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TRANSCRITOS RELACIONADOS COM A MATURAÇÃO OOCITÁRIA EM BOVINOS: EXPRESSÃO EM OÓCITOS DE FOLÍCULOS PRÉ-ANTRAIS E ANTRAIS E EFEITOS *IN VITRO* DE EGF E PROGESTERONA

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Tese de Doutorado submetida à avaliação da banca examinadora do Programa de Pós-Graduaçã em Biotecnologia da Rede Nordeste de Biotecnologia da Universidade Federal do Ceará como parte dos requisitos para obtenção do título de Doutor em Biotecnologia. Área de concentração: Biotecnologia em Agropecuária.

Orientador: Prof. Dr. José Roberto Viana Silva

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A Deus.
Aos meus pais, Jocélio e Aparecida.
Minha tia, Francisca Rodrigues.
Meus irmãos, Taisa e Tércio.
Minha esposa, Lizandra Albuquerque.
Com amor, dedico.

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“Tudo o que fizerem, sem em palavra seja em ação, façam-no em nome do Senhor Jesus, dando por meio dele graças ao Pai.” Colossences 3:17

RESUMO

Durante o crescimento, sob influencia de hormônios e fatores de crescimento, como a Progesterona (P4) e o Fator de Crescimento Epidermal (EGF), os oócitos sintetizam grandes quantidades de RNAm que se acumulam para formar um grande estoque de RNAm materno, dentre esses, estão o fator de crescimento e diferenciação 9 (GDF9), a quinase cMOS, a ribonuclease específica poli (a) (PARN), o fator de iniciação da tradução (eIF4E), a ciclina B1 (CCNB1) e a histona com ligante específico para oócito (H1FOO). Dessa forma, os objetivos deste estudo foram: (1) avaliar os níveis de RNAm para eIF4E, PARN, H1FOO, cMOS, GDF9 e CCNB1 em oócitos de folículos secundários e antrais em diferentes estágios de desenvolvimento e (2) avaliar a influência de fator de crescimento epidermal (EGF) e progesterona (P4) no crescimento, retomada da meiose e nos níveis dos referidos transcritos em oócitos de folículos antrais pequenos e médios após pré-maturação e maturação *in vitro*. Para este fim, oócitos frescos derivados de folículos secundários bovinos, bem como de folículos antrais pequenos, médios e grandes foram coletados para análise dos níveis de RNAm para os transcritos citados acima. Além disso, oócitos de folículos secundários cultivados por 18 dias ou de complexos cumulus oócito (CCO) de folículos antrais após crescimento e pré-maturação também foram coletados para análise dos níveis de RNAm. Em seguida, CCOs derivados de folículos antrais pequenos e médios foram cultivados em meio contendo EGF (10 ng / mL), P4 (100 µM) ou ambos. Durante o cultivo, foram avaliadas a taxa de crescimento, a retomada da meiose e os níveis de RNAm acima citados. Os níveis de RNAm em oócitos de folículos secundários e antrais foram analisados pelo teste de Kruskal-Wallis. O percentual de oócitos em VG foi analisado pelo teste Mann Whitney. Os resultados mostraram que os níveis de RNAm para H1FOO, GDF9, CCNB1 e PARN foram maiores em oócitos de folículos antrais pequenos, médios e grandes do que em oócitos folículos secundários. O cultivo *in vitro* folículos secundários aumentou significativamente os níveis todos os transcritos estudados. A pré-maturação de oócitos de folículos antrais pequenos aumentou os níveis de RNAm para GDF9, PARN e eIF4E. Além disso, níveis mais altos de cMOS e H1FOO foram identificados em oócitos de folículos antrais médios pré-maturados. Adicionalmente, EGF e P4 influenciaram o crescimento de oócitos submetidos à pré-maturação *in vitro* e a expressão de transcritos em oócitos de folículos antrais pequenos e médios. EGF ou P4 também aumentou os níveis de RNAm do cMOS ($P<0,05$), enquanto que tanto o EGF como a P4 aumentaram os níveis de mRNA para os níveis de H1FOO. Em conclusão, o crescimento folicular está associado a um aumento na expressão de H1FOO, GDF9, CCNB1 e PARN. O cultivo *in vitro* de folículos secundários, pré-maturação e a

maturação dos oócitos dos folículos antrais aumentam a expressão de eIF4E, PARN, H1FOO, cMOS, GDF9 e CCNB1. Além disso, P4 sozinho ou associado ao EGF aumentam a expressão de RNAm para cMOS, CCNB1 e H1FOO.

Palavras-chave: Crescimento de oócitos. Pré-maturação de oócitos. Maturação de oócitos. Estoque de RNAm.

ABSTRACT

During growth, under the influence of hormones and growth factors, such as Progesterone (P4) and Epidermal Growth Factor (EGF), oocytes synthesize large amounts of mRNA that are accumulated to form a large stock of maternal mRNA, are the growth factor and differentiation 9 (GDF9), a cMOS kinase, a specific poly (a) ribonuclease (PARN), the translation initiation factor (eIF4E), a cyclin B1 (CCNB1) and a history with specific oocyte ligand (H1FOO). Thus, the objectives of this study were: (1) to evaluate the levels of mRNA for eIF4E, PARN, H1FOO, cMOS, GDF9 and CCNB1 in oocytes of secondary and antral follicles at different stages of development and (2) to evaluate the factor factor epidermal growth (EGF) and progesterone (P4) without growth, resuming the mean and levels of levels transcribed in oocytes of small and medium antral follicles after pre-maturation and in vitro maturation. For this purpose, fresh oocytes derived from secondary bovine follicles, as well as small, medium and large antral follicles were collected to analyze the levels of mRNA for transcripts mentioned above. In addition, secondary follicles grown for 18 days or the cumulus oocyte complex (CCO) of antral follicles after growth and pre-maturation were also collected for analysis of mRNA levels. Then, the CCOs derived from small and medium antral follicles were cultured in medium containing EGF (10 ng / mL), P4 (100 µM) or both. During cultivation, growth rates were evaluated, taken from the average and levels of mRNA mentioned above. The levels of mRNA in oocytes from secondary and anal follicles were analyzed by the Kruskal-Wallis test. The percentage of oocytes in the VG was analyzed using the Mann Whitney test. The results showed that mRNA levels for H1FOO, GDF9, CCNB1 and PARN were higher in oocytes of small, medium and large antral follicles than in secondary follicle oocytes. The cultivation of secondary follicles in vitro significantly increased the levels of all studied transcripts. Pre-maturation of oocytes from small antral follicles increased the levels of mRNA for GDF9, PARN and eIF4E. In addition, higher levels of cMOS and H1FOO were identified in oocytes from pre-matured middle antral follicles. Additionally, EGF and P4 influenced the growth of oocytes submitted to in vitro pre-maturation and the expression of transcripts in oocytes of small and medium antral follicles. EGF or P4 also increased cMOS mRNA levels ($P < 0.05$), while both EGF and P4 increased mRNA levels to H1FOO levels. In conclusion, follicular growth is associated with an increase in the expression of H1FOO, GDF9, CCNB1 and PARN. In vitro culture of secondary follicles, pre-maturation and oocyte maturation of antral follicles increase the expression of eIF4E, PARN, H1FOO, cMOS, GDF9 and CCNB1. In addition, P4 alone or associated with EGF increases mRNA expression for cMOS, CCNB1 and H1FOO.

Keywords: Oocyte growth. Oocyte pre-maturation. Oocyte maturation. mRNA stock

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

AI	Anáfase I
AMPc/cAMP	Adenosina monofosfato cíclico
CCO	Complexo Cumulus Oócito
CGP	Células germinativas primordiais
OD	Oócito desnudo
EGF	Fator de crescimento epidermal
ERK-1	Sinal Extracellular Regulado – 1
ERK -2	Sinal Extracellular Regulado – 2
FIV	Fertilização *in vitro*
FSH	Hormônio folículo estimulante
FSHr	Receptor do hormônio folículo estimulante
GVBD	Quebra da Vesícula Germinativa
GPR-3	Proteina-G acoplado ao receptor -3
HCl	Ácido Clorídrico
IGF-1	Fator de crescimento semelhante à insulina – 1
IGFPB	Proteína ligadora de IGF
IBMX	3-Isobutil-1-methilxantina
IU	Unidades Internacionais
KD	Quilodalton
LH	Hormônio Luteinizante
LHr	Receptor do Hormônio Luteinizante
M	Molar

MAPK	Proteina Quinase Ativada por Mitógeno
MI	Metáfase I
MII	Metáfase II
P4	Progesterona
RNA	Ácido ribonucleico
RNAm	Ácido ribonucléico mensageiro
RVG	Ruptura da vesícula germinativa
SAS	Software de Análise Estatística
SD	Desvio Padrão
TI	Telófase I
VG/GV	Vesícula germinativa
XIAP	X-ligação inibidor de apoptose

SUMÁRIO

1 INTRODUÇÃO

Durante o desenvolvimento, os folículos ovarianos sofrem constantes modificações em sua morfologia, tendo como principais características o crescimento do oócito, a proliferação e a diferenciação das células da granulosa e da teca, bem como o aparecimento da cavidade antral (GREEN & SHIKANOV, 2016). Essas modificações são influenciadas pela ação de vários hormônios e fatores de crescimento, como a Progesterona (P4) e o Fator de Crescimento Epidermal (EGF), que atuam no controle do crescimento de oócitos e folículos ovarianos (HUANG *et al.,* 2013). Durante o crescimento, os oócitos sintetizam grandes quantidades de RNAm que se acumulam para formar um grande estoque de RNAm materno, dentre esses, estão o fator de crescimento e diferenciação 9 (GDF9), a quinase cMOS, a ribonuclease específica poli (a) (PARN), o fator de iniciação da tradução (eIF4E), a ciclina B1 (CCNB1) e a histona com ligante específico para oócito (H1FOO). Quando os oócitos atingem o tamanho máximo, a redução do nível de AMPc no interior do oócito (KAWAMURA *et al.,* 2011) induz alterações na morfologia nuclear resultando na condensação da cromatina (VIEUX & CLARKE, 2018) e consequente retomada da meiose.

Estudos demonstraram que o uso de substâncias que mantenham altas concentrações de AMPc no interior do oócito, como a cilostamida (BILODEAU-GOESEELS, 2012; ALBUZ *et al.,* 2010; SHU *et al.,* 2008) tem contribuído para o bloqueio da retomada da meiose *in vitro*. Os efeitos de cilostamida no desenvolvimento *in vitro* tem sido descrito em humanos (SHU *et al.,* 2008; VANHOUTTE et al., 2009; VANHOUTTE *et al.,* 2009), bovinos (LUCIANO *et al.,* 2011; ALBUZ *et al.,* 2010), ovinos (ROSE *et al.,* 2013) e camundongos (NOGUEIRA *et al.,* 2003; VANHOUTTE et al., 2009; ALBUZ *et al.,* 2010; JEE *et al.,* 2009).

Dessa forma, esse através desse estudo será possível avaliar se a adição de fatores de crescimento (EGF) e hormônios (P4), tem impactos positivos na expressão de RNAs mensageiros para proteínas que regulam o estoque citoplasmático dos RNAs, bem como com os níveis de RNAs mensageiros estocados no oócito.

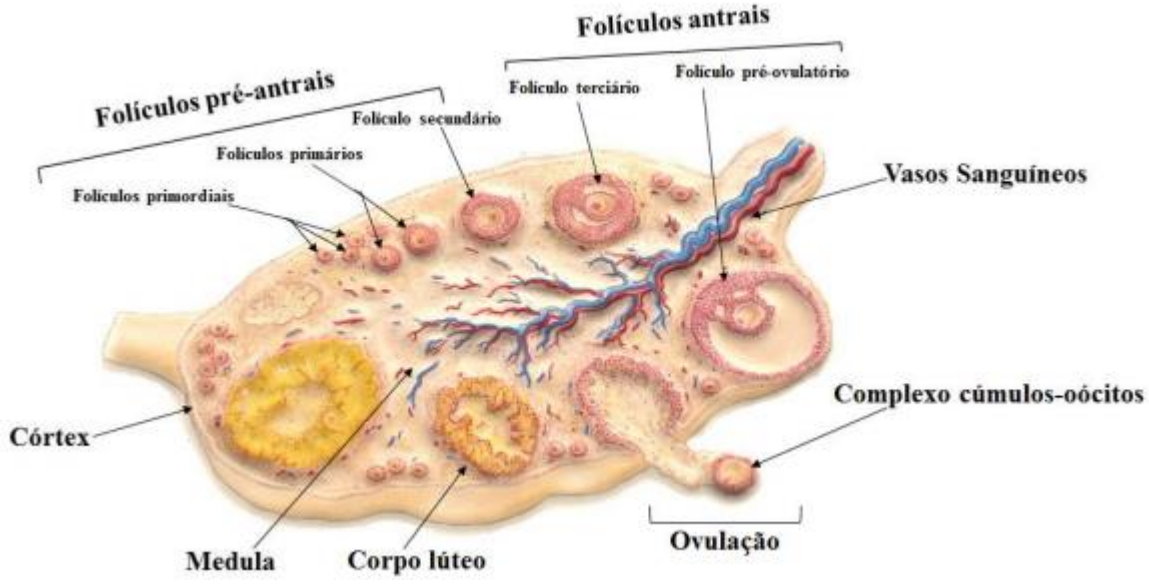
Para um melhor entendimento da importância deste trabalho, segue uma revisão de literatura onde serão abordados aspectos relacionados aos folículos ovarianos pré-antrais, maturação oocitária *in vitro* e estoque de RNAs durante a maturação oocitária.

2 REVISÃO DA LITERATURA

2.1 O ovário

O ovário mamífero é um órgão composto por vários tipos de células, incluindo oócitos, células da granulosa, da teca, do estroma e do epitélio da superfície ovariana. O ovário apresenta a região cortical, onde estão os folículos ovarianos e a região vascularizada, que está localizada na porção mais interna do ovário na maioria das espécies e constituída por tecido conjuntivo, nervos, vasos sanguíneos e linfáticos responsáveis pela nutrição e sustentação do ovário (PORRAS-GÓMEZ e MORENO-MENDONZA, 2017). Além dos nutrientes e hormônios provenientes da corrente sanguínea, fatores produzidos pelos diferentes tipos celulares contribuem para a formação de um sistema bastante complexo que regula as funções do ovário, ou seja, a produção de gametas e hormônios (ERICKSON e SHIMASAKI *et al*., 2003). Estas funções são exercidas pela interação de dois fenômenos que ocorrem no ovário, a oogênese e a foliculogênese; estes correspondem a processos biológicos complexos e coordenados que requerem uma série de eventos que induzem as mudanças morfológicas e funcionais dentro do folículo, conduzindo a diferenciação de células e o desenvolvimento dos oócitos (BONNET *et al*., 2008). A figura 1 demonstra a organização do ovário mamífero.

Figura 1. Organização do ovário mamífero: região cortical e medular.



Fonte: Pessoa (2013., com adaptações).

2.2. Avanços no cultivo *in vitro* de folículos pré-antrais e na compreensão da folículogênese inicial

Na última década, houve um progresso substancial na elucidação de fatores que regulam o crescimento e desenvolvimento de oócitos e folículos, bem como a maturação dos oócitos, através do estudo das ações de um grande número de proteínas expressas ao longo da oogênese, em particular durante os estágios iniciais da folículogênese (SANCHEZ e SMITHZ, 2012).

O uso de técnicas de biologia molecular juntamente com sistemas de cultivo *in vitro* de foliculos pré-antrais e/ou maturação de oócitos *in vitro*, por exemplo, permitiu o estudo da influência de uma série de fatores (como hormônios, proteínas recombinantes e/ou fatores de crescimento) no desenvolvimento folicular, sobrevivência, produção de esteróides, crescimento e maturação de oócitos. Além disso, o cultivo de células da granulosa e o co-cultivo de oócitos com células da granulosa tornaram-se modelos aceitos para estudar a interação entre oócitos e suas células somáticas circundantes, fornecendo um foco sobre o potencial dos oócitos para regular uma variedade de funções de células da granulosa (SANCHEZ e SMITHZ, 2012).

Na tentativa de entender esses mecanismos para folículos primordiais, primários e secundários, a expressão e quantificação de RNAm para fatores de crescimento local e citocinas e seus receptores têm recebido crescente atenção nos últimos anos (SILVA *et al.*, 2010). A esse respeito, diferentes estudos foram realizados com cabras (SARAIVA *et al*., 2010; FROTA *et al*., 2011a; FROTA *et al*., 2011b; FROTA *et al*., 2011c; COSTA *et al*., 2012; LEITAO *et al.*, 2014), ovelhas (SILVA *et al.*, 2010; ROSSI *et al.*, 2015) e vacas (VASCONCELOS *et al.*, 2012; SILVA et al., 2013; PASSOS *et al.*, 2013*;* REBOUÇAS *et al.*, 2013; PASSOS *et al.*, 2016; PAULINO *et al.*, 2019; CAVALCANTE *et al.*, 2019) como modelos animais.

No tocante à espécie bovina, estudos realizados nos últimos anos têm contribuído para se compreender o papel de hormônios e fatores de crescimento durante o cultivo *in vitro* de folículos pré-antrais. Além disso, contribuiu para o desenvolvimento de um sistema *in vitro* de cultivo capaz de promover o crescimento e desenvolvimento de folículos pré-antrais até o estágio de folículo antral. Dentre esses, no estudo realizado por VASCONCELOS *et al.* (2012), demonstrou-se que a adição de FSH e/ou GDF-9 ao meio de cultura promove o crescimento *in vitro* de folículos pré-antrais. O GDF-9 é um regulador importante da

proliferação e diferenciação de vários tipos celulares (DIJK *et al.*, 2000). Apesar de FSH e/ou GDF-9 não interagirem sinergicamente neste processo, a adição de GDF-9 estimulou a formação do antro folicular, aumentou a expressão das proteínas versican e perlecan no fluido antral e, como resultado de uma interação positiva com FSH, aumentou a expressão *in vitro* de outras proteínas do líquido antral, como versican e HAS2.

Nessa mesma categoria folicular, também se avaliou o efeito de FSH em combinação com a ativina-A, uma glicoproteína importante para o desenvolvimento dos folículos ovarianos em mamíferos (SILVA et al., 2013), e demonstrou-se que que ativina-A é importante para o início do desenvolvimento folicular (até 6 dias), mas reduz a estimulação do efeito do FSH nos folículos pré-antrais bovinos após 18 dias de cultivo *in vitro* (SILVA et al., 2013). Silva et al. (2013) também demonstrou que após 18 dias de cultivo *in vitro*, a combinação de ativina-A e FSH reduz os níveis de RNAm para ActR-IB, ActR-IIB, FSH-R e PCNA, sugerindo que o FSH isolado ou associado a ativina-A diminui a sensibilidade dos folículos à ativina-A.

Por outro lado, identificou-se que a associação de FSH e BMP-15 ao meio de cultivo promoveram o crescimento folicular e a formação de antro, e mantiveram a viabilidade folicular durante o cultivo (PASSOS *et al.*, 2013). Outros trabalhos já demonstraram a importância de BMPs para o controle da oogênese e foliculogênese em espécies de mamíferos. BMP-2 e -4 modulam a produção hormonal de células da granulosa e teca contribuindo para o crescimento folicular (ROSSI *et al.*,, 2015; ROSSI *et al.*, 2016). BMP-5, -6, -7 e -8 atuam no desenvolvimento folicular e contribuições da BMP-15 ao oócito e maturação folicular (ROSSI *et al.*,, 2015).

Além de ativina-A e BMPs, sabe-se que o sistema IGF está envolvido no crescimento e diferenciação de folículos ovarianos (SILVA *et al.*, 2009). O sistema IGF inclui dois ligantes, IGF1 (IGF-I) e IGF2 (IGF-II), dois receptores (IGF1R e IGF2R) e uma família de proteínas de ligação a IGF de alta afinidade (IGFBP-1, IGFBP-2. IGFBP-3, IGFBP-4, IGFBP-5 e IGFBP-6) (LE ROITH *et al.*, 2001). Em ovários bovinos, IGFBP-2 e IGFBP-5 são sintetizados localmente na parede folicular por células da granulosa e IGFBP-4 e IGFBP-6 nas células da teca (ARMSTRONG *et al*., 1998).

Nosso grupo de pesquisa demonstrou que IGFs, seus receptores e IGFBPs apresentam expressão variável em folículos pré-antrais. Após 12 dias de cultivo de folículos pré-antrais do tamanho de 0,2 mm, os níveis de RNAm para IGF1, IGF1R, IGFBP-3, IGFBP-5 e IGFBP-6 foram reduzido quando folículos atingiram um diâmetro de 0,5 mm. Quando comparado

com folículos frescos de 0,5 mm, folículos cultivados do mesmo tamanho mostram redução na sua expressão de IGFBP-1, IGFBP-2, IGFBP- 3 e IGFBP-6 (REBOUÇAS *et al.*, 2013).

