats (Capra hircus). *Reprod. Biol.* **13**, 325-32. doi: 10.1016/j.repbio.2013.09.005

Thammasiri, J., Navanucraw, C., Uriyapongson, S., Khanthusaeng, V. and Kamollirt, C. (2016). Expression of cumulus-oocyte complex genes and embryonic development in goats subjected to progestogen-based estrus synchronization. *Theriogenology* **86**, 612-18. doi: 10.1016/j.theriogenology.2016.02.011

Torner, H., Ghanem, N., Ambros, C., Hölker, M., Tomek, W., Phatsara, C., ALM, H., Sirard, M. A., Kanit, W., Schellander, K. and Tesfaye, D. (2008). Focus on mammalian embryo genomics molecular and subcellular characterisation of oocytes screened for their developmental competence based on glucose-6-phosphate dehydrogenase activity. *Reproduction* **135**, 197–212. doi: 10.1530/REP-07-0348

Uzbekova, S., Roy-Sabau, M., Dalbiès-Tran, R., Perreau, C., Papillier, P., Mompart, F., Thelie, A., Pennetier, S., Cognie, J., Cadoret, V., Royere, D., Monget, P. and Mermillod, P. (2006). Zygote arrest 1 gene in pig, cattle and human: evidence of different transcript variants in male and female germ cells. *Reprod. Biol. Endocrinol*. **4**, 12. doi:10.1186/1477-7827-4-12

Wei, S., Shen, X., Gong, Z., Deng, Y., Lai, L. and Liang, H. (2017). FSHR and LHR expression and signaling as well as maturation and apoptosis of cumulus-oocyte complexes following treatment with FSH receptor binding inhibitor in sheep. *Cell Physiol. Biochem*. **43**, 660-669. doi: 10.1159/000480650

Ulloa-Aguirre, A., Reiter, E. and Crépieux, P. (2018). FSH receptor signaling: complexity of interactions and signal diversity. *Endocrinology* **159**, 3020–3035. doi:10.1210/en.2018-00452

Yang, F., Wang, M., Zhang, B., Xiang, W., Zhang, k., Chu, M., and Wang, P. (2018). Identification of new progestogen associated networks in mammalian ovulation using bioinformatics. *BMC Syst. Biol.* **12**, 36. doi: 10.1186/s12918-018-0577-7

Zörnig, M., Hueber, A., Baum, W. and Evan, G. (2001). Apoptosis regulators and their role in tumorigenesis. *Biochim. Biophys. Acta.* **1551**, 1-37. doi: 10.1016/S0304-419X(01)00031-2

5. CONSIDERAÇÕES FINAIS

A dose de 80 mg de FSH em ambos os regimes foi suficiente para promover o desenvolvimento de múltiplos folículos e obter CCOs imaturos com crescimento exponencial finalizado (BCB+). Contudo, o regime de múltipla aplicação aumentou a proporção de CCOs de boa qualidade morfológica (grau I/II) e demonstrou ser mais apropriado na modulação gênica da qualidade oocitária. A concentração de estradiol no fluido folicular não foi alterada pela dose ou forma de aplicação de FSH, sugerindo que as células somáticas foliculares desenvolvem estratégias para manter a produção deste hormônio equilibrada evitando assim os efeitos deletérios causados pelo seu excesso. P4 e MAP não alteraram a resposta folicular e nem a qualidade morfológica dos CCOs, porém promoveram efeitos dissimilares no *status* molecular dos CCOs. A P4 exógena impactou positivamente a abundância dos genes com importância na aquisição da competência, provavelmente devido à elevação das concentrações séricas deste hormônio, que interage com FSH alterando o perfil de expressão desses genes. Deixa-se como proposta de futuras investigações dentro desta linha, a avaliação do tempo de *coasting* do FSH (intervalo entre a última dose e a coleta dos CCOs), além da comparação entre a administração do FSH em dose decrescente (usada aqui) e dose constante, o que contribuiria bastante com o estabelecimento de um protocolo de estimulação efetivo não apenas na elevação da população folicular, mais também que gerasse oócitos imaturos potencialmente de alta competência ao desenvolvimento. Por fim, o uso de 80 mg de FSH associado a um dispositivo intravaginal liberador de P4 incrementa a população folicular e a obtenção de CCOs imaturos preditos como mais competentes para uso em ARTs em ovelhas.

Serviço Público Federal
Universidade Federal Fluminense
Pró-Reitoria de Pesquisa, Pós-Graduação e Inovação
Comissão de Ética no Uso de Animais

Certificamos que o projeto nº 979, intitulado "Desenvolvimento de protocolos de aspiração folicular em ovelhas da raça Santa Inês visando a produção in vitro de embriões", sob a orientação do Profª Felipe Zandonadi Brandão da Faculdade de Veterinária da Universidade Federal Fluminense, está de acordo com os Princípios Éticos na Experimentação Animal da SBCAL e obteve a aprovação da Comissão de Ética no Uso de Animais em 8 de fevereiro de 2018. A quantidade total de animais aprovada pela CEUA para este projeto foi de 62 (sessenta e dois) ovinos, e este certificado é válido por três anos após sua aprovação.

Niterói, 8 de fevereiro de 2018.

Fabio Otero Ascoli

Prof. Dr. Fabio Otero Ascoli
Coordenador da CEUA