As citocinas também exercem um papel importante na regulação do desenvolvimento folicular ovariano (SHIMIZU *et al.,* 2012). As interleucinas (ILs) são citocinas pró-inflamatórias participam ativamente do processo de ovulação (FIELD *et al.,* 2014). PASSOS *et al.* (2016) demonstrou que o crescimento de folículos secundários para pequenos folículos antrais é seguido por um aumento nos níveis de RNAm para IL-1β e IL-1RA. Além disso, com o crescimento de folículo antral pequeno para grande, verificou-se um aumento na expressão de IL-1 β, IL-1RA e IL-1RII. *In vitro*, a IL-1b a (10 e 50 ng / mL) promove ativação de folículos primordiais e desenvolvimento folicular após 6 dias de cultivo.

Recentes estudos demonstraram que o fator de crescimento epidermal (EGF) e progesterona (P4) são candidatos para melhorar a sobrevivência folicular e crescimento folicular *in vitro* (PAULINO *et al.*, 2019). Estes autores demonstraram que o EGF promove um crescimento folicular e melhora a expressão de RNAm para GDF9 e cMOS em folículos bovinos cultivados *in vitro*, enquanto a progesterona não influencia o crescimento folicular *in vitro*, mas aumenta a expressão de GDF9 e ciclina B1 em oócitos (PAULINO *et al.,* 2019). Apesar dos avanços obtidos com o cultivo de folículos pré-antrais bovinos, ainda não foram produzidos embriões a partir de oócitos de folículos cultivados *in vitro*. Isto mostra a importância de se estudar os níveis de RNAs mensageiros que são estocados nos oócitos, como por exemplo, GDF9, ciclina B1 e cMOS, e que tem um papel importante durante a maturação e desenvolvimento embrionário inicial.

2.3. Avanços na Maturação Oocitária *In vitro* em Bovinos

Mais recentemente, protocolos de pré-maturação e a descoberta de novos aditivos para meios de cultivo demonstraram o potencial de maximizar a competência oocitária e o desenvolvimento inicial de embriões (GILCHRIST *et al.,* 2011; BILODEAU-GOESEELS, 2012; BEZERRA *et al.,* 2016; DA ROSA *et al.,* 2017). Apesar disso, a eficiência da produção *in vitro* de embriões bovinos (PIV) permanece em torno de 40% em oócitos de doadores não superovulados, apesar do grande esforço feito para entender os efeitos das condições de cultivo, composição do meio e suplementação hormonal (MARINHO *et al.,* 2015). Embora bezerros vivos derivados de oócitos recuperados de folículos antrais pequenos tenham sido relatados, a baixa viabilidade de oócitos durante o crescimento *in vitro*, bem como a competência de desenvolvimento de oócitos cultivados *in vitro* permanecem problemáticas (HUANG *et al.,* 2013).

Por outro lado, estudos demonstraram que é possível aumentar a competência de desenvolvimento oocitário durante a MIV por meio de condições ideais de cultura e/ou aditivos apropriados com impacto na competência plena de desenvolvimento (SHABANKAREH *et al.,* 2015). Como a maioria dos RNAs / proteínas que suportam as fases iniciais da embriogênese são acumuladas durante o crescimento dos oócitos, conforme determinado em estudos com camundongos (BIASE *et al.,* 2014). Conhecer as mudanças na expressão gênica durante o crescimento e competência dos oócitos desempenhará um papel crucial no desenvolvimento de técnicas adequadas de reprodução assistida.

Acredita-se que a inibição ou atraso da maturação nuclear *in vitro* permite mais tempo para os oócitos realizarem o acumulo de moléculas importantes para o desenvolvimento embrionário inicial, o que poderia potencialmente melhorar a eficiência de produção *in vitro* de embriões (BILODEAU-GOESEELS, 2012). O reinício espontâneo da meiose ocorre principalmente devido a uma diminuição em concentração de adenosina monofosfato cíclico intraoócito (cAMP), que tem um papel importante no controle da maturação de oócitos de mamíferos (THOMAS et al., 2002; LUCIANO et al., 2011).

A Cilostamida é um inibidor de PDE3 específico do oócito (humano (VANHOUTTE et al., 2009, 2009a; SHU et al., 2008); bovino (ALBUZ et al., 2010; LUCIANO et al., 2011); ovino (ROSE et al., 2013) e murino (NOGUEIRA et al., 2003a; VANHOUTTE et al., 2009; JEE et al., 2009; ALBUZ et al., 2010) e sua eficiência depende das espécies e do tempo de incubação do CCO. BEZERRA et al. (2016) demonstrou que Cilostamida na concentração de

10 μM e metades foliculares interagem e são eficazes para prevenir a retomada meiótica de oócitos e manter os níveis de AMPc elevados em CCOs bovinos cultivados por 12h.

Outros estudos demonstraram que a inibição de PDE3 sustenta a parada meiótica e a junção de gap de oócitos de bovinos em crescimento *in vitro* (ALAM *et al*., 2018). A presença de junções gap funcionalmente abertas, mediando a comunicação entre células oocitárias e cumulus, é responsável por manter a cromatina sem condensação (GV0). Quando essa comunicação é interrompida, a cromatina condensa-se rapidamente e a síntese de RNA cessa repentinamente (LUCIANO *et al.*, 2011).

Esses autores também observaram que o prolongamento do acoplamento da junção de gap durante a cultura de oócitos antes de sua maturação *in vitro* aumenta a capacidade dos oócitos de pequenos folículos antrais de sofrer meiose. Assim, uma interação complexa de eventos de sinalização autócrina e parácrina entre o oócito e suas células cumulus e granulosa circundantes é responsável por manter a sincronia altamente regulada da maturação *in vivo* e é fundamental para a aquisição da competência de desenvolvimento do oócito (SILVESTRE *et al.*, 2012).

Várias substâncias, como IL1β e TNFα, podem influenciar o crescimento de oócitos e armazenamento de transcritos *in vitro* (LIMA et al., 2018). Sabe-se que IL-1 e seus receptores são expressos em diferentes compartimentos (oócitos e granulosa) de folículos antrais bovinos e que 10 ng / ml de IL1β estimula o desenvolvimento folicular inicial (PASSOS et al., 2016). O TNFα e seus receptores também são expressos em folículos antrais bovinos e foi demonstrado que TNFα manteve a ultraestrutura de células do cumulus durante o cultivo *in vitro* de CCOs bovino (SILVA et al., 2017a). LIMA et al. (2018) demonstraram que o TNFα e a IL1β promovem o crescimento *in vitro* de oócitos durante um período de cultivo de 48 horas. No entanto, os oócitos não atingiram o tamanho mínimo para garantir a competência oocitária. Portanto, esse crescimento não estava vinculado a um aumento da taxa de maturação de oócitos de folículos antrais pequenos. Além disso, essas citocinas não influenciaram a expressão de RNAm para GDF-9, c-Mos, CCNB1 e H1FOO nos oócitos.

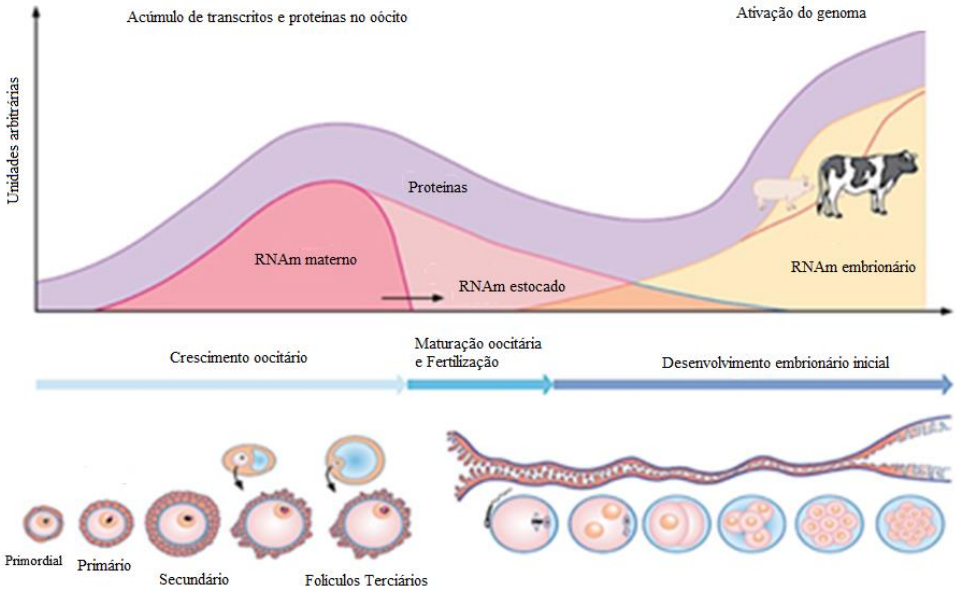
2.4. RNAs mensageiros estocados durante crescimento oocitário

MAMO et al. (2011) descreveram as mudanças globais do perfil transcriptômico que ocorrem durante a maturação meiótica de oócitos bovinos imaturos e após a maturação *in vitro*. Estes autores indentificaram um grande número de diferentes genes em oócitos bovinos,

dos quais aproximadamente 75% foram mais expressos em oócitos imaturos, reforçando a ideia de que a maioria dos transcritos é acumulada quando os oócitos se encontram no estágio de vesícula germinativa.

Estes transcritos irão coordenar o subsequente desenvolvimento oocitário até a ativação do genoma embrionário. Sabe-se que alguns RNAm são traduzidos em oócitos entre a metáfase I (MI) e MII, enquanto outros são traduzidos durante a fertilização ou durante os estágios iniciais da embriogênese. A figura 2 demonstra o acúmulo de transcritos e proteínas durante o crescimento oocitário e desenvolvimento embrionário inicial.

Figura 2. Acúmulo de transcritos e proteínas durante o crescimento oocitário e desenvolvimento embrionário inicial



Fonte: BOBE *et al.(*2006)

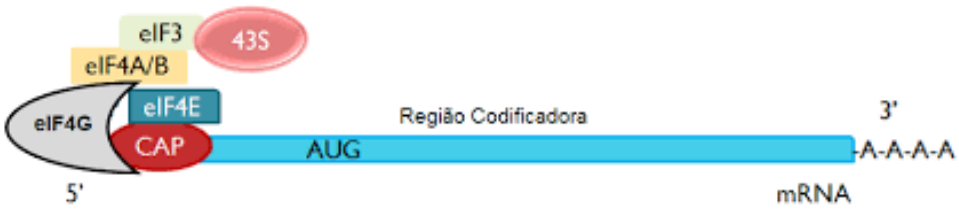
Durante o período de bloqueio meiótico, apesar da aparente quiescência, os oócitos aumentam expressivamente de volume e sofrem importantes transformações citoesqueléticas e moleculares, que conferem potencial para suportar os demais estágios de desenvolvimento. Tais transformações envolvem não somente a diferenciação e o deslocamento de organelas citoplasmáticas, mas, principalmente, a transcrição e o estoque de RNAm, que serão mobilizados em momentos específicos para síntese proteica (BREVINI-GANDOLFI E GANDOLFI, 2001; SIRARD *et al.*, 2006).

Concomitantemente, as células da granulosa se proliferam, diferenciam-se e estabelecem comunicação com os oócitos por meio das junções GAP, com transferência bidirecional de fatores de baixo peso molecular, constituindo o complexo cumulus-oócito. A possibilidade de transcrição gênica durante o estágio diplóteno da prófase I confere aos oócitos capacidade de síntese e de estoque de transcritos essenciais para suportar todo o processo de maturação oocitária até a embriogênese inicial (BREVINI-GANDOLFI E GANDOLFI, 2001).

BIASE et al. (2014) demonstraram ainda que o potencial de desenvolvimento embrionário e a qualidade dos blastocistos são determinados pela adequada concentração oocitária de mRNA. Embora o completo mecanismo implicado nesse processo ainda não esteja totalmente desvendado, sabe-se que a redução em comprimento da cauda poli-A determina a inatividade traducional e a estabilidade do RNAm, garantindo seu estoque citoplasmático (VASSALI E STUTZ, 1995). Deste modo, a qualquer momento, conforme a necessidade celular, ocorre a readenilação e consequente tradução dos transcritos estocados (HUARTE *et al.*, 1992).

Durante a maturação meiótica de oócitos bovinos, o fator de iniciação da tradução, eIF4E gradualmente se torna fosforilado. Essa fosforilação substancial começa no momento da ruptura da vesícula germinativa (RVG) e continua até o estágio de MII. O início da fosforilação do eIF4E ocorre paralelamente a um aumento significativo na síntese protéica global (TOMEK et al., 2002). Uma vez no citoplasma, o CBC (complexo de ligação-cap) nuclear é substituído por eIF4F, compreendendo fatores de iniciação da tradução eucariótica eIF4E, eIF4G e eIF4A. O eIF4F liga a subunidade ribossômica 40S via eIF3. O eIF4F se associa ao PABP, criando um estrutura pseudo-circular que favorece a tradução eficiente e protege o RNAm da degradação (MAQUAT *et al.*, 2010).

Figura 3. Cascata de sinalização de eIF para início da tradução.



Fonte: Machado (2014).

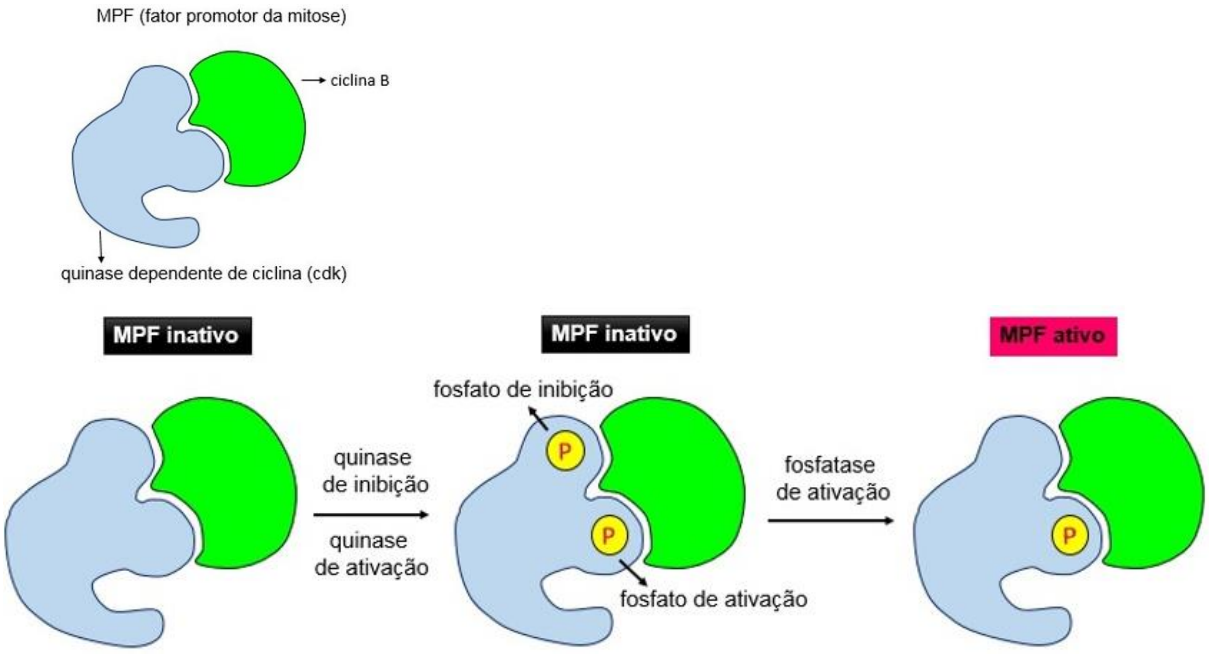
PARN (ribonuclease poli (A)) é uma enzima responsável pela poliadenilação (COPELAND & WORMINGTON, 2001; KORNER et al., 1998) e, apesar de outras enzimas estarem ativas, PARN é o mais ativo, a cauda poli (A) originalmente adquirida no núcleo é agora reduzida, para cerca de 20-40 bases. Em outras palavras, a poliadenilação é controlada pela presença ou ausência de PARN, e não pelo recrutamento de outra polimerase poli (A) (RICHTER, 2011).

Adicionalmente, genes envolvidos no processo de transcrição e tradução nos oócitos são muito importantes para os oócitos em desenvolvimento (BEZERRA *et al.*, 2019). Entre esses transcritos, está o ligante específico de oócitos histona (H1FOO) localizada nos cromossomos dos oócitos durante o processo de maturação meiótica (YAN *et al*., 2015). H1FOO é sintetizado e se acumula durante oogênese, e sua presença é mantida até estágio do embrião inicial, segue a degradação (FU *et al*., 2003; TANAKA *et al.*, 2005).

O fator Maturação de oócitos Mos (cMOS) é uma cinase expressa exclusivamente em células germinativas, estudos mostram um papel importante na maturação de oócitos (MCPHERRON, 2015), uma vez que a via cMOS-MAPK1 / 3 é importante para manutenção da parada da MII (PHILLIPS *et al*., 2002).

Ciclina B1 (CCNB1) é a unidade reguladora do fator de promoção da maturação (MPF) e desempenha um papel importante na maturação de oócitos (WU *et al*., 1997). De acordo com DE VANTERY *et al.* (1996), CCNB1 é preferencialmente armazenada como RNAm em oócitos imaturos e só é traduzida com a retomada da meiose, uma vez que a associação da ciclina B1 com a proteína p34cdc2 é necessária para a ativação do MPF e, consequentemente, a transição do G2 / Fase M.

Figura 4. Ativação do Fator Promotor da Meiose (MPF)



Fonte: https://i1.wp.com/www.ciclocelular.com.br/wp-content/uploads/2015/06/ativacao-do-mpf.jpg?ssl=1 (com adaptações).

2.5 Fator de crescimento epidermal (EGF) e Progesterona (P4) na maturação oocitária

A família do fator de crescimento epidermal (EGF) compreende 11 proteínas, incluindo EGF, fator de crescimento semelhante ao EGF de ligação à heparina (HB-EGF), fator de crescimento transformador-α (TGFα), epígeno (EPGN), neuregulinas 1 a 4 (NRG1-4), anfiregulina (AREG), epiregulina (EREG) e betacelulina (BTC), todos com propriedades estruturais e propriedades funcionais (SCHNEIDER E WOLF, 2009) que exercem influência nos mais variados eventos biológicos relacionados à fisiologia ovariana em mamíferos, como proliferação de células da granulosa, esteroidogênese e maturação de oócitos (PEREIRA *et al.*, 2018).

Os fatores de crescimento pertencentes à família EGF podem se ligar a diferentes tipos de receptores transmembranares. Estudos recentes não apenas implicaram sinalização peptídica do tipo EGF em células da granulosa e do cúmulus, essenciais para a maturação meiótica do oócito, mas também forneceram informações sobre como essa rede de sinalização coordena a maturação citoplasmática do oócito e, portanto, influencia a capacidade de desenvolvimento do oócito (CONTI *et al.*, 2006).

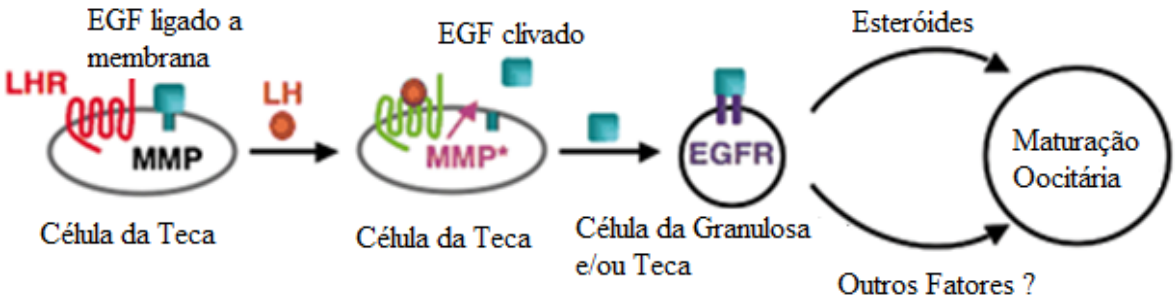
A ativação dos receptores EGFR com seu ligante EGF, presente na superfície celular, promove sua dimerização, que é acompanhada pela fosforilação dos resíduos de tirosina, responsáveis pelo início da transdução de sinal e pela cascata de eventos intracelulares, bem como pelo recrutamento de várias enzimas e proteínas responsável pela proliferação e sobrevivência celular (CONTI *et al.*, 2006).

O EGF pode atuar de várias maneiras no folículo ovariano, tais como: estimulação da mitose das células da granulosa e teca (CELESTINO *et al*., 2009), indução do crescimento de oócitos (GALL et al., 2004), preservação da viabilidade folicular (CELESTINO *et al.*, 2009), produção de esteróides (JAMNONGJIT *et al.*, 2005), expansão de células cumulus (BOLAMBA *et al.*,2006) e desempenhando um papel preponderante nas atividades nucleares e maturação citoplasmática (HATOYA *et al.*, 2009; PEREIRA *et al.*, 2014).

Parte da função da rede EGF é induzir a expansão cumulus, um processo pelo qual as células cumulus secretam e montam uma matriz rica em ácido hialurônico que facilita a ovulação, penetração e fertilização espermática (RICHANI and GILCHRIST, 2018). Os

peptídeos do tipo EGF induzem a expressão temporal de RNAm a partir de genes necessários para a formação da matriz cumulus (Has2, Ptx3, Tnfaip6, Ptgs2) *in vitro* em folículos pré-ovulatórios e de células cumulus (ASHKENAZI *et al*., 2005; SHIMADA *et al.*, 2006; RICHANI and GILCHRIST, 2018), o que facilita a expansão cumulus.

Figura 5. Modelo para maturação de oócitos induzida por EGF



Fonte: Hatoya et al (2008).

A retomada meiótica de oócitos induzida por FSH e a expansão do cumulus *in vitro* são mediadas pelos peptídeos do tipo EGF (SHIMADA *et al.*, 2006; DOWNS E CHEN, 2008; PROCHAZKA *et al.*, 2011). A exposição do CCO a altos níveis de cAMP também induz a retomada meiótica por regulação positiva de peptídeos do tipo EGF (SHIMADA *et al*., 2006; DOWNS E CHEN, 2008). Anfiregulina (AREG) exógeno, epiregulina (EREG) e EGF também induzem a retomada meiótica e a expansão cumulus de CCOs em uma ampla gama de espécies de mamíferos, incluindo humano (PROCHAZKA *et al.*, 2011; RICHANI *et al*., 2013).

A progesterona, por sua vez, é um hormônio essencial na oogênese e desempenha um papel no crescimento folicular, ovulação e luteinização (KIM *et al., 2017)*. P4 também é necessária para o apoio materno da sobrevivência e desenvolvimento do concepto durante a gravidez em mamíferos (KIM & GREENWALD, 1987; PELUSO, 2006).

Em bovinos, a P4 induz a transcrição de genes e afeta a competência oocitária, sinalizando através de receptores nucleares (PGRs) e de membrana (APARICIO *et al.*, 2011; O´SHEA *et al.*, 2012). Os COCs bovinos expressam receptores nucleares (PGR-A, PGR-B) e P4 ligados à membrana (mPRa, mPRb, componente da membrana do receptor de progesterona (PGRMC) 1 e PGRMC2) (APARICIO *et al.*, 2011). Além disso, a expressão

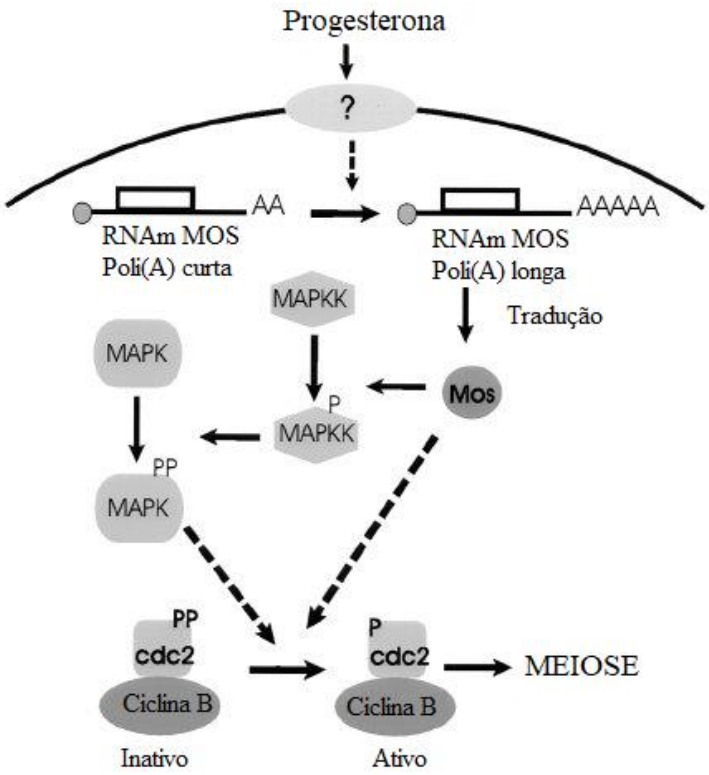
proteica desses receptores muda dinamicamente após a maturação *in vitro* em resposta a LH, FSH ou P4 (LUCIANO *et al.*, 2010).

Os efeitos da P4 no ovário são mediados indiretamente, via eixo hipotalâmico-hipofisário, ou diretamente, via interações com os seus receptores no ovário (SLOMCZYNSKA *et al.*, 2000). Existem duas formas funcionais de receptores para a P4: PRA e PRB (VALADEZ-COSMES *et al.*, 2016). Uma forma adicional desses receptores, o PRC, foi detectada em linhagens de células de câncer de mama e pode inibir a atividade dos outros dois receptores na presença de P4 (VALADEZ-COSMES *et al.*, 2016).

A presença dos PR foi demonstrada no ovário de mulheres (IWAI *et al.*, 1990), macacas (HILD-PETITO *et al.*, 1988), vacas (VAN DEN BROECK *et al.*, 2002; D´HAESELEER *et al.*, 2007), porcas (SLOMCZYNSKA *et al.*, 2000), coelhas (IWAI *et al.*, 1991), cadelas (VERMEIRSCH *et al.*, 2001) e camundongas (GAVA *et al.*, 2004). Em humanos e outros primatas, PRA e PRB são expressos igualmente nas CG de folículos pré-ovulatórios (SUZUKI *et al.*, 1994; DUFFY E STOUFFER, 1995; LUCIANO *et al.*, 2010).

Em bovinos, foi demonstrado que a quantidade de PR aumenta conforme o folículo se desenvolve, indicando que a P4 pode regular o crescimento folicular durante os estádios iniciais do desenvolvimento folicular (D´HAESELEER *et al.*, 2007). Também foi verificado que os PR atuam mediando os efeitos protetores da P4 contra a apoptose em células da granulosa de folículos pré-ovulatórios bovinos (VALADEZ-COSMES *et al.*, 2016). No entanto, em alguns casos, o efeito de fatores pró-apoptóticos supera o efeito protetor da P4, e a degeneração folicular acontece. A figura abaixo demonstra o mecanismo de atuação de progesterona, que influencia a poliadenilação de cMOS e fosforilação de quinases que irão ativar a ciclina B culminando na retomada da meiose.

Figura 6. Sinalização de progesterona e sua influência na retomada da meiose



Fonte: D´HAESELEER *et al.* (2007).

2.6 Características moleculares de oócitos e células somáticas de folículos em diferentes estágios de desenvolvimento que influenciam a maturação oocitária *in vitro* e a produção de embriões

ARTIGO 1

Molecular characteristics of oocytes and somatic cells of follicles at different sizes that influence in vitro oocyte maturation and embryo production

Artigo de revisão aceito para publicação em Domestic Animal Endocrinology (Qualis A1 / Fator de Impacto: 2,30)

Molecular characteristics of oocytes and somatic cells of follicles at different stages of development that influence *in-vitro* oocyte maturation and embryo production

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Abstract

During the last 10-15 years, in-vitro research to predict antral follicle growth and oocyte maturation has delivered interesting advances in the knowledge of processes regulating follicle growth and developmental competence of oocytes. This review discusses the contribution of cumulus and mural granulosa cells in the process of oocyte maturation and cumulus expansion in cumulus oocyte complexes (COCs) from follicles of different sizes and shows that differences in gene expression in oocytes, granulosa and theca cells of small and large follicles impact the success of in-vitro blastocyst development. In addition, the molecular mechanisms by which COC metabolism and antioxidant defense provide oocyte

competence are highlighted. Furthermore, new insights and perspectives on molecular and cellular regulation of in-vitro oocyte maturation are emphasized.

Key-words: *oocyte, follicle size, meiotic competence, developmental competence, in-vitro culture*

1. **Introduction**

The study of *in vitro* maturation (IVM) of oocytes has provided evidence of molecular events related to oocyte competency and early embryo development. More recently, pre-maturation protocols and the discovery of new additives for culture media have demonstrated the potential to maximize oocyte competence and early embryo development [1,2,3,4] . However, the efficiency of bovine in-vitro embryo production (IVP) remains around 40% in oocytes from non-superovulated donors, despite extensive effort that has been made to understand the effects of culture conditions, media composition and hormone supplementation [5]. In goats and sheep, there are relatively few studies in this area, but the percentage of blastocysts ranges from 20% to 30% [6]. Part of the problem is due to the complexity of the molecular events, in which various hormones and growth factors are involved in the control of oocyte and follicle growth. It is also important to consider that bovine follicles ranging from 4.0 to 16.0 mm in diameter can be aspirated using the conventional ovum pick-up (OPU) system and, as a consequence, oocytes at different stages of cytoplasmic / molecular maturation are recovered and used for IVP [7]. Although live calves derived from oocytes recovered from small antral follicles have been reported by several research groups, the low viability of oocytes during in-vitro growth as well as the developmental competence of in-vitro grown (IVG) oocytes remain problematic [7,8,9]. The outcome of *in vitro* fertilization (IVF) of

oocytes recovered from small antral follicles can be an alternative to improve oocyte maturation and IVP of embryos [10], but the percentage of live births is still low [11]. On the other hand, studies have demonstrated that it is possible to increase developmental competence during IVM through optimal culture conditions and⁄or appropriate additives with impact on the establishment of epigenetic marks and acquisition of full developmental competency [12]. Since most of the RNAs/proteins that support the initial phases of embryogenesis are accumulated during oocyte growth, as determined from studies in the mouse [13], knowing the changes in gene expression during oocyte growth and competence will play a crucial role in the development of adequate assisted reproduction techniques.

In this sense, understanding the requirements of oocytes recovered from different sized follicles is very important to optimize IVM and IVF of oocytes, which can have an excellent impact on the number of embryos produced *in vitro*. The review highlights the progress made on *in vitro* maturation of oocytes and discusses the influence of oocyte and follicle sizes, as well as, the molecular characteristics associated with the acquisition of developmental competence during the IVM of oocytes from small and large antral follicles both at the cellular and molecular level.

2. **Influence of oocyte and follicle sizes on oocyte maturation**

It has been shown the size-specific follicle selection, rather than absolute culture time, seems to result in the identification of oocytes that will have the best meiotic and developmental outcomes *in vitro* [14]. Our review of the literature on this relationship is provided in Table 1. In cows, the relationship between follicle size and the quality of the enclosed oocyte has been clearly demonstrated [12,15]. Studies have reported that transcriptome analysis of oocytes from distinct follicle sizes can help to better understand the steps required for an oocyte to

acquire developmental competence [15]. Small antral follicles (1.0 - 3.0mm) have oocytes with a diameter of 100 to 110 µm and, at this size, the oocyte gradually achieves the capacity to undergo meiotic maturation, which includes a series of modulations of organelles and inclusions that are necessary for the oocyte to achieve developmental competence [16]. In this species, chromatin morphology of oocytes from small (0.5–2.0 mm) and medium-sized antral follicles (2.0–6.0 mm) have four discrete stages of germinal vesicle (GV), i.e., from GV0 to GV3, in which chromatin becomes progressively condensed [17,18]. The presence of functionally open gap junctions, mediating the communication between oocyte and cumulus cells, is responsible for keeping the chromatin uncondensed (GV0). When this communication is interrupted, the chromatin rapidly condenses and RNA synthesis suddenly ceases [19]. The GV2 and GV3 stages show the highest developmental capability [17]. These authors also remarked that the action of gap-junction communication on chromatin structure and function is mediated by cyclic AMP and that prolongation of gap junction coupling during oocyte culture before its in-vitro maturation enhances the ability of oocytes from small antral follicles to undergo meiosis [20]. Thus, a complex interplay of autocrine and paracrine signaling events between the oocyte and its surrounding cumulus and granulosa cells is responsible for maintaining the highly regulated synchrony of in-vivo maturation, and is critical for acquisition of oocyte developmental competence [20]. Figure 1 shows that reduced levels of cyclic AMP in bovine oocytes (>110µm) disrupt gap junctions among the encircling somatic cells, as well as between cumulus cells and oocyte [21, 22]. As a consequence, maturation promoting factor (MPF) destabilization triggers oocyte meiotic resumption. High levels of cyclic nucleotides in oocytes of ~100µm prevent destabilization of MPF and keep the oocyte at GV stage [22].

In caprine species, the ability of oocytes to complete meiotic maturation is acquired in 1.5 mm follicles [23]. Caprine oocytes gain the ability to assure embryo development until the

blastocyst stage in follicles larger than 2 mm, when most of them take on a GV3 configuration [23-24]. In sheep, studies have shown that about 48% of the oocytes obtained from small antral follicles (0.5–1.0 mm) are able to resume and complete meiotic maturation up to MII, when matured in co-culture with cumulus cells from medium-sized (3.0–4.0 mm) antral follicles [25]. Research reported that the integrity of cumulus cells is important to assure the developmental competence of ovine immature oocytes from antral follicles of 2.0 – 6.0 mm in diameter [26]. The low competence of development of *in vitro* cultured COCs collected from small antral follicles may be related to the inability of cumulus cells to secrete factors capable of activating signaling pathways that promote meiotic maturation in ovine species [27]. For example, other studies demonstrated that in rhesus macaques, specific concentration of amphiregulin (10 ng/ml) enhances nuclear maturation, but not cytoplasmic maturation in oocytes from small antral follicles (0.5 – 2.0 mm) [28].

In pigs, the ability of oocytes to complete meiotic maturation is acquired in antral follicles of about 2 mm in diameter [29,30] and the GV0 configuration of chromatin disappeared completely in follicles of about the same size [31]. Studies have shown that both equine chorionic gonadotrophin (eCG) and epidermal growth factor (EGF) decrease the effectiveness of gap junctional communication (GJC) during IVM of porcine COCs, which indicates that regulation of GJC between cumulus cells would then be specifically regulated during oocyte IVM [32]. In addition, porcine oocytes (113.0 µm) isolated from small antral follicles (1.2 – 1.5 mm) grew and acquired meiotic competence in medium supplemented with cyclic AMP and follicle stimulating hormone FSH after a 5-day culture period [33]. Studies showed that continuous high PKA activity is one of the primary causes of meiotic incompetence of oocytes (95-105 µm) from small porcine follicles (0.4 - 1.0 mm) and that this high PKA activity cannot simply be attributed to the cAMP concentration [34].

In human oocytes, gain of meiosis ability starts at the antral follicle stage, while its size reaches up to 100-120 μm [35]. Theoretically, antral follicles with diameter of 2.0-5.0 mm contain oocytes with nuclear and cytoplasmic competence [35]. However, the minimum size of follicles required for developmental competence in humans is estimated to be 5.0 - 7.0 mm in diameter [36]. It is possible that the selection of the dominant follicle induces changes in the remaining follicles that are detrimental for subsequent oocyte fertilization and embryonic development [37]. This information shows that oocyte competency is influenced by molecular events that are triggered in the different stages of follicular development. Table 1 presents the main changes in oocytes according to follicle size and species.

3. Gene expression in oocytes and granulosa cells from follicles of different sizes

It is well known that while the oocyte progresses in growth and development, it acquires maternal stores (mRNAs and proteins) which are essential to support the development of the embryo during the early cleavage stages [11]. Studies reported that in cattle, various mRNAs and miRNAs expressed in the oocyte and/or granulosa cells are involved in follicular recruitment, selection, dominance and atresia [38]. The interaction between mRNAs and miRNAs eventually determine ovarian follicle growth or atresia. Among them, FOXO1 and miR~183~96~182 cluster are novel molecular factors associated with ovarian follicle development. Higher expression of these miRNAs in granulosa cells of preovulatory follicles corresponds with lower expression of FOXO1 in dominant follicles and increased levels in subordinate follicles, suggesting that this gene has a role in follicle regression [38].

Evaluation of transcriptomes of oocytes from bovine follicles of different sizes (<3, 3-5, >5-8, and >8mm) showed very few differences between oocytes from small follicles (<3 versus 3-5mm), but various differences were detected in the levels of mRNA between oocytes from

larger follicles [15]. Changes in several genes involved in important functions, like transcriptional regulation (TAF2), chromatin remodeling (PPP1CB), energy production (SLC25A31), and transport of key molecules within the cell (NAGPA, CYHR1) were identified [15].

Higher abundance of TAF2 mRNA in oocytes with higher developmental competence suggests that it participates in post-fertilization activities perhaps involving embryonic genome activation [15]. The mitochondrial solute carrier family SLC25 is involved in the translocation of ATP and ADP across the inner mitochondrial membrane [39]. Several studies have correlated the oocyte ATP content and its developmental competence, both in humans [40] and bovine species [41,42,43,44]. NAGPA is a gene involved in the transport of hydrolases to lysosomes [45], a process that reflects the importance in translation and protein transport. Optimal control of protein degradation mechanisms likely ensures high developmental competence [15]. CYHR1 transcript abundance shows a constant decrease in oocytes as follicle size increases [46]. The products of CYHR1 gene may play a role during meiotic resumption in the bovine oocyte, possibly together with importins, which transports transcription and chromatin-remodeling factors into the nucleus [46]. In this way, depending on the size of the follicle, the abundance of transcripts stored in the oocytes has a crucial impact in the efficiency of oocyte maturation and early embryo development. Figure 2 presents the oocyte functions that are affected during follicular growth as well as genes related to these functions. Table 2 shows the role of genes, and their respective proteins, that are differentially expressed during follicular growth.

Within an antral follicle, the oocyte is surrounded by several layers of cumulus and mural granulosa cells. It is known that the characteristics of these cells vary according to the size of the follicle and this can directly affect the ability of the enclosed oocyte to undergo maturation. Studies reported the down-regulation of genes involved in cumulus expansion

(TNFAIP6, PTGS2 and PTX3) as well as of genes related to human oocyte maturation, including several EGF-like growth factors (EREG, AREG and BTC) in cumulus cells (CCs) from in-vitro matured oocytes in comparison with CCs from in-vivo matured oocytes [47]. These encircling somatic cells provide nutrients, survival factors, growth factors, and various signal molecules that are required for meiotic cell cycle progression from diplotene arrest to metaphase-II (M-II) arrest and to achieve meiotic competency in follicular oocytes [48]. Other studies showed that mouse oocytes are deficient in enzymes/pathways involved in synthesizing cholesterol and require cumulus cells to provide products of the cholesterol biosynthetic pathway [49]. Additionally, since oocytes are not able to take up L-alanine and to metabolize glucose for energy production, they obtain these amino acids and products of glycolysis from cumulus cells [50]. On the other hand, oocyte-derived bone morphogenetic protein 15 (BMP15) and growth differentiation factor 9 (GDF9) control cumulus cell metabolism, particularly glycolysis and cholesterol biosynthesis, by modulating the expression of enzymes required for cholesterol synthesis [49].

In the sheep model, a bidirectional talk within the follicle between the oocyte and its encircling granulosa cells determine the fate of both cell types. Analysis of the canonical pathways identified molecular events involved in these communications, including IGF1, VEGF, FGF, Notch and gap junction signaling pathways (CX43, CX37)[51]. Studies have demonstrated high expression of the IGFR1 gene in oocytes and high expression of the IGF-1 gene during oocyte-GC cross-talk [51]. In the same way, VEGFA was highly expressed in GCs while its receptor FLT1 was overexpressed in oocytes. The involvement of FGF2 in oocyte-GC dialog was also evidenced by the overexpression of FGF2 in oocytes and of FGFR1 and FGFR2 in granulosa cells (GCs) [51].

Peddinti et al. [52] reported that cumulus cells attached to oocytes from follicles with a diameter of 2.0–8.0 mm have higher expression of proteins involved in cell communication,

generation of precursor metabolites and energy than oocytes. Oocytes of small antral follicles are smaller in diameter and have thinner cumulus cell layers than those from medium antral follicles. Therefore, the gene expression profile of cumulus cells may be a valuable source of genetic markers for oocyte competence and thus may provide clues about how to improve assisted reproductive technologies in the future [53].

Hatzirodos et al. [54] showed that a large number of genes are expressed in the granulosa of antral follicles of different sizes, but small follicles have more genes that are variably expressed, particularly genes that are at least >2-fold more expressed in small follicles when compared to large follicles. These authors also reported that gene expression becomes less variable in large follicles. Genes related to developmental processes like those stimulated by KIT, IHH and MEST are most active in small follicles. Additionally, CNEPF, CCNE2, WNT2B genes undergo up-regulation in small follicles and down-regulation in large follicles, whereas the genes FRZB, CLDN11, AOX1, LTBP1, RARRES1 are up-regulated in large follicles and down-regulated in small follicles [54]. The main function of genes expressed in granulosa cells are shown in Table 2. In summary, somatic cells provide nutrients, growth factors, as well as regulate the expression of genes important for oocyte maturation and that this relationship is influenced by the characteristics of somatic cells which in turn are influenced by follicle size. Figure 3 presents changes in gene expression in oocyte and granulosa cell during antral follicle growth and in-vitro oocyte maturation.

4. Cellular and molecular organization in oocytes from follicles at different sizes

Oocyte cytoplasmic maturation comprises molecular changes, organelle reorganization and cytoskeletal component changes that contribute to acquisition of oocyte developmental competence [55]. In mammals, the number of mitochondria increases dramatically as the

oocyte grows. In cattle, a 45-fold increase in mitochondria occurs from the primordial germ cell to the preovulatory follicular stage [56], with a mean number of 260,000 mitochondrial copies in mature oocytes [57]. Mitochondrial respiration is a central determinant of developmental competence in mature and immature oocytes [58,59]. Therefore, the amount of mtDNA in the mature oocyte is a rate-limiting factor for the number of electron transport chain components formed. Human oocytes with high numbers of mitochondria are associated with high developmental competence [43]. On the other hand, others studies reported that the number of mtDNA copies in pigs mainly affects embryonic development potential, but has little effect on oocyte maturation and IVF [60]. It is accepted that the correct changes in the localization, morphology and biochemical properties of organelles and cytoskeleton must occur for the oocyte to acquire high developmental potency [61].

Studies conducted with the organelle reorganization in bovine oocytes during dominant follicle growth and regression and found that oocytes from follicles in the growing phase (<10 mm of diameter) display the least area of mitochondrial contact with lipid droplets, and a peripheral distribution of lipids [62]. Oocytes from follicles of 10.0-12.0 mm of diameter are similar to those from preovulatory phase follicles (≥17.0 mm of diameter): they have mitochondria in the peripheral region and increased contacts between mitochondria and lipid droplets compared to other phases [62]. Oocytes from regressing phase follicles (12.0 mm of diameter) are characterized by an increase in mitochondrial number, and an even distribution of mitochondria, and a greater percent lipid volume. Lipids and fatty acids constitute the main energetic source for protein synthesis during oocyte nuclear maturation and embryonic development [62]. Studies showed that genes involved in the cholesterol biosynthesis pathway (DHCR7, LDLR and VLDLR) are downregulated in in-vitro matured CCs in comparison with those in-vivo matured [51]. The data suggest that the IVM conditions alter

lipid metabolism in CCs, thus affecting their ability to provide the oocyte with lipid products and consequently guarantee oocyte nuclear maturation [53].

Based on previous studies, in all animals, oocytes must undergo a proper calcium response during fertilization in order for fertilization and subsequent development to proceed normally [63]. The oocyte-cell cycle progression to metaphase I (MI) and II (MII) stages is also related to transient fluctuation in cytosolic Ca2+. *In vitro*, extracellular Ca2+ performs a specific function for the initiation of meiotic resumption in caprine oocytes [64]. These results suggest that the occurrence of GVBD and cell cycle progression to the MI and MII stages is closely related to Ca2+, and that extracellular Ca2+ plays a specific role in initiating meiotic resumption in goat oocytes. Gonadotrophins and growth hormone (GH) cause an increase in Ca2+ concentration in cumulus cells of follicles from 2.0 to 6.0 mm in diameter, which is an important event during bovine meiotic maturation [20]. Studies suggest that STIM1, a transmembrane protein with a Ca2+ sensor domain located in the luminal space of the endoplasmic reticulum, is essential for normal fertilization, as it is involved in the maintenance of the long-lasting repetitive Ca2+ signal in mouse oocytes [65]. At fertilization, release in the cytosol is mediated by inositol 1,4,5 trisphosphate (IP3). It is believed that modifications of endoplasmic reticulum and IP3 receptors represent an increased sensitivity of mature oocytes to the mechanisms of release of Ca2+ that regulate a fertilization. Human oocytes display a similar pattern of changes in endoplasmic reticulum distribution and IP3 abundance [66]. These results show that at least one cytoplasmic change occurs during *in vitro* maturation of human oocytes that might be important for fertilization and subsequent embryonic development, but they suggest that a low developmental competence of *in vitro*-matured oocytes could be the result of deficiencies in the ability to release Ca2+ at fertilization. Therefore, during oocyte maturation, the endoplasmic reticulum undergoes significant changes in its distributions that are associated to an enhanced ability to release

Ca2+ [67]. In general, changes in the location, morphology and biochemical properties at the cellular and molecular level in oocytes of different follicular categories can explain these alterations and are related to the successful fertilization of the oocytes.

5. Influence of oocyte and follicle sizes on COC metabolism and oocyte antioxidant defense

Several studies have focused on the basic pattern of metabolism during follicle development and oocyte maturation and it is demonstrated that the antioxidant defense mechanism can impact the efficiency of oocyte maturation and early embryo development. Among the metabolic factors in mature porcine oocytes, low levels of intracellular reactive oxygen species (ROS) and high levels of intracellular glutathione (GSH) were demonstrated to be important for promoting oocyte maturation and supporting early embryonic development [68,69]. GSH is synthesized exclusively in the cytosol and becomes compartmentalized into separate redox pools in the endoplasmic reticulum, nucleus and mitochondria. GSH is synthesized during oocyte maturation, and its highest concentration is found at the MII stage, while it drops during the preimplantation development and reaches its lowest concentration at the blastocyst-stage [70]. In this same species, studies showed that oocytes from small follicles (1.0-2.0 mm of diameter) had a lower level of GSH than oocytes from medium follicles, indicating more incomplete cytoplasmic maturation [71].

Previous studies showed that bovine oocytes cultured in maturation medium supplemented with follicular fluid (FF) from large follicles (9.0-13.0 mm of diameter) had lower ROS levels and higher GSH levels [72]. Thus, it is speculated that higher superoxide dismutase (SOD) activity in the FF of large follicles might suggest a higher radical-scavenging activity, fewer ROS and a higher GSH level. It was also found that FF from large follicles improved

cytoplasmic maturation by increasing oocyte GSH levels [72]. However, the effects of glucose metabolism on the GSH and ROS content of porcine oocytes are not yet completely understood [69]. In this studyGSH levels inside cultured porcine oocytes decreased significantly when 200 μM of the pentose phosphate pathway (PPP) inhibitor dehydroepiandrosterone (DHEA) or 2μM of the glycolysis inhibitor iodoacetate (IA) were added to normal maturation medium, while DHEA also increased intracellular ROS levels. These results provide further evidence that the glucose metabolic pathway plays a role in the maintenance of high level of GSH and low level of ROS in oocytes.

Several studies have focused on human follicular fluid, a sample that is rather readily available and that represents the liquid milieu bathing and influencing the developing oocytes. Follicular fluid contains not only ROS but also antioxidants, with the total antioxidant capacity itself having been investigated as a predictive marker of successful IVF [73]. The beneficial effect of follicular fluid against oxidative damage may be in part due to an elevated activity of SOD isoenzymes, which act as important scavengers of free radicals [74]. Notably, a study in pig proposed a relationship between increased SOD activity in follicular fluid and an enhanced cytoplasmic maturation of the oocyte [74]. In contradiction, others studies documented that significantly higher SOD activities were found in human follicular fluid samples associated with oocytes that failed to become fertilized, emphasizing differences between species [75]. Presence of a minimal level of ROS can induce bovine oocytes to acquire developmental competence during IVM [76].

The basic pattern of metabolism during development and maturation of the oocyte is demonstrated as a dynamic process with the consumption of oxygen and the signaling of nutrients present in culture media; mainly glucose, pyruvate and lactate [77]. Induced-IVM systems that enhance oocyte quality, lead to increased lactate production by COCs over the course of IVM, suggesting stimulation of cumulus cell glycolysis [78]. Significantly, treating

COCs during pre-IVM with forskolin plus IBMX leads to intra-oocyte GSH accumulation in a pre-IVM duration-dependent manner, which is ablated when gap junctions are blocked [78,79]. This cAMP-mediated increase in GSH is associated with lower levels of H2O2, which suggests that a key benefit of cAMP-mediated IVM may be an improvement in the oocyte's antioxidant defenses, requiring GSH supplied by cumulus cells [79]. Cyclic AMP-modulated pre-IVM treatments also increase COC oxygen consumption and oocyte oxidative metabolism, associated with an increase in the oocyte redox ratio and a higher ATP:ADP ratio [78,80]. Glutathione ethyl ester (GEE) is a cell permeable GSH donor, that was shown to promote IVM or fertilization of bovine or macaque oocytes [81,82,83]. Therefore, activation of cAMP signaling pathways during oocyte maturation not only impacts on oocyte metabolism, but also on oocyte antioxidant defense, in a gap junction-dependent manner [79]. Taken together, the information described above provides evidence that there is a relationship between the production of molecules responsible for oocyte antioxidant defense and follicle / oocyte size in the effectiveness of this defense mechanism.

6. **Strategies to improve maturation rate of oocytes from antral follicles at different sizes**

The oocyte undergoes a remarkable long and complex journey within the follicle prior to reaching MII in a state that renders oocyte fully competent for fertilization, which is a process that relies on maternal control mechanisms established during oocyte maturation [84]. Studies have suggested that inhibition of spontaneous nuclear in-vitro maturation would allow more time for the oocytes to accumulate molecules. Thus, oocyte competence is acquired before IVM [1]. On the other hand, several studies demonstrated that it is possible to increase developmental competence during IVM through optimal culture conditions and⁄or appropriate

additives with impact on the establishment of epigenetic marks and acquisition of full developmental competency [21].

In pigs, studies have shown that oocytes from small *in vitro* grown antral follicles on medium containing cilostamide, a cAMP blocker, showed effective meiotic arrest, oocyte growth and better cumulus expansion [85]. Studies also reported that cilostamide and follicular hemisections inhibit premature meiotic maturation by maintaining cAMP concentration at an appropriate level in bovine COCs [3]. In ovine species, the combination of COC selection and prematuration with cilostamide and forskolin for 6 h improves both yield and quality of embryonic development [86]. In bovine oocytes derived from medium antral follicles, studies revealed that the inhibition of the EGFR pathway in the presence of FSH can effectively arrest bovine oocytes at GV stage during 15 h of culture *in vitro* without compromising their meiotic and developmental capacity [4]. This study shows that extending the duration of cAMP-mediated pre-IVM, to approximate a large part of the GV-GVBD interval, leads to improvements in subsequent oocyte quality. This adds to the growing body of evidence that cAMP-mediated pre-IVM/IVM has great benefit to the oocyte in terms of its capacity to support preimplantation embryo development [87]. Such IVM approaches are intended to better simulate *in vitro,* as close as possible, the natural process of oocyte maturation in vivo. A fundamental mode of action of cAMP-mediated pre-IVM systems appears to be the retention of CC-oocyte gap-junctional coupling during the critical first half of oocyte meiosis *in vitro*. This enhanced functional CC-oocyte coupling affects oocyte chromatin remodeling and transcription [19,88], oocyte metabolism [78,80], and accumulation of intra-oocyte GSH. In regard the human, it has been indicated that increase in time of hCG injection up to 38 h before oocyte retrieval results in more in-vivo matured oocytes with dispersed CC pattern, an increase in IVM rate and improved clinical outcome [89]. Thus, inhibition of the spontaneous

resumption of meiosis associated with the use of appropriate means may be a strategy for the acquisition of full developmental competent oocytes.

7. **Final considerations**

Since the first studies in the 1980s, significant advances in the development of methods for maturation of COCs from large follicles have been achieved. The relationship between the size of the follicle and the quality of the oocyte was clearly demonstrated in cows, so the follicle-size model helped to better understand the steps necessary for the oocyte to acquire competence. In other species, such as swine and sheep, several advances related to gene expression and to the process of molecular maturation were also achieved.. In addition, the maturation stage of granulosa cells is very important for the acquisition of competence of oocytes, especially in oocytes of small and medium-sized follicles, whose process of obtaining competence is limited. Further technological advances to optimize culture environments are needed to improve in-vitro maturation systems that can be effectively used in large animals for obtaining oocytes of higher competence, which may have impacts on the multiplication of genetically superior animals or on the treatment of ovarian diseases in humans.

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The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Figures and Tables

Figure 1. Schematic drawing of bovine granulosa/cumulus–oocyte complex presenting the processes that may lead to resumption of meiosis. (AR: Areg; BC: Betacelulin; ER: Ereg; CDC25:

phosphatases present in oocyte; Wee1b: oocyte-specific kinase; ERK-P: protein kinase present in oocyte; AP-1/ELK: transcription factor present in oocyte).

Figure 2. Genes and oocyte functions that are affected during follicular growth. During follicular development and oocyte growth is acquired maternal stocks (mRNAs and proteins) that are essential to support the development of the embryo. YTHDF2 gene post-transcriptionally regulates transcript dosage during oocyte maturation. FOXO1 and miR~183~96~182 cluster are novel molecular factors associated with ovarian follicle development. Higher expression of these miRNAs in granulosa cells of preovulatory follicles corresponds with lower expression of FOXO1 in dominant follicles and increased levels in subordinate follicles suggesting that this gene has a role in follicle regression. Changes in several genes involved in crucial functions, such as transcriptional regulation (TAF2), chromatin remodeling (PPP1CB), energy production (SLC25A31), as well as transport of key molecules within the cell (NAGPA, CYHR1) were identified during oocyte growth.

Figure 3. Changes in the genes expression (Down-regulated and Up-regulated) in oocyte and granulosa cell in small follicle and large follicle, as well as, genes involved in cumulus expansion and oocyte-CG cross-talk during *in vitro* oocyte maturation.

Table 1. Characteristics of oocytes according to follicle size and species.

Table 2. Genes involved with oocyte maturation and the function of their respective proteins.

Figure 1

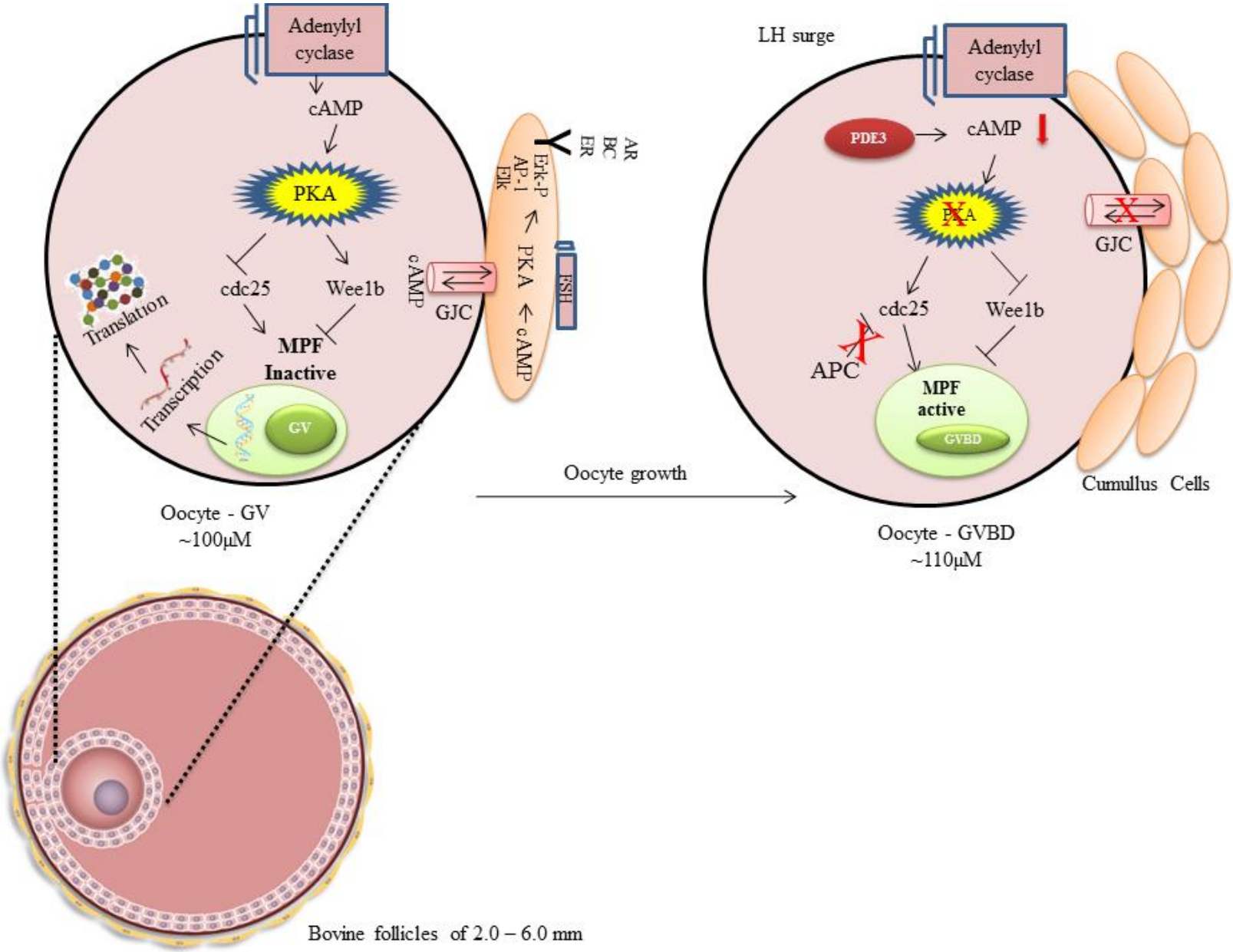

Adenylyl cyclase
cAMP
PKA
cdc25
Wee1b
MPF Inactive
Translation
Transcription
GV
cAMP
GJC
Erk-P
AP-1
Elk
PKA
cAMP
AR
BC
ER
FSH
Oocyte - GV
~100µM
Oocyte growth
LH surge
Adenylyl cyclase
PDE3
cAMP
PKA
GJC
cdc25
Wee1b
APC
MPF active
GVBD
Cumullus Cells
Oocyte - GVBD
~110µM
Bovine follicles of 2.0 – 6.0 mm

Figure 2

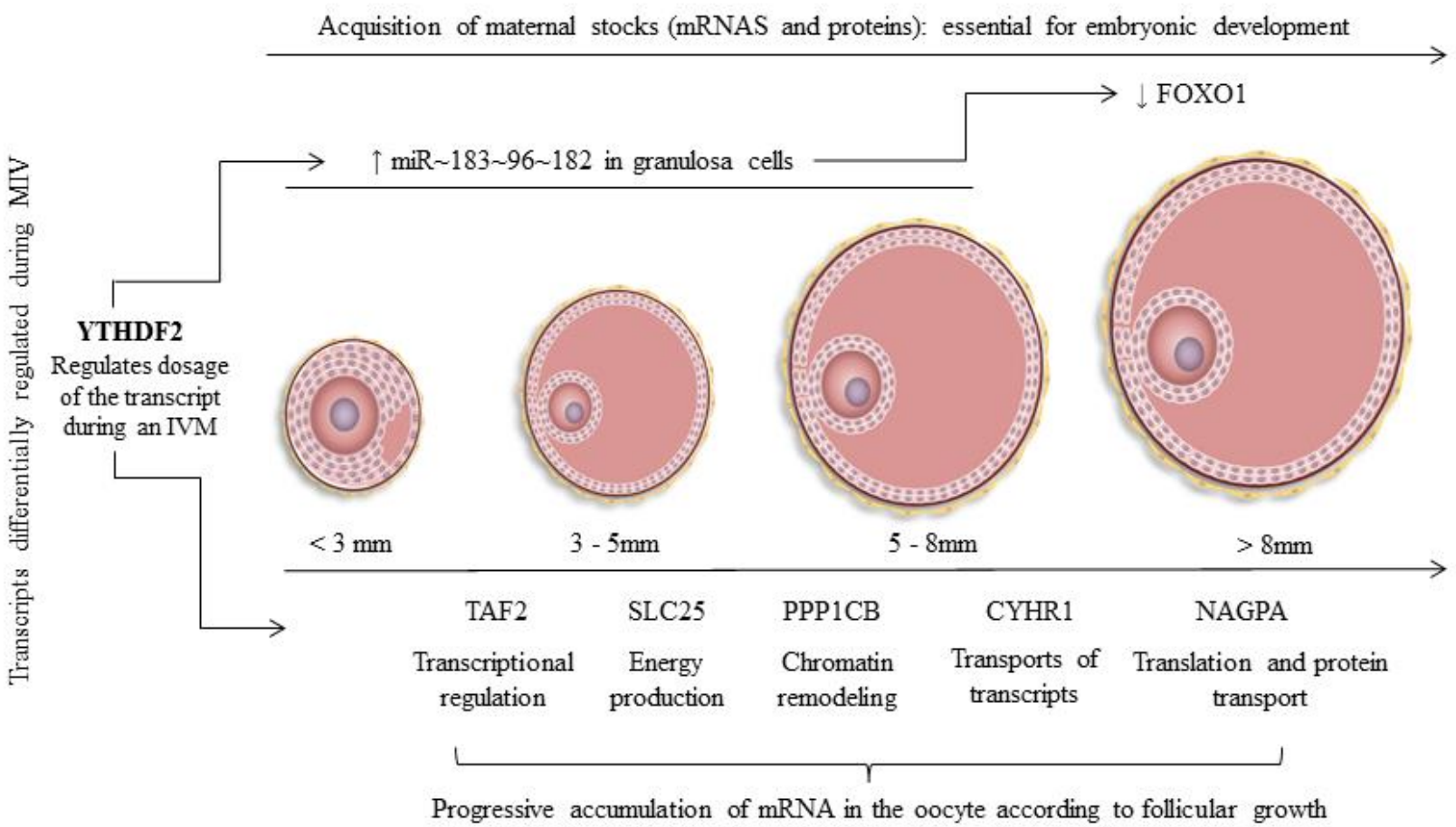
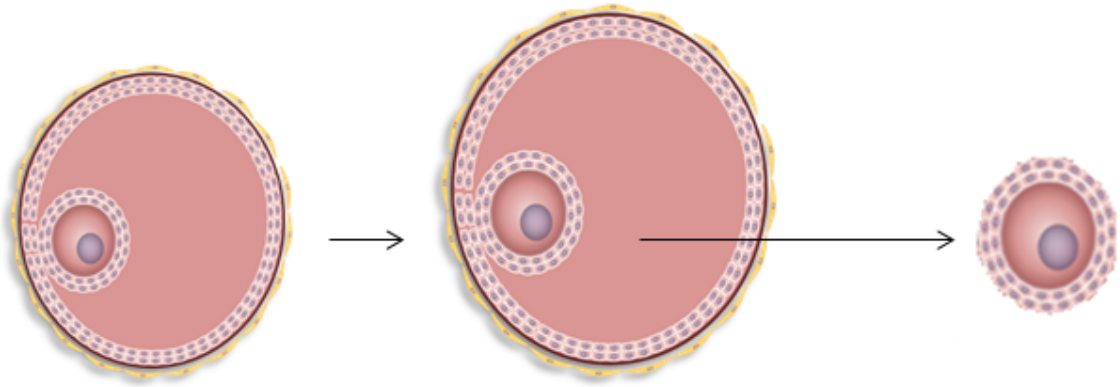

Acquisition of maternal stocks (mRNAS and proteins): essential for embryonic development
↓ FOXO1
↑ miR~183~96~182 in granulosa cells
Transcripts differentially regulated during MIV
YTHDF2
Regulates dosage of the transcript during an IVM
< 3 mm
3 - 5mm
5 - 8mm
> 8mm
TAF2
Transcriptional regulation
SLC25
Energy production
PPP1CB
Chromatin remodeling
CYHR1
Transports of transcripts
NAGPA
Translation and protein transport
Progressive accumulation of mRNA in the oocyte according to follicular growth

Figure 3



Small follicle (3- 5mm)

Large follicle (9 - 12mm)

Cumulus-oocyte complex

Up-regulated genes in small follicle

- CNEPF
- CCNE2
- WNT2B

Down-regulated genes in small follicle

- FRZB
- CLDN11
- AOX1
- LTBP1
- RARRES1

Up-regulated genes in large follicle

- FRZB
- CLDN11
- AOX1
- RARRES1
- LTBP1

Down-regulated genes in large follicle

- WNT2B
- CCNE2
- CENPF

Over expression genes in oocyte during in vitro oocyte maturation.

- IGFR1
- FLT1
- FGF2

Down-regulation Genes and growth factors involved in cumulus expansion of oocyte in MII

- TNFAIP6
- PTGS2
- PTX3
- EREG
- AREG
- BTC

Pathways identified molecular events involved in oocyte-GC cross-talk

- IGF1
- VEGF
- FGF
- NOTCH

Table 1. Characteristics of oocyte according to follicle size and species.

Species	Follicle size	Characteristics of oocytes	Reference
Ovine	**0.5–1.0mm**	Oocytes are able to resume and complete meiotic maturation up to MII, when matured in co-culture with gonadotropin-stimulated cumulus cells from medium-sized antral follicles (3.0–4.0 mm).	[25]
Caprine	**1.5 mm**	Oocytes become MII-competent in 1.5 mm follicles and gain the ability to assure embryo development until the blastocyst stage in follicles larger than 2.0 mm.	[22, 24]
Swine	**0.4-1.0mm**	Continuous high PKA activity is one of the primary causes of meiotic incompetence of oocytes (95-105µm) from porcine small follicles.	[27, 31, 33]
	2.0 mm	The ability of oocytes to complete meiotic maturation is acquired. eCG and EGF decrease the effectiveness of GJC during IVM; The increase of MPF activity at both meiotic resumption and development to MII requires phosphorylation of CDC2 during porcine oocyte maturation.	
Bovine	**0.5 – 6.0 mm**	At a diameter of 100 to 110 µm, the oocyte gradually achieves the capacity to undergo meiotic maturation, which includes a series of modulations of organelles and inclusions that are necessary for the oocyte to achieve developmental competence.	[17-20]
Human	**2.0 – 5.0 mm**	Oocytes (100-120µm) have the ability to resume meiosis	[36, 37]
	5.0 – 7.0 mm	It is estimated the minimum size of follicle required for developmental competence.	

Table 2. Genes involved with oocyte maturation and the function of their respective proteins.

Genes	Functions	References
Aldehyde oxidase 1 (AOX1)	Produces hydrogen peroxide and, under certain conditions, can catalyze the formation of superoxide	[101]
Amphiregulin (AREG)	Propagates the LH signal throughout the follicle and controls oocyte maturation.	[83]
Betacellulin (BTC)	Play roles in oocyte maturation, cellular growth and differentiation.	[102]
Centromere protein F, 350/400 kDa (mitosin) (CNEPF)	Nuclear protein transiently associated with the outer kinetochore plate in M phase and is involved in M phase progression.	[65]
Claudin 11(CLDN11)	It is a tight junction protein that regulates cellular adesion.	[103]
Cyclin E2 (CCNE2)	It belongs to the cyclin family and regulates the cell cycle.	[104]
Cysteine and histidine rich 1 (CYHR1)	Interacts with importin alpha2 and regulates protein transport.	[105]
Epiregulin (EREG)	Controls oocyte maturation and embryo development.	[106]
Fms related tyrosine kinase 1 (FLT-1)	Acts as a receptor for VEGFA and controls angiogenesis and the communication between oocyte and GC	[57]
Forkhead Box O1(FOXO1)	It is a transcription factors that regulates apoptosis, cell-cycle arrest, oxidative stress resistance, DNA repair, glucose metabolism, and cell differentiation.	[107]
Frizzled related protein (FRZB)	Regulates early embryonic development.	[108]
Indian hedgehog (IHH)	Plays a role in cellular growth and differentiation and control follicle development.	[109]
Insulin like growth factor 1 receptor (IGFR1)	Regulates oocyte-GC cross-talk and has a crucial role in granulosa cells function.	[110]
Insulin like growth factor 1(IGF-1)	Amplify gonadotropin action and increases steroidogenesis by granulosa cells.	[111]

Latent transforming growth factor beta binding protein 1 (LTBP1)	Involved in the assembly, secretion and targeting of TGFB1 to sites at which it is stored and/or activated. May have a structural role in the extracellular matrix.	[112]
Mesoderm specific transcript (MEST)	It is a member of the alpha/beta hydrolase superfamily that is highly expressed in the oocyte. Involved in the control of adult behavior.	[113]
microRNA 133a (miR-133a)	Regulates the expression of cyclin B gene and controls oocyte maturation.	[114]
microRNA 152 (miR-152) / microRNA 142-5p (miR-142-5p)	Regulates oocyte metabolism, it is stored in the oocyte to act during early embryo development	[115]
microRNA 205-5p (miR-205-5p)	Regulates oocyte maturation and the early stages of embryo development.	[116]
N-Acetylglucosamine-1-phosphodiester alpha-N-acetylglucosaminidase (NAGPA)	It is involved in the transport of hydrolases to lysosomes and controls protein degradation.	[16]
NOTCH	Key signaling pathway for cell proliferation and differentiation.	[117]
Pentraxin 3 (PTX3)	Stimulates synthesis of hyaluronan-rich extracellular matrix and cumulus cells expansion.	[118]
Prostaglandin-endoperoxide synthase 2 (PTGS2)	Reduces to apoptosis and it is involved with cumulus expansion.	[119]
Protein phosphatase 1 catalytic subunit beta (PPP1CB)	Regulates condensation of chromatin in oocytes during maturation.	[51]
Proto-oncogene receptor tyrosine kinase (KIT)	Regulates oocyte growth and follicular development.	[120]
Retinoic Acid Receptor Responder 1 (RARRES1)	The encoded protein was identified as a retinoid acid receptor-responsive gene.	[121]
Solute Carrier Family 25 Member 31 (SLC25A31)	Controls translocation of ATP across mitochondrial membrane and regulates ATP content in oocytes.	[122]
TATA-Box Binding Protein Associated Factor 2 (TAF2)	Participates in post-fertilization activities and it is involved in embryonic genome activation.	[16]
Tumor necrosis factor alpha-inducible protein 6 (TNFAIP6)	The protein encoded by this gene contains a hyaluronan-binding domain that regulates cumulus cells expansion.	[123]
Vascular endothelial growth factor A	Controls ovary angiogenesis and the communication between oocyte and	[124]

(VEGFA)	GC.	
Wingless-type MMTV integration site family, member 2B (WNT2B)	Regulation of cell growth and differentiation	[65]
YTH N6-Methyladenosine RNA binding protein 2 (YTHDF2)	It is required to produce MII oocytes which are competent to support initial zygotic development.	[43]

3 JUSTIFICATIVA

O estudo dos eventos moleculares que ocorrem durante o crescimento e maturação oocitária é de grande importância para o desenvolvimento de estratégias que visem aumentar a eficiência da produção *in vitro* de embriões a partir de oócitos oriundos de folículos secundários, bem como, de pequenos e médios folículos antrais.

A produção *in vitro* de embriões bovinos tem contribuído de forma significativa para aumentar o número de descendentes de fêmeas de alto valor genético, pois é uma técnica aplicada em escala comercial que possibilita a obtenção de 50 a 100 embriões/fêmea/ano. No entanto, a percentagem de oócitos que não alcança o estágio de blastocisto ainda é relativamente alta. Desta forma, é essencial a realização de estudos para avaliar se ineficiência observada durante os processos de maturação nuclear e desenvolvimento embrionário inicial estão relacionadas com os níveis de RNAs mensageiros para proteínas (PARN e IF4E) que regulam o estoque citoplasmático dos RNAs, bem como com os níveis de RNAs mensageiros que são estocados no oócito (GDF-9, histona H1FOO, CCNB1 e quinase cMOS).

É importante ressaltar que muitos destes RNAs mensageiros estocados codificam proteínas que regulam diretamente a maturação meiótica e desenvolvimento embrionário inicial. Assim, esse estudo pode contribuir para o desenvolvimento de novas metodologias com o objetivo de potencializar o período de maturação oocitária *in vitro* e possibilitar uma rápida multiplicação de bovinos geneticamente superiores contribuindo para o desenvolvimento da economia nacional.

Além disso, as informações da biologia molecular podem contribuir para o desenvolvimento de sistemas de cultivo *in vitro*, pois será possível avaliar se a adição de fatores de crescimento (EGF) e hormônios (P4), tem impactos positivos na expressão de RNAs mensageiros para proteínas que regulam o estoque citoplasmático dos RNAs, bem como com os níveis de RNAs mensageiros estocados no oócito.

Associado a isso, o crescimento, a pré-maturação e a maturação *in vitro* dos oócitos oriundos de pequenos e médios folículos antrais pode contribuir para otimizar o desenvolvimento de sistemas de cultivo para folículos pré-antrais. O estudo do crescimento *in vitro* de folículos antrais e pré-antrais é de grande importância tanto para a pesquisa fundamental, quanto para a reprodução animal. Além disso, o presente trabalho fornece base para novas pesquisas que tem como objetivo entender os eventos moleculares que ocorrem

durante a maturação *in vitro*, aumentanto assim a eficiência da técnica de produção *in vitro* de embriões no Brasil.

4 HIPÓTESES

1) Os níveis de RNAm para eIF4E, PARN, H1FOO, cMOS, GDF9 e CCNB1 aumentam durante o desenvolvimento de folículos secundários até os estágios de folículos antrais;

2) O cultivo in vitro de folículos secundários por 18 dias eleva os níveis de RNAm para eIF4E, PARN, H1FOO, cMOS, GDF9 e CCNB1;

3) A presença de EGF e/ou P4 durante o cultivo in vitro de oócitos derivados de pequenos e médios folículos antrais, aumentam os níveis de RNAm para eIF4E, PARN, H1FOO, cMOS, GDF9 e CCNB1, bem como, as taxas de retomada de meiose.

5 OBJETIVOS

5.1 Objetivo Geral

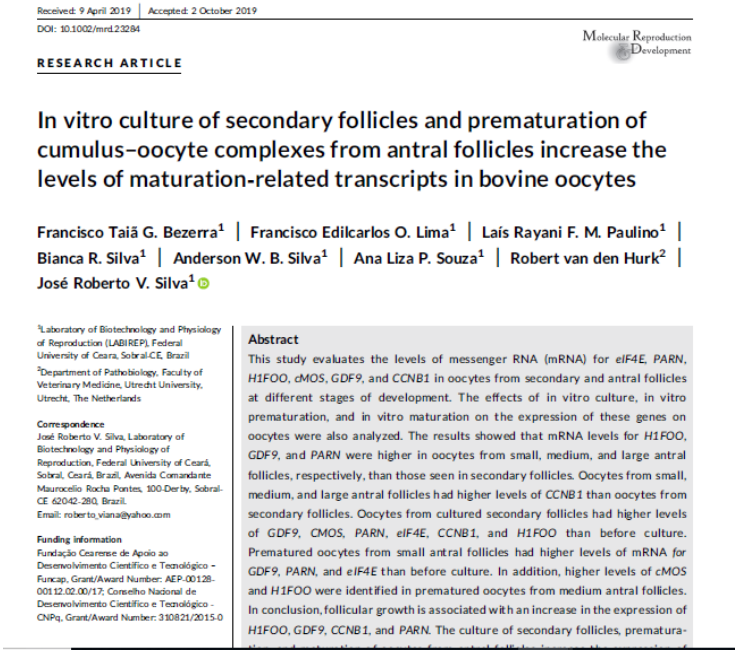
Avaliar os níveis de RNAm para eIF4E, PARN, H1FOO, cMOS, GDF9 e CCNB1 em oócitos de folículos secundários e em oócitos derivados de pequenos e médios folículos antrais, submetidos às etapas de crescimento e pré-maturação na presença de EGF e/ou P4.

5.2 Objetivos Específicos

- Avaliar os níveis de RNAm para eIF4E, PARN, H1FOO, cMOS, GDF9 e CCNB1 em oócitos de folículos secundários e de folículos antrais pequenos, médios e grandes e em folículos secundários cultivados *in vitro* por 18 dias;
- Avaliar os níveis de RNAm para eIF4E, PARN, H1FOO, cMOS, GDF9 e CCNB1em oócitos derivados de pequenos e médios folículos antrais submetidos ao período de crescimento oocitário *in vitro* na presença de EGF e/ou P4*;*
- Avaliar a taxa de crescimento *in vitro* de oócitos derivados de pequenos folículos antrais na presença de EGF e/ou P4;
- Avaliar a taxa de VG e retomada da meiose (RVG) em oócitos submetidos ao período de crescimento, pré-maturação e maturação *in vitro.*

6 CAPÍTULO I – ARTIGO II

In vitro culture of secondary follicles and prematuration of cumulus oocyte complexes from antral follicles increase the levels of maturation-related transcripts in bovine oocytes



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RESEARCH ARTICLE

In vitro culture of secondary follicles and prematuration of cumulus–oocyte complexes from antral follicles increase the levels of maturation-related transcripts in bovine oocytes

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Abstract
This study evaluates the levels of messenger RNA (mRNA) for *eIF4E*, *PARN*, *H1FOO*, *cMOS*, *GDF9*, and *CCNB1* in oocytes from secondary and antral follicles at different stages of development. The effects of in vitro culture, in vitro prematuration, and in vitro maturation on the expression of these genes on oocytes were also analyzed. The results showed that mRNA levels for *H1FOO*, *GDF9*, and *PARN* were higher in oocytes from small, medium, and large antral follicles, respectively, than those seen in secondary follicles. Oocytes from small, medium, and large antral follicles had higher levels of *CCNB1* than oocytes from secondary follicles. Oocytes from cultured secondary follicles had higher levels of *GDF9*, *CMOS*, *PARN*, *eIF4E*, *CCNB1*, and *H1FOO* than before culture. Prematured oocytes from small antral follicles had higher levels of mRNA for *GDF9*, *PARN*, and *eIF4E* than before culture. In addition, higher levels of *cMOS* and *H1FOO* were identified in prematured oocytes from medium antral follicles. In conclusion, follicular growth is associated with an increase in the expression of *H1FOO*, *GDF9*, *CCNB1*, and *PARN*. The culture of secondary follicles, prematura-

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In vitro culture of secondary follicles and prematuration of cumulus oocyte complexes from antral follicles increase the levels of maturation-related transcripts in bovine oocytes

In vitro culture increases the levels of transcripts in bovine oocytes

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Abstract

This study evaluates the levels of mRNA for *eIF4E, PARN, H1FOO, cMOS, GDF9* and *CCNB1* in oocytes from secondary and antral follicles at different stages of development. The effects of *in vitro* culture, *in vitro* prematuration and *in vitro* maturation on the expression of these genes on oocytes were also analyzed. The results showed that mRNA levels for *H1FOO, GDF9* and *PARN* were higher in oocytes from small, medium and large antral follicles, respectively, than those seen in secondary follicles. Oocytes from small, medium and large antral follicles had higher levels of *CCNB1* than oocytes from secondary follicles.

Oocytes from cultured secondary follicles had higher levels of *GDF9, CMOS, PARN, eIF4E, CCNB1* and *H1FOO* than before culture. Pre-matured oocytes from small antral follicles had higher levels of mRNA *for GDF9, PARN* and *eIF4E* than before culture. In addition, higher levels of *cMOS* and *H1FOO* were identified in pre-matured oocytes from medium antral follicles. In conclusion, follicular growth is associated with an increase in the expression of *H1FOO, GDF9, CCNB1* and *PARN*. The culture of secondary follicles, prematuration and maturation of oocytes from antral follicles increase the expression of *eIF4E, PARN, H1FOO, cMOS, GDF9* and *CCNB1*.

Keywords: oocyte growth, follicular development, *in vitro* prematuration, *in vitro* maturation

1. Introduction

In vitro culture of preantral follicles, as well as prematuration and *in vitro* maturation of oocytes (IVM) are reproductive tools of great potential and high applicability. The application of these biotechnologies has enabled investigations of factors related to oocyte development and follicular atresia. Once optimized, these techniques can improve *in vitro* embryo production from oocytes enclosed in preantral and early antral follicles (Max *et al.*, 2018).

Continuous follicular growth is controlled both by hormones and growth factors that act, directly or indirectly, in an autocrine and/or paracrine manner (Figueiredo *et al.*, 2011). Studies have shown that the extent of oocyte growth, which may be represented by follicular size, is closely related to success of *in vitro* oocyte maturation. Many studies have demonstrated that as follicular volume increases, the oocyte maturation rate, fertilization rate, and blastocyst rate will increase (DIAS *et al.*, 2013; YOON *et al.*, 2015). During growth, oocytes synthesize large quantities of mRNA that accumulates to form a large stock of maternal mRNA. When oocytes reach their full size, they undergo changes in nuclear

morphology due to large-scale chromatin condensation (Vieux & Clarke, 2018). Histone marks associated with active chromatin are replaced by repressive histone modifications in the nucleus of the fully grown oocyte and the nucleus becomes transcriptionally inactive (Tanaka *et al.,* 2005). When fully grown oocytes enter meiotic maturation, many previously active mRNAs become translationally silenced, whereas previously silent mRNA becomes activated (Vieux & Clarke, 2018).

It is known that some mRNAs are translated in oocytes between metaphase I (MI) and MII, whereas others are translated during fertilization or during the early stages of embryogenesis. Regarding the mechanisms that regulates the storage and translation of mRNAs, one that is particularly important is cytoplasmic polyadenylation (Richter & Lasko, 2011). During meiotic maturation of bovine oocytes, the translation initiation factor, *eIF4E* (the cap binding protein), gradually becomes phosphorylated. This substantial phosphorylation begins at the time of germinal vesicle breakdown (GVBD) and continues to the metaphase II stage. The onset of *eIF4E* phosphorylation occurs in parallel with a significant increase in overall protein synthesis (Tomek *et al.,* 2002). *PARN* (poly(A) ribonuclease) is the second factor; as the name suggests, it is a deadenylating enzyme (Korner *et al.,* 1998; Copeland & Wormington, 2001). Others enzymes are active, but because *PARN* is the more active, the poly(A) tail originally acquired in the nucleus is now shortened, to about 20–40 bases. In other words, polyadenylation is controlled by the presence or absence of *PARN*, and not by the recruitment of another poly(A) polymerase (Richter, 1999). In addition to these genes that are involved with the translation process, transcripts that are stored in the oocytes are quite important for oocyte development. Among these transcripts is oocyte-specific linker histone (*H1FOO*) that is localized on the oocyte chromosomes during the process of meiotic maturation (Yan *et al.,* 2015). *H1FOO* is synthesized and accumulates during oogenesis, and its presence is maintained until some stage of the early embryo, at

which rapid degradation follows (Tanaka *et al.,* 2001, 2005; Fu *et al.,* 2003). Oocyte maturation factor Mos (*cMOS*) is kinase expressed exclusively in germ cells, studies show an important role in oocyte maturation (McPherron *et al.,* 1993), since the *cMOS*-MAPK1/3 pathway is important for maintaining MII arrest (Phillips *et al.,* 2002). Cyclin B1 (*CCNB1)* is regulatory unit of maturation-promoting factor (MPF) and plays an important role in oocyte maturation (Wu *et al.,* 1997). Studies show that growth differentiation factor-9 (*GDF9*) may be secreted by oocyte and granulosa cells (Silva *et al.,* 2004; Spicer *et al.,* 2008) and promotes theca and granulosa cell proliferation, stimulates the expression of genes in cumulus cells (Elvin *et al.,* 1999a, 2000) and is involved in the process of oocyte maturation by regulating the function of cumulus cells from preovulatory follicles (Gui *et al.,* 2005). In this sense, it is hypothesized that preantral follicle culture, *in vitro* prematuration and maturation of oocytes, increase the levels of these mRNAs and have a positive impact on oocyte maturation *in vitro*. Additionally, it is unknown if there is a difference in the expression of these genes among oocytes of preantral follicles and of small, medium and large antral follicles. Monitoring maternal mRNA accumulation during oocyte growth is very important to understand if changes in oocyte transcript levels can provide a signature for developmental competence. Knowing the levels of mRNAs in oocytes of follicles at different stages of growth is very useful for the development of *in vitro* culture systems to improve oocyte competence.

The objectives of this study is to evaluate the levels of mRNA for *eIF4E*, *PARN*, *H1FOO*, *cMOS*, *GDF9* and *CCNB1* in oocytes derived from different follicular categories, i.e., secondary follicles, and small, medium and large antral follicles. The levels of these mRNA were also evaluated in preantral follicles before and after 18 days of culture, as well as in oocytes of small and medium antral follicles before and after prematuration and maturation *in vitro*.

2. Materials and methods

Unless indicated otherwise, culture media and other chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.1. Experiment 1: Levels of mRNAs for GDF9, CCNB1, H1FOO, cMOS, PARN and eIF4E in oocytes from in vivo grown secondary follicles and small, medium and large antral follicles

Cow ovaries (n= 40) were obtained from a local abattoir and transported within 1 h to the laboratory in saline solution (0.9% NaCl; 30°C) containing 100 IU/mL penicillin and 50 mg/mL streptomycin sulfate. In the laboratory, the ovaries were washed in saline solution and an average of 50 oocytes from different categories, i.e., secondary follicles (~0.2 mm in diameter), small antral follicles (1.0 – 3.0 mm in diameter), medium antral follicles (3.0 – 6.0 mm in diameter) and large antral follicles (> 6 mm in diameter) were collected.

To obtain the oocytes from secondary follicles, ovarian cortical slices (1–2 mm thick) were cut from the ovarian surface using a surgical blade under sterile conditions. The ovarian cortex was subsequently placed in fragmentation medium, consisting of TCM-199 supplemented with 100 mg/mL penicillin and 100 mg/mL streptomycin. Secondary follicles (~0.2mm) were then visualized under a stereo-microscope (SMZ645 Nikon, Tokyo, Japan) and manually micro-dissected, using 26 gauge (26G) needles, attached to 1 mL syringes. After isolation, follicles were transferred to 100 µL drops, containing fresh medium to evaluate the follicular quality further. Then, a rupture was performed in the follicle and the collection of four groups containing ten oocytes was performed. Oocytes were stored at -80 ° C for RNA extraction. To obtain oocytes from small, medium and large antral follicles, bovine ovaries (n = 160) were obtained from a slaughterhouse and transported to the laboratory in saline solution (0.9 % NaCl) containing antibiotics (100 IU / mL penicillin and

50 μg / mL streptomycin sulfate) at 30° C, within a maximum period of 1 h. In the laboratory, the ovaries were washed in saline solution and COCs were aspirated from small (1.0–3.0 mm in diameter), medium (3.0–6.0 mm) and large (> 6.0 mm) antral follicles using a 21 gauge needle connected to a sterile syringe. Then, the cumulus cells were removed by vortexing and the oocytes were stored at -80° C for RNA extraction. Oocytes collected from large antral follicles (> 6.0 mm in diameter) were used as fresh control in the comparison of oocytes from secondary follicle and oocytes from small, medium and large antral follicles.

Total RNA was extracted using the Trizol reagent (Invitrogen, São Paulo, Brazil). According to the manufacturer's instructions, 1 mL of Trizol solution was added to each frozen samples and the lysate was aspirated through a 20-gauge needle before centrifugation at 10,000 g for 3 min at room temperature. Thereafter, all lysates were diluted 1:1 with 70% ethanol and subjected to a mini-column. After binding of the RNA to the column, DNA digestion was performed using RNAse-free DNAse (340 K unitz units/mL) for 15 min at room temperature. After washing the column three times, the RNA was eluted with 30 mL RNAse-free water.

The RNA concentration was estimated by reading the absorbance at 260 nm and was checked for purity at 280 nm in a spectrophotometer (Amersham, Biosciences Cambridge, England). Before the reverse transcription reaction, samples of RNA were incubated for 5 min at 70°C and then cooled in ice. Reverse transcription was performed in a total volume of 20 μL composed of 10 μL of sample containing 2 mg of RNA, 4 μL reverse transcriptase buffer (Invitrogen, São Paulo, Brazil), 8 units RNAsin, 150 units of reverse transcriptase Superscript III, 0.036U random primers, 10 mM DTT and 0.5 mM of each dNTP (Invitrogen, São Paulo, Brazil). The mixture was incubated at 42 1C for 1 h, subsequently at 80 °C for 5 min, and finally stored at -20 °C. The negative control was prepared under the same conditions, but without addition of reverse transcriptase.

Quantification of the mRNA for *GDF9, CCNB1, H1FOO, cMOS, PARN* and *eIF4E* was performed by using SYBR Green. Each reaction in real time (20 μL) contained 10 μL of SYBR Green Master Mix (Applied Biosystems, Warrington, UK), 7.3 μL of ultrapure water, 1 μL of cDNA and 5 mM of each primer. Real time PCR was performed in at thermocycler (Mastercyclers ep Realplex, Eppendorf, Germany). The primers designed to perform amplification of mRNA for *CCNB1, H1FOO, GDF9, cMOS, PARN* and *eIF4E* are shown in Table 1. This table also shows glyceraldehyde3-phosphatedehydrogenase (*GAPDH*), which was used as endogenous controls for normalization of messenger RNA expression (Rebouças *et al.,* 2013; Rossi *et al.,* 2015; Rossi *et al.,* 2016; Bezerra *et al.,* 2016; Silva *et al.,* 2017). The specificity of each primer pair was confirmed by melting curve analysis of PCR products. The thermal cycling profile for the first round of PCR was initial denaturation and activation of the polymerase for 10 min at 95 ℃, followed by 40 cycles of 15 s at 95 ℃, 30s at 58 ℃, and 30 s at 72 ℃. The final extension was for 10 min at 72 ℃. Primer efficiency was determined using serial dilutions of the target cDNA. All reactions were performed in triplicate in a real time PCR Mastercyclers Realplex (Eppendorf Germany). The negative control was prepared under the same conditions but without addition of cDNA. The delta–delta-CT method was used to transform CT values in to normalized relative messenger RNA expression levels (Livak & Schmittgen, 2001).

The data of expression of mRNA in oocytes from in vivo grown secondary follicles and small, medium and large antral follicles were analyzed by the Kruskal-Wallis test, followed by the post hoc non-parametric test: the Dunn's Multiple Comparison Test. Differences were considered significant when $P<0.05$.

2.2 *Experiment 2: Effects of in vitro culture on the levels of mRNAs for GDF9, CCNB1, H1FOO, cMOS, PARN and eIF4E in oocytes from secondary follicles*

Cow ovaries (n= 40) were obtained from a local abattoir and transported within 1 h to the laboratory in saline solution (0.9% NaCl; 30 °C) containing 100 IU/mL penicillin and 50 mg/mL streptomycin sulfate. In the laboratory, ovarian cortical slices (1–2 mm thick) were cut from the ovarian surface using a surgical blade under sterile conditions. The ovarian cortex was subsequently placed in fragmentation medium, consisting of TCM-199 supplemented with 100 mg/mL penicillin and 100 mg/mL streptomycin. Preantral follicles (~0.2mm) were then visualized under a stereo-microscope (SMZ645 Nikon, Tokyo, Japan) and manually micro-dissected, using 26 gauge (26G) needles, attached to 1 mL syringes. The isolation of the secondary follicles was performed as described in experiment 1. An average of 20 follicles was isolated from each animal. After isolation, follicles were transferred to 100 µL drops, containing fresh medium to evaluate the follicular quality further. Follicles with a visible oocyte, surrounded by two or more layers of granulosa cells, an intact basement membrane and without antral cavity were selected for culture. For *in vitro* studies, selected follicles (n= 100) were individually cultured in 100 µL drops of culture medium in Petri dishes (60 x 15mm; Corning, USA) under mineral oil. The basic culture medium consisted of TCM-199 (pH 7.2 – 7.4) supplemented with supplemented with 10 µg/mL insulin, 5.5µg/mL transferrin and 5 ng/mL selenium (ITS), 3.0 mg/mL bovine serum albumin (BSA), 2mM glutamine, 2 mM hypoxanthine, 50 µg/mL of ascorbic acid and 100 ng/mL FSH) (ovine FSH, Sigma, St. Louis, MO, USA). Incubation was done at 39ºC, 5% CO_2 in air for 18 days. Every other day, 60 µL of the culture media were replaced with fresh medium. Fresh medium was prepared and incubated for 1 h prior to use. At days 0 and 18, follicle diameters were evaluated by performing two perpendicular measurements using an inverted microscope with Nis elements 2.4 software (Nikon, Nikon Instruments Inc., Japan). After 18 days of culture, follicles were ruptured and oocytes were collected and stored in micro-centrifuge tubes at -80

°C, until RNA extraction. Quantification of the mRNA for *GDF9, CCNB1, H1FOO, cMOS, PARN* and *eIF4E* was performed as described in experiment 1.

The data of expression for *GDF9*, *CCNB1*, *H1FOO*, *cMOS*, *PARN* and *eIF4E* in oocytes from secondary follicles were analyzed by the Kruskal-Wallis test, followed by the post hoc non-parametric Dunn's Multiple Comparison Test. Differences were considered significant when $P<0.05$.

2.3 *Experiment 3: Effects of in vitro growth and prematuration on the levels of mRNAs for GDF9, CCNB1, H1FOO, cMOS, PARN and eIF4E in oocytes from small antral follicles (1.0 – 3.0 mm)*

Cow ovaries (n= 40) were obtained from a local abattoir and transported within 1 h to the laboratory in saline solution (0.9%NaCl; 30°C) containing 100 IU/mL penicillin and 50 mg/mL streptomycin sulfate. In the laboratory, the ovaries were washed in saline solution and cumulus–oocyte complexes (COCs) were aspirated from small antral follicles (1–3 mm in diameter) using an 21 gauge needle connected to a sterile syringe. Under a stereomicroscope, COCs were classified according to the morphology of oocyte and cumulus cells. After morphological evaluation, COCs presenting compact cumulus cells and oocytes with no signs of cytoplasmic degeneration were considered normal and destined to *in vitro* culture.

For the growth, prematuration and maturation of oocytes *in vitro*, the medium was TCM-199 supplemented with 4% polyvinylpyrrolidone (PVP), 1µg/mL estradiol, 4 mM hypoxanthine, 0.2 mM pyruvic acid, 2.2 mg/mL sodium bicarbonate, 5.0 mg/mL LH (Bioniche, Belleville, ON, Canada), 0.5 mg/mL FSH (Bioniche, Belleville, ON, Canada), 5% fetal bovine serum and 100 IU/mL penicillin and 50 mg/mL streptomycin sulfate. The growth medium composition was according to a protocol described previously by Huang (2014), but with addition of FSH and LH. The COCs were cultured individually for 48 hours in growth

medium alone. After the growth period, the morphology, oocyte diameters and meiotic progression were evaluated and the COCs were destined to *in vitro* prematuration. In addition, four groups containing 10 oocytes were stored at -80 ° C for RNA extraction.

The *in vitro* prematuration medium (pre-IVM) was TCM-199 containing Earle's salts and L-glutamine (Sigma) supplemented with 0.2 mM pyruvic acid, 5.0 mg/mL LH (Bioniche, Belleville, ON, Canada), 0.5 mg/mL FSH (Bioniche, Belleville, ON, Canada), 0.4% BSA, 10 μM of cilostamide and 100 IU/mL penicillin and 50 μg/mL streptomycin sulfate. The COCs were cultured individually for 20 h and after the prematuration period, the morphology, oocyte diameters and meiotic progression were evaluated and the COCs were destined to *in vitro* maturation. In addition, four groups containing 10 oocytes were stored at -80 ° C for RNA extraction.

The maturation medium was the same medium used during prematuration without cilostamide. The COCs were cultured individually for 22 hours. After the period of culture, the meiotic progression was evaluated and oocytes were stored in micro-centrifuge tubes at -80 °C, until RNA extraction.

The percentages of GV in the different treatments were evaluated using Mann Whitney test. The data of expression of mRNAs for *GDF9, CCNB1, H1FOO, cMOS, PARN* and *eIF4E* in oocytes from small antral follicles (1.0 – 3.0 mm) were analyzed by the Kruskal-Wallis test, followed by the post hoc non-parametric Dunn's Multiple Comparison Test. Differences were considered significant when $P<0.05$.

2.4 *Experiment 4: Effects of in vitro prematuration on the levels of mRNAs for GDF9, CCNB1, H1FOO, cMOS, PARN and eIF4E in oocytes from medium antral follicles (3.0 – 6.0 mm)*

Cow ovaries (n= 40) were obtained from a local abattoir and transported within 1 h to the laboratory in saline solution (0.9% NaCl; 30°C) containing 100 IU/mL penicillin and 50 mg/mL streptomycin sulfate. In the laboratory, the ovaries were washed in saline solution and cumulus–oocyte complexes (COCs) were aspirated from middle antral follicles (3.0–6.0 mm in diameter) using an 21 gauge needle connected to a sterile syringe. Under a stereomicroscope, COCs were classified according to the morphology of oocyte and cumulus cells. After morphological evaluation, COCs presenting compact cumulus cells and oocytes with no signs of cytoplasmic degeneration were considered normal and destined to *in vitro* culture.

For prematuration and maturation of oocytes *in vitro*, grade 1 and 2 COCs with a visible compact and intact cumulus and a dark cytoplasm were selected for culture and randomly distributed into 200 µL of IVM medium with or without follicular hemi-sections and cultured in an incubator at 39°C in a saturated humidity atmosphere containing 5% CO_2 and 95% air. A number of 100 COCs were cultured to evaluate the effects of either prematuration or both prematuration and maturation.

To obtain follicular hemi-sections, follicles were isolated from the ovaries and dissected free of stromal tissue and transparent follicles measuring 3–6 mm in diameter were selected (Richard & Sirard, 1996a, 1996b). The follicles were sectioned into equal halves with a scalpel and the oocytes were removed and discarded. Then, the follicular hemi-sections were washed in TCM-199 containing 0.4% bovine serum albumin (BSA) and randomly distributed into 4-well culture plates (Nunc, Roskilde, Denmark) containing culture medium. Two follicular hemi-sections were each added to 50 µL of medium, and then cultured for 2 h before incubation with COCs, as described by De Cesaro et al. (2013).

The *in vitro* maturation medium (IVM) was designated TCM-199*, which consists of TCM-199 containing Earle's salts and L-glutamine (Sigma) supplemented with 25 mM

HEPES, 0.2 mM pyruvic acid, 2.2 mg/mL sodium bicarbonate, 5.0 mg/mL LH (Bioniche, Belleville, ON, Canada), 0.5 mg/mL FSH (Bioniche, Belleville, ON, Canada), 0.4% BSA and 100IU/mL penicillin and 50 mg/mL streptomycin sulfate. The prematuration medium (pre-IVM medium) consisted of TCM-199* supplemented with 10 µM cilostamide and follicular hemi-sections (n=8). After culture, to evaluate meiotic progression, the cumulus cells were removed by vortexing and the oocytes were fixed in 4% paraformaldehyde for 15 min and transferred to 0.5% TritonX-100. The chromatin configuration during meiosis were assessed by 10 µg/mL bisbenzimide (Hoechst33342) and analyzed under an epi-fluorescent inverted microscope (Leica, DMI4000B). Oocytes having nuclear membrane were considered at germinal vesicle (GV) stage, while those without nuclear membrane, i.e., that were at GVBD, metaphase I, anaphase I, telophase I, or metaphase II stages, were considered to have resumed meiosis.

After period of culture, oocytes were collected both after the prematuration and maturation periods and stored in micro-centrifuge tubes at -80 °C, until RNA extraction. Quantification of the mRNA for *GDF9, CCNB1, H1FOO, cMOS, PARN* and *eIF4E* was performed as described in experiment 1.

The percentages of GV in the different treatments were evaluated using Mann Whitney test. The data of expression of mRNAs for *GDF9, CCNB1, H1FOO, cMOS, PARN* and *eIF4E* in oocytes from medium antral follicles (3.0 – 6.0 mm) were analyzed by the Kruskal-Wallis test, followed of the post hoc nonparametric Dunn's Multiple Comparison Test. Differences were considered significant when $P<0.05$.

3.0 Results

3.1 Experiment 1: Expression of mRNA in oocytes from secondary follicles and from small, medium and large antral follicles

Figure 1A shows that the levels of mRNA for *GDF9* in oocytes of large antral follicles were higher than those seen in oocytes of secondary follicles ($P<0.05$). The levels of mRNA for *CMOS* (Figure 1B) and *eIF4E* (Figure 1D) in oocytes from secondary follicles and from small, medium and large antral follicles were not different. On the other hand, levels of mRNA for *PARN* (Figure 1C) in oocytes of medium antral follicles were higher than those seen in oocytes of secondary and small antral follicles ($P<0.05$). The levels of transcripts for *CCNB1* in oocytes of small, medium and large antral follicles were higher than those in oocytes of secondary follicles ($P<0.05$). In addition, the levels of mRNA for *H1FOO* (Figure 1F) in oocytes of small antral follicles were significantly higher than those seen in oocytes of secondary follicles ($P<0.05$).

3.2 Experiment 2: Effects of in vitro culture on the levels of mRNAs for GDF9, CCNB1, H1FOO, cMOS, PARN and eIF4E in oocytes from secondary follicles

In vitro culture of secondary follicles for 18 days promoted a significant increase in follicular diameter, i.e., from 183.19$\pm$15.44µm at day 0 to 232.29$\pm$16.36µm at day 18. These *in vitro* grown follicles (Figure 2) had an increase in the levels of mRNA for *GDF9* (Figure 3A), *cMOS* (Figure 3B), *PARN* (Figure 3C), *eIF4E* (Figure 3D), *CCNB1* (Figure 3E) and *H1FOO* (Figure 3F) in their oocytes when compared to those before culture.

3.3 Experiment 3: Effects of in vitro growth and prematuration on the levels of mRNAs for GDF9, CCNB1, H1FOO, cMOS, PARN and eIF4E in oocytes from small antral follicles (1.0 – 3.0 mm)

In vitro culture of COCs from small antral follicles for 48h promoted an increase in their oocyte diameter from 107.06 ± 1.35μm to 114.28 ± 0.28μm. After growth and prematuration of COCs from small antral follicles (Figure 4A), only 17.0% of the oocytes had meiosis resumption, but after IVM, a meiosis resumption rate of 80% was observed (Table 2).

These pre-matured oocytes had increased levels of mRNA for *GDF9* (Fig. 5A), *PARN* (Figure 5C) and *eIF4E* (Figure 5D) when compared to those before culture. There was, however, no significant increase in the level of mRNAs for *cMOS* (Figure 3B), *CCNB1* (Figure 5E) and *H1FOO* (Figure 5F) after the prematuration.

3.4 Experiment 4: Effects of in vitro prematuration on the levels of mRNAs for GDF9, Cmos, PARN, eIF4E, CCNB1 and H1FOO in oocytes from medium antral follicles (3.0 – 6.0 mm)

Prematuration and IVM of oocytes from medium antral follicles (Figure 4B) increased the levels of mRNA for *cMOS* (Figure 6B) and *H1FOO* (Figure 6F) in their oocytes when compared to oocytes before the IVM period. The levels of mRNA for *GDF9* (Figure 6A), *PARN* (Figure 6C) and *eIF4E* (Figure 6D) did not show significant differences, but *CCNB1* (Figure 6E) presented a significant increase after pre-IVM period, when compared to oocytes before prematuration. After prematuration of COCs from medium antral follicles, only 35.0% of the oocytes had meiosis resumption, whereas after IVM a meiosis resumption rate of 90% was observed (Table 3). Figure 7 shows the changes in the levels of mRNA for *GDF9, CMOS, PARN, eIF4E, CCNB1* and *H1FOO* during the growth of bovine secondary follicles up to small, medium and large antral follicles, as well as after *in vitro* culture of secondary follicles and prematuration of COCs of small and medium antral follicles.

4. Discussion

This study shows an increase in the expression of *H1FOO, GDF9, CCNB1* and *PARN* during the growth of secondary follicles to the antral stages. During development, growing oocytes engage in a prolonged phase of intensive RNA synthesis required to produce transcripts essential for oocyte growth and pre-implantation embryo development (Bachvarova, 1985). In the micro-environment of an antral follicle, the cellular communication between oocytes and cumulus cells is complex, and both sides have active regulatory roles. Macro-molecules, such as RNAs (Macaulay *et al.,* 2014; 2016), can also be transported from cumulus cells to oocytes. Despite the molecular interactions associated with meiotic competence are not well defined at the moment, we report here the increase in expression levels of genes related to oocyte competence. The results of co-expression analyze in oocytes performed by Biase & Kimble (2018) showed that regulatory mechanisms promote the transcription and accumulation of gene products, among them dozens of genes with transcriptional regulatory functions or functions related to translation, including *GDF9*. Alam et al. (2018) showed that GDF9 mRNAs from small bovine antral follicles were expressed only in oocytes but not in granulosa cells. The oocyte secretes specific factors GDF9 and thus stimulates follicular cells to proliferate and secrete steroids and other factors, which, in turn, regulate oocyte growth (Gilchrist *et al.,* 2004; Binelli *et al.,* 2009). In bovine oocytes, *CCNB1* was detected throughout the cytoplasm with granular appearance, without large variations between immature and mature oocytes (Quetglas *et al.,* 2009). The levels of *CCNB1* proteins are required at specific times during meiosis, and although they remain constant, cyclin protein concentrations generally fluctuate during the cell cycle due to synthesis and periodic degradation (De Vantery *et al.,* 1996). This protein kinase is involved in cell signaling and any change in synthesis and/ or storage can cause severe consequences during oocyte maturation (Pereira *et al.,* 2018). The PARN gene is important during oocyte maturation, embryogenesis, early development, DNA damage and cell cycle progression (Balatsos *et al.,*

2012). Recent studies on the maturation of mammalian RNAs revealed a novel step involving PARN (Berndt *et al.,* 2012). H1FOO, an oocyte-specific histone H1, is expressed during development in fully meiotically competent germinal vesicle-stage oocytes until the late two-cell embryonic stage (Bustin *et al.,* 2005; Tanaka *et al.,* 2003). This expression is essential for oocyte maturation in mice (Furuya *et al.,* 2007) and has beneficial effects on developing oocytes and the fertilized egg (Hayakawa *et al.,* 2012). In the bovine species, the results of this work provide clear evidence that *GDF9, CCNB1, PARN* and *H1FOO* are required throughout oocyte growth.

In vitro culture of secondary follicles also increases the expression of mRNAs for *GDF9, CCNB1, PARN, cMOS, EIF4E* and *H1FOO*. Secondary follicles are excellent sources of potentially fertilizable oocytes. However, the *in vitro* development of these follicles has been a great challenge to produce fully grown and competent oocytes (Mbemya *et al.,* 2018). The success in culture of preantral follicles resulting in live births was only achieved in mice (Dela Pena *et al.,* 2002; O'brien *et al.,* 2003) to date. In larger species, the results reached only at variable production of number of embryos in goats (Saraiva *et al.,* 2010; Magalhães *et al.,* 2011), sheep (Arunakumari *et al.,* 2010) and buffalo (Agarwal *et al.,* 2014). In cattle, secondary follicular grow *in vitro* up to antrum formation (Mbemya *et al.,* 2018), but competent oocytes were still not reported after culturing these follicles. In this study, we observed a significant increase in mRNA levels for *GDF9, CCNB1, PARN, cMOS, eIF4E* and *H1FOO* in oocytes derived from secondary follicles grown for 18 days. Several variables may affect the outcome of follicle culture *in vitro* (Mbemya *et al.,* 2018), such as the culture base media composition (McNatty *et al.,* 2007; Santos *et al.,* 2014) and supplementation (Ferreira *et al.,* 2016), the animal model (Magalhães *et al.,* 2011), the culture system (Araujo *et al.,* 2014), the follicular category (Magalhães-Padilha *et al.,* 2013), reactive oxygen species (ROS) production (Leiva-Revilla *et al.,* 2017), and activation or silencing of genes encoding

antioxidant defense enzymes, growth and progression of meiosis (Dalton *et al.,* 1999). Therefore, increase of the expression of these genes in oocytes may be a good indicator of viability of *in vitro* culture secondary follicles, providing support to oocyte development.

Prematuration and maturation of oocytes from antral follicles also increased the expression of *cMOS, CCNB1 and H1FOO*. Although *in vitro* maturation rates (IVM) are relatively high, approximately 90% (Lonergan & Fair, 2014), low rates of embryonic development are generally attributed to the inability of oocytes to support cell multiplication to the blastocyst stage (Camargo *et al.,* 2006; Meirelles *et al.,* 2013), suggesting that factors necessary for the proper development of the embryo are absent in the culture media (Da Silveira *et al.,* 2017). During IVM, oocytes need to undergo nuclear and cytoplasmic maturation (Ferreira *et al.,* 2009). However, before they become fully competent oocytes, they also need to undergo molecular maturation. Transcriptional activity is supposed to be low during maturation (Bettegowda & Smith, 2007), and much of the mRNA transcribed and stored in the cytoplasm during oocyte growth is degraded, while part of it is protected from degradation and imparts stability to the untranslated regions 3 ' (Brevini *et al.,* 2007). In this study, oocytes undergoing prematuration and *in vitro* maturation increased mRNA levels for *GDF9, CCNB1, PARN, cMOS, eIF4E* and *H1FOO*, which indicate that these RNAs are stored in the oocyte throughout the oocyte growth. Different forms of mRNA and proteins are present in the ooplasm and may be required in early cleavage (Meirelles *et al.,* 2004; Li *et al.,* 2010), playing a role on embryonic genome activation (Schultz, 2002). Genetic regulation of female fertility (follicular development, oocyte maturation, and early pre-implantation embryo development) involves the spatiotemporal regulation of these genes that play key roles at various stages of the female reproductive axis (Tesfaye *et al.,* 2018). Further studies are required to clarify the molecular mechanisms of *in vitro* mammalian oocyte maturation, which depend on a precise sequence of changes in maternal gene expression.

In conclusion, follicular growth from secondary to antral follicles is associated with an increase in the expression of *H1FOO, GDF9, CCNB1* and *PARN*. *In vitro* culture of secondary follicles, as well as prematuration and maturation of oocytes from antral follicles increase expression of *eIF4E, PARN, H1FOO, cMOS, GDF9* and *CCNB1*.

CONFLICT OF INTEREST STATEMENT

There was no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Figures and tables

Figure 1. Levels of mRNA for *GDF9* (A), *cMOS* (B), *PARN* (C), *eIF4E* (D), *CCNB1* (E), *H1FOO* (F) in oocytes from secondary follicles (S. F), small antral follicles (S.A.F), medium antral follicles (M.A.F) and large antral follicle (L.A.F).

Figure 2. Bovine secondary follicle after 18 days of culture.

Figure 3. Levels of mRNA in oocytes from secondary follicles before and after 18 days of culture. (A) *GDF9*, (B) *cMOS*, (C) *PARN*, (D) *eIF4E*, (E) *CCNB1*, (F) *H1FOO*.

Figure 4. COCs of small (1 - 3 mm) and medium (3 – 6 mm) antral follicles after *in vitro* prematuration. (A) COC of small antral follicles after growth and prematuration. (B) COC of medium antral antral follicles after *in vitro* prematuration.

Figure 5. Levels of mRNA in oocytes in oocytes from small antral follicles (1.0 – 3.0 mm in diameter). (A) *GDF9*, (B) *cMOS*, (C) *PARN*, (D) *eIF4E*, (E) *CCNB1*, (F) *H1FOO*.

Figure 6. Levels of mRNA in oocytes from medium antral follicles (3.0 – 6.0 mm in diameter) after prematuration and IVM. (A) *GDF9*, (B) *cMOS*, (C) *PARN*, (D) *EIF4E*, (E) *CCNB1*, (F) *H1FOO*.

Figure 7. Changes in the levels of mRNA for *GDF9, CMOS, PARN, eIF4E, CCNB1* and *H1FOO* during the growth of bovine secondary follicles up to small, medium and large antral follicles, as well as after culture of secondary follicles and prematuration of COCs of small and medium antral follicles.

Table 1. Primer pairs used in real-time PCR for quantification of mRNA in oocytes.

Table 2. Percentage of oocytes from follicles of 1.0 – 3.0 mm in the germinal vesicle (GV) stage and after resumption of meiosis before and after the prematuration period.

Table 3. Percentage of oocytes from follicles of 3.0 - 6.0 mm in the germinal vesicle (GV) stage and after resumption of meiosis before and after the prematuration period.

Figure 1

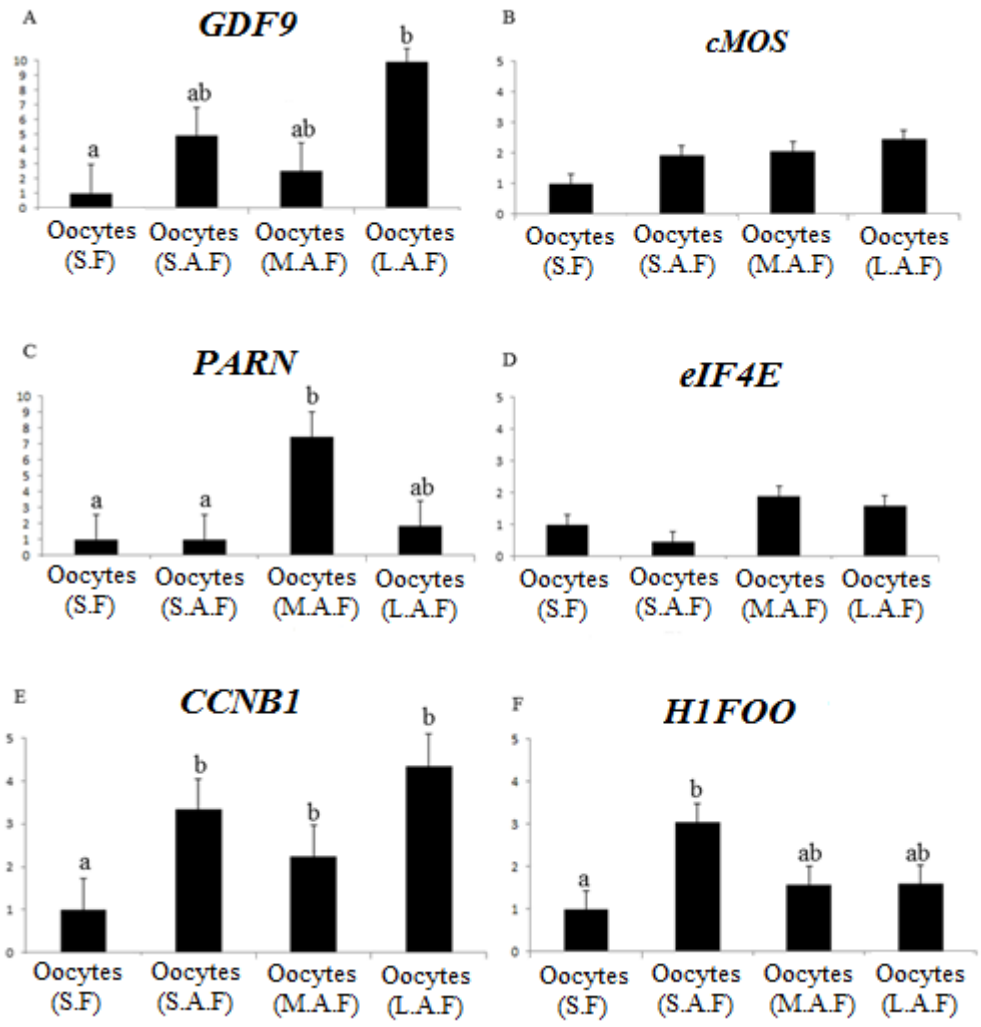
A
GDF9
a
ab
ab
b
Oocytes (S.F)
Oocytes (S.A.F)
Oocytes (M.A.F)
Oocytes (L.A.F)
B
cMOS
C
PARN
a
a
b
ab
D
eIF4E
E
CCNB1
a
b
b
b
F
H1FOO
a
b
ab
ab

Figure 2

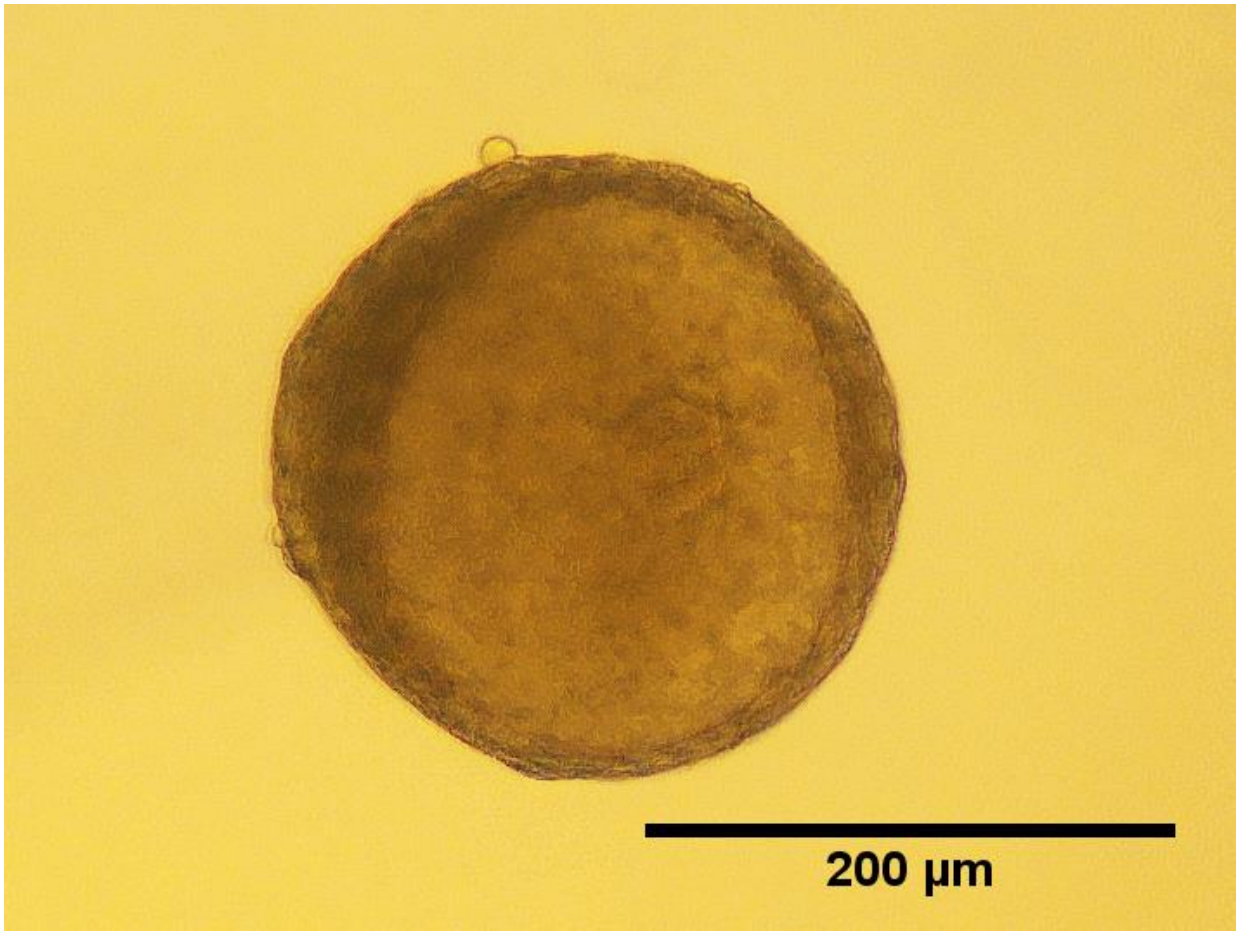

Figure 3

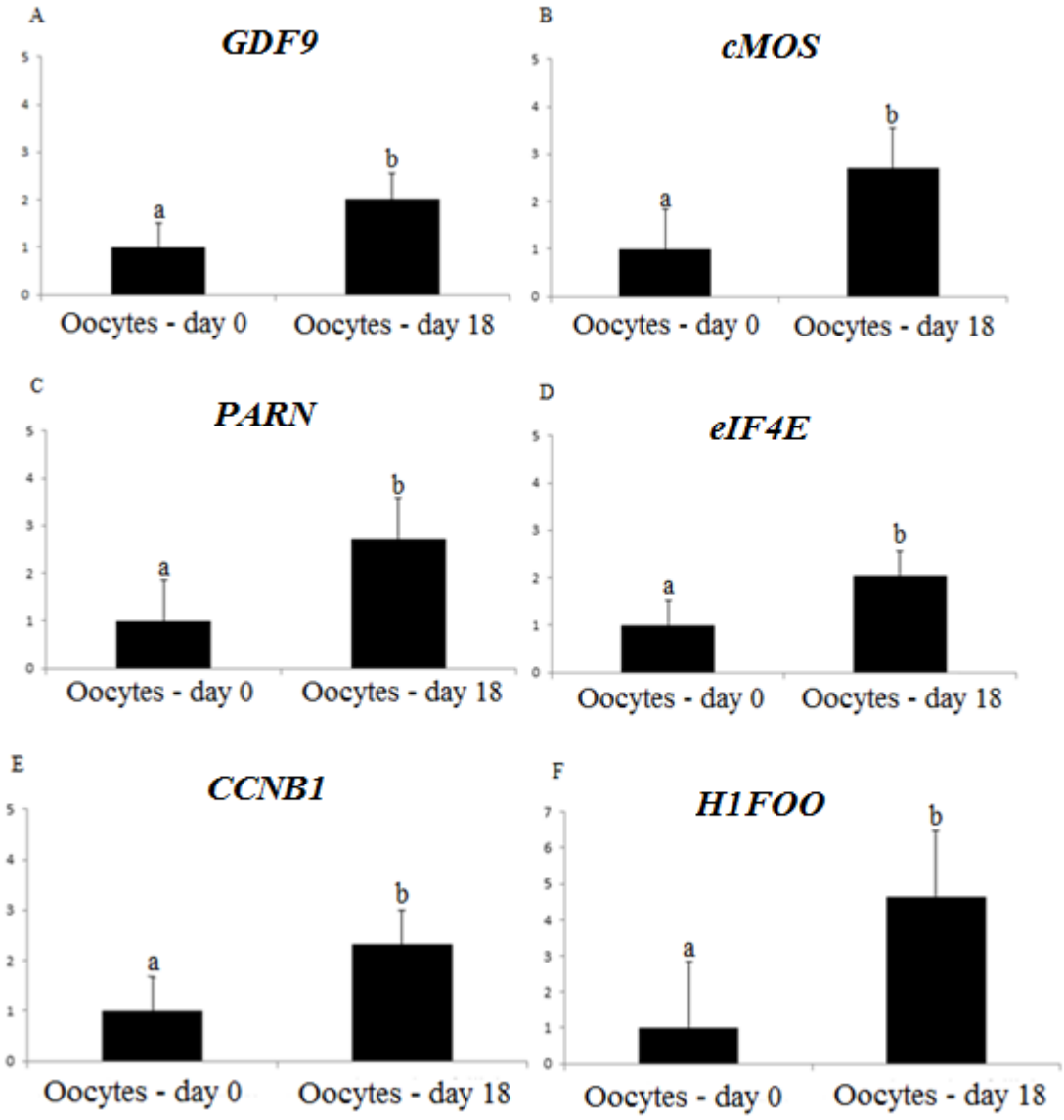
A
GDF9
a
b
Oocytes - day 0
Oocytes - day 18
B
cMOS
a
b
Oocytes - day 0
Oocytes - day 18
C
PARN
a
b
Oocytes - day 0
Oocytes - day 18
D
eIF4E
a
b
Oocytes - day 0
Oocytes - day 18
E
CCNB1
a
b
Oocytes - day 0
Oocytes - day 18
F
H1FOO
a
b
Oocytes - day 0
Oocytes - day 18

Figure 4

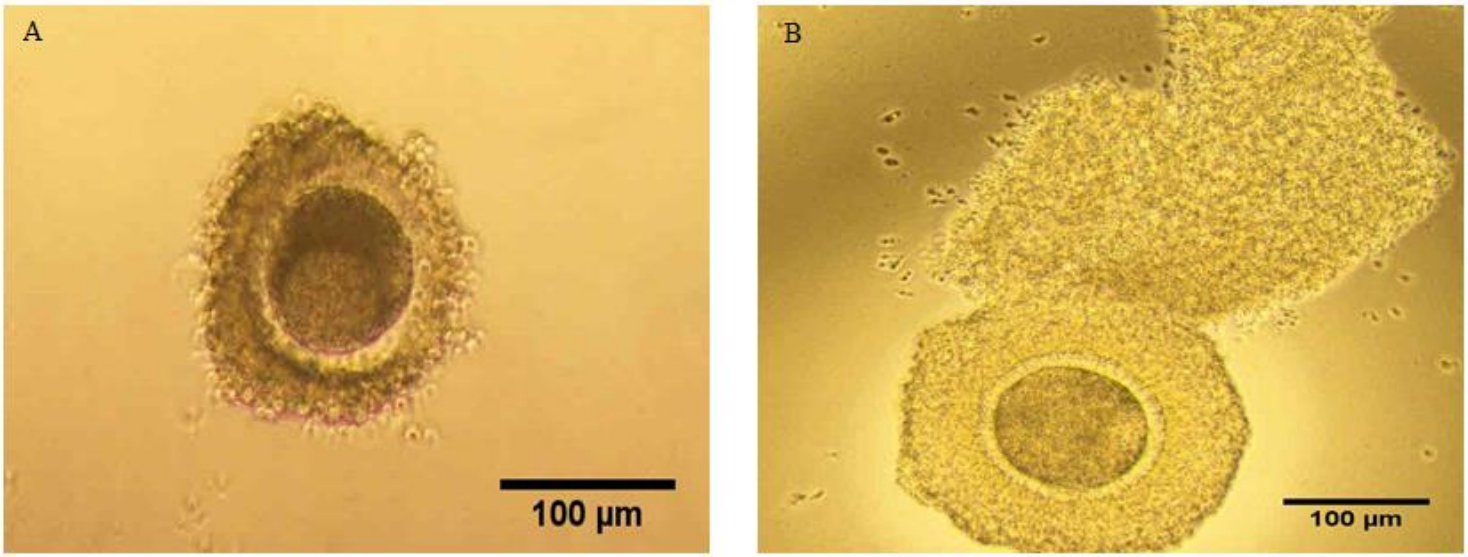

Figure 5

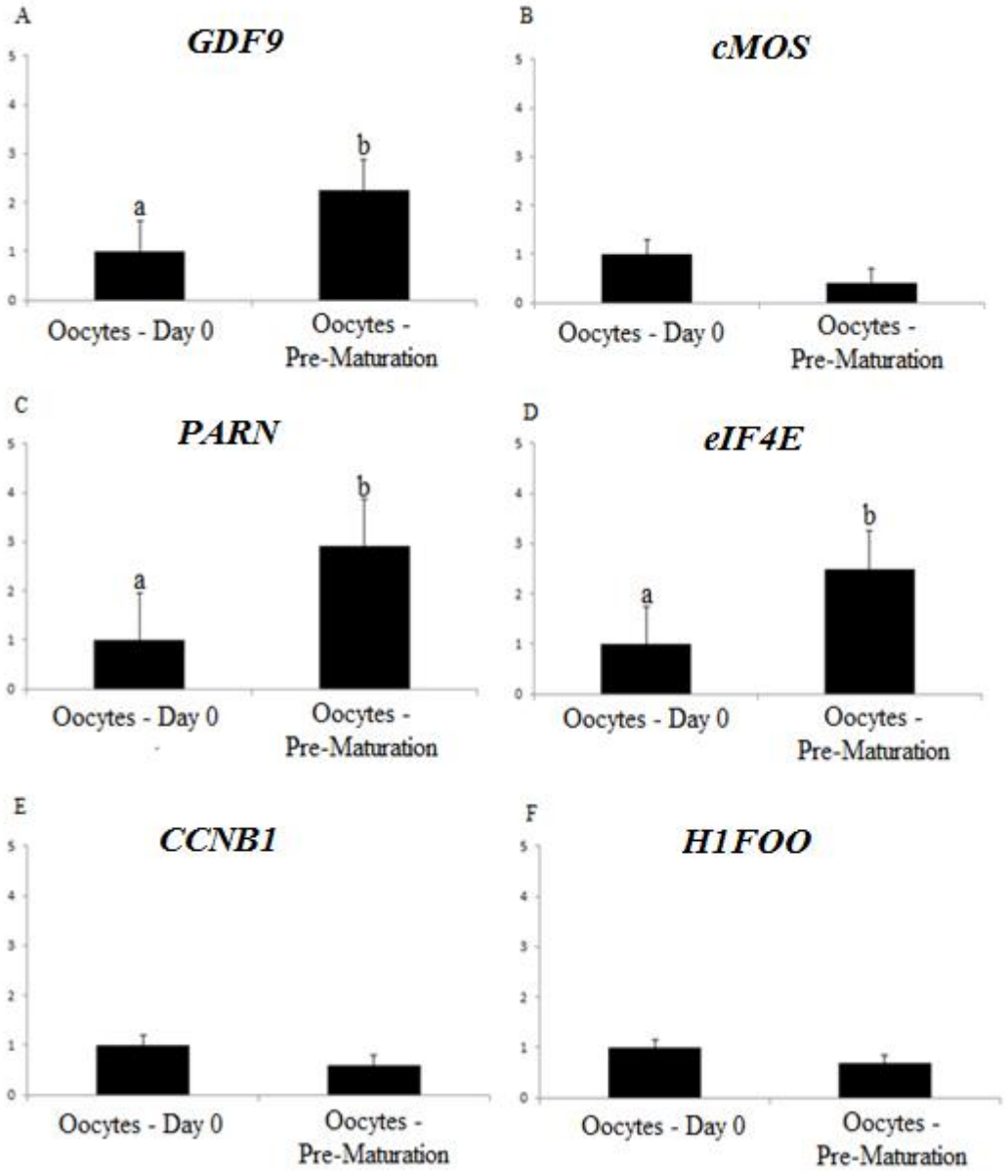
A
GDF9
a
b
Oocytes - Day 0
Oocytes - Pre-Maturation
B
cMOS
Oocytes - Day 0
Oocytes - Pre-Maturation
C
PARN
a
b
Oocytes - Day 0
Oocytes - Pre-Maturation
D
eIF4E
a
b
Oocytes - Day 0
Oocytes - Pre-Maturation
E
CCNB1
Oocytes - Day 0
Oocytes - Pre-Maturation
F
H1FOO
Oocytes - Day 0
Oocytes - Pre-Maturation

Figure 6

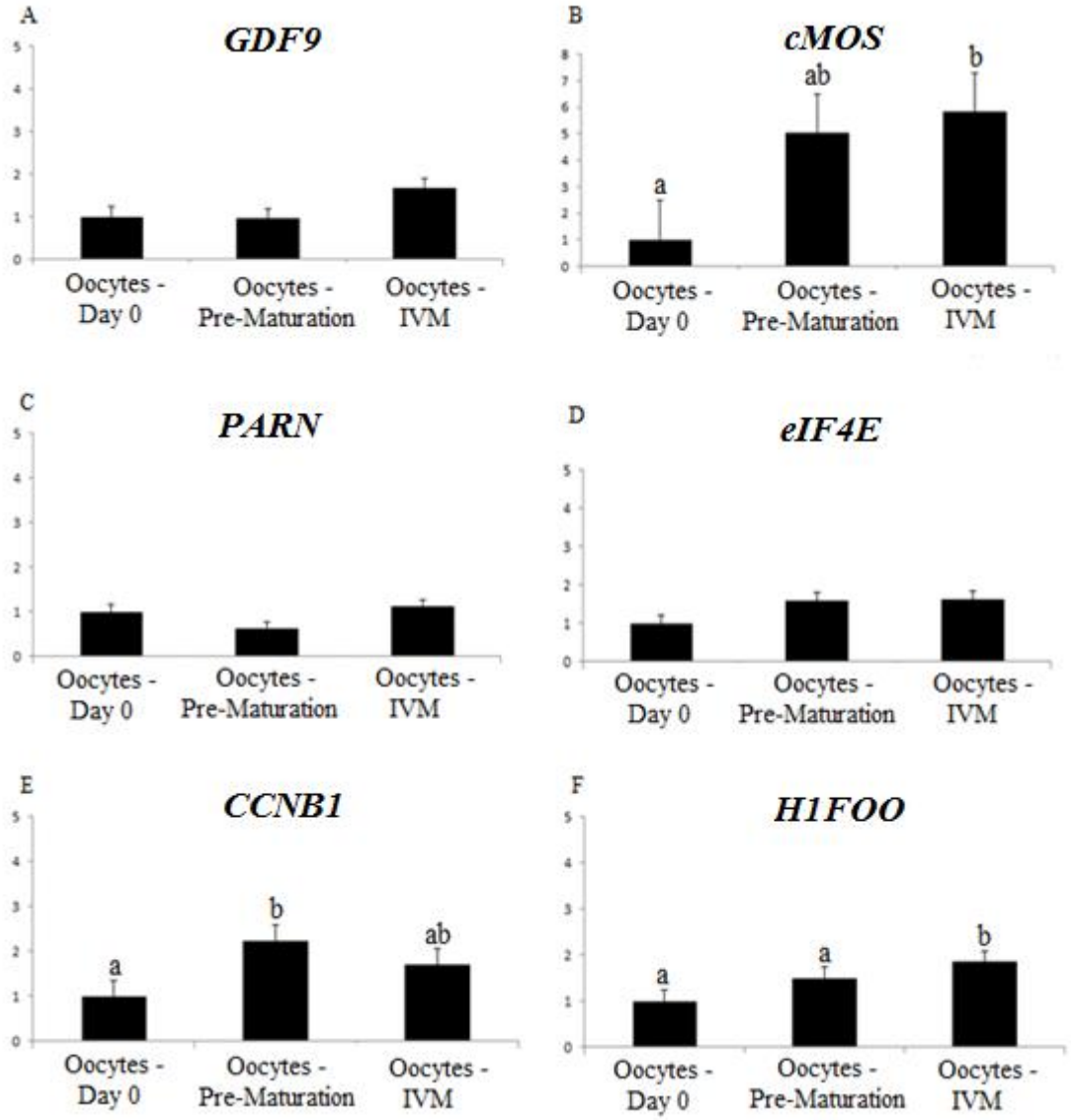
A
GDF9
Oocytes - Day 0
Oocytes - Pre-Maturation
Oocytes - IVM
B
cMOS
a
ab
b
C
PARN
D
eIF4E
E
CCNB1
a
b
ab
F
H1FOO
a
a
b

Figure 7

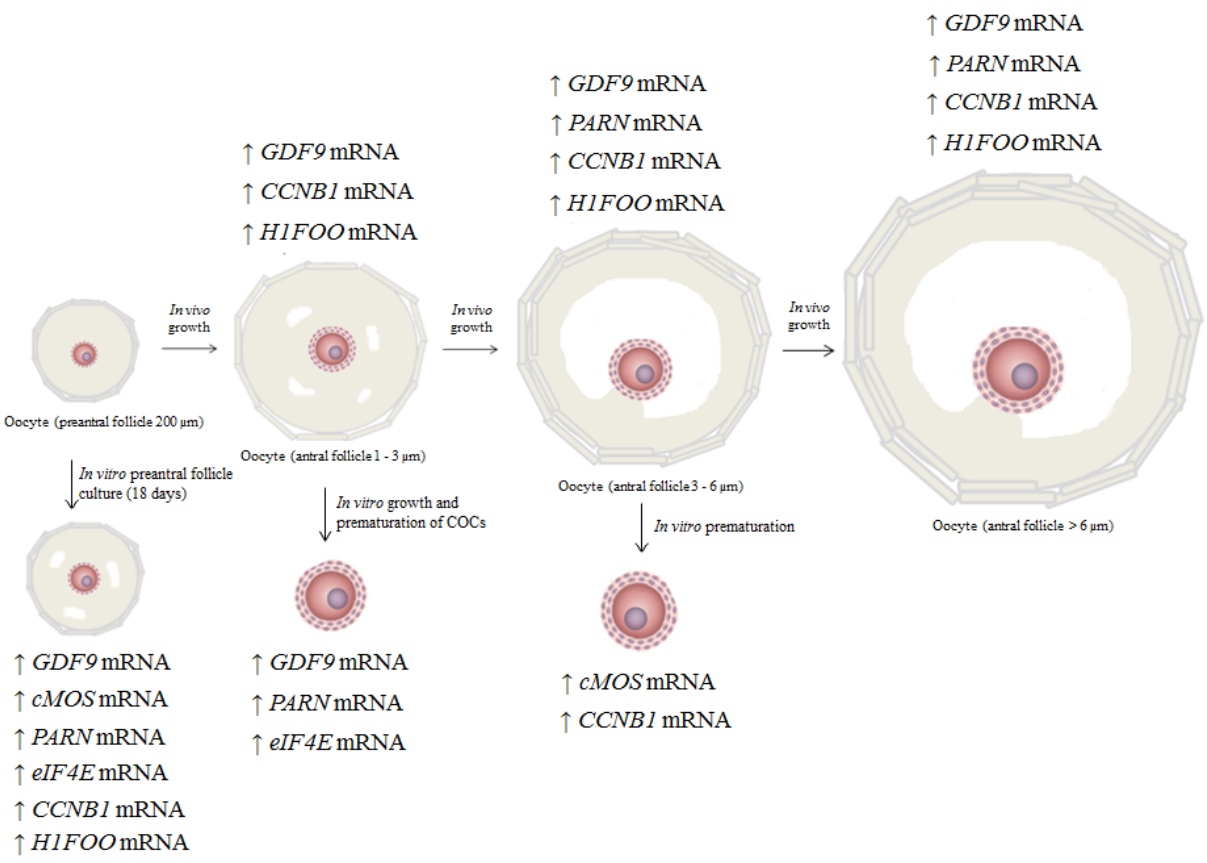
↑ GDF9 mRNA
↑ PARN mRNA
↑ CCNB1 mRNA
↑ H1FOO mRNA
↑ GDF9 mRNA
↑ CCNB1 mRNA
↑ H1FOO mRNA
↑ GDF9 mRNA
↑ PARN mRNA
↑ CCNB1 mRNA
↑ H1FOO mRNA
In vivo growth
In vivo growth
In vivo growth
Oocyte (preantral follicle 200 µm)
Oocyte (antral follicle 1 - 3 µm)
Oocyte (antral follicle 3 - 6 µm)
Oocyte (antral follicle > 6 µm)
In vitro preantral follicle culture (18 days)
In vitro growth and prematuration of COCs
In vitro prematuration
↑ GDF9 mRNA
↑ cMOS mRNA
↑ PARN mRNA
↑ eIF4E mRNA
↑ CCNB1 mRNA
↑ H1FOO mRNA
↑ GDF9 mRNA
↑ PARN mRNA
↑ eIF4E mRNA
↑ cMOS mRNA
↑ CCNB1 mRNA

Table 1. Primer pairs used in real-time PCR for quantification of mRNA in oocyte and cumulus cells.

GENES	STRAND	SEQUENCE	SIZE (nt)	Gene ID:
GAPDH	F R	5'-TGTTTGTGATGGGCGTGAACCA-3' 5'-ATGGCGTGGACAGTGGTCATAA-3'		GI:27525390
CCNB1	F R	5'-CTCCAGTGCTCTCCTCCTCACT-3' 5'-CTAATCTTCGTGTTCCTGGTGATCC-3'	84	NM_001045872.1
CMOS	F R	5'- CTGCAAGATCGGGGACTTCG - 3' 5'- CTCGGTGAGTGTAGGTGCCA– 3'	95	AY_168496.1
GDF9	F R	5'-GACCCCTAAATCCAACAGAA-3' 5'AGCAGATCCACTGATGGAA3'	120	GI: 51702523
H1FOO	F R	5'- AGTCGAAGGTCAAAGAAAGAGGGAGC-3' 5'- TGAACTCTGACTTCCAGGCTGTGT -3'	80	NM_001035372.1
PARN	F R	5'- GAACAAGTGCCGATTGCTGTC -3' 5'- CGACATAGTCAGCGTAGGTCT -3'	76	NM_001101118.2
eIF4E	F R	5'- TGGCAAGCAAACCTTCGAT -3' 5'-GGATATGGTTGTACAGAGCCC -3'	73	NM_174310

Table 2. Percentage of oocytes from follicles of 1.3 – 3.0 mm in the germinal vesicle (GV) stage and after resumption of meiosis before and after the pre-maturation period

	GERMINAL VESICLE (GV)		**RESUMPTION OF MEIOSIS**	
	Pre-Maturation (%)	IVM (%)	Pre-Maturation (%)	IVM (%)
TCM-199	83 (28/34)*	20(6/30)	17(6/34)	80(24/30)*

(*P<0,0005)

Table 3. Percentage of oocytes of 3.0 - 6.0 mm in the germinal vesicle (GV) stage and after resumption of meiosis before and after the pre-maturation period

	GERMINAL VESICLE (GV)		**RESUMPTION OF MEIOSIS**	
	Pre-Maturation (%)	IVM (%)	Pre-Maturation (%)	IVM (%)
TCM-199	65 (26/40)	10(2/20)	35(14/40)	90(18/20)

7 CAPÍTULO 2 – ARTIGO III

Effects of EGF and progesterone on oocyte meiotic resumption and expression of maturation-related transcripts during prematuration of oocytes from small and medium-size bovine antral follicles

EGF, P4 and the levels of transcripts in oocytes

Artigo submetido à Reproduction Fertility and Development (Qualis A1 / Fator de Impacto: 1,73)

Effects of EGF and progesterone on oocyte meiotic resumption and expression of maturation-related transcripts during prematuration of oocytes from small and medium-size bovine antral follicles

EGF, P4 and the levels of transcripts in oocytes

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Abstract

This study evaluated the influence of EGF and progesterone (P4) on growth, resumption of meiosis and on expression of mRNA for *eIF4E, PARN, H1FOO, cMOS, GDF9* and *CCNB1* in oocytes from small and medium antral follicles after prematuration and maturation *in vitro*. Oocytes from small (< 2.0mm) and medium (3.0 – 6.0mm) antral follicles were cultured in medium containing EGF (10 ng/mL), P4 (100 µM) or both. After culturing, growth rate, meiosis resumption and mRNA levels for *eIF4E, PARN, H1FOO, cMOS, GDF9* and *CCNB1* were evaluated. The results showed that P4 increase the mRNA levels for *cMOS, H1FOO* and *CCNB1* after culture of oocyes from small antral follicles. EGF also increased mRNA levels

for *CCNB1* in these oocytes. For medium antral follicles, P4 alone or the association of EGF and P4 increased oocyte diameters after prematuration in vitro. In these oocytes, the presence of either EGF or P4 in culture medium increased *cMOS* mRNA levels. In conclusion, P4 increase the mRNA levels for *cMOS*, *H1FOO* and *CCNB1* after culture of oocyes from small antral follicles. P4 and both EGF and P4 promotes the growth of oocytes from medium antral follicles, while either EGF or P4 increased *cMOS* mRNA levels.

Keywords: oocyte growth; follicular development; *in vitro* prematuration; *in vitro* maturation

1. Introduction

Ultrasound-guided ovum pick-up (OPU) combined with *in vitro* fertilization (IVF) technique is now widely used to produce embryos in cattle. However, the cumulus–oocyte complexes (COCs) are prematurely removed from their follicles and, consequently, the oocytes are less competent than those matured in vivo (Galli *et al.*, 2014). Thus, oocyte prematuration systems aim to improve oocyte quality by temporarily preventing spontaneous nuclear maturation and by prolonging oocyte-cumulus cells gap-junctional communication *in vitro* to allow continued mRNA and protein accumulation within the ooplasm (Albuz *et al.*, 2010). It is known that the events that occur during the oocyte growth and maturation are coordinated by different factors that may be involved in the storage of mRNA. It has been reported that progesterone (P4) and epidermal growth factor (EGF) may alter a synthesis of mRNA and, consequently, increase the storage of proteins necessary for oocyte maturation and early embryonic development (Wijayagunawardane *et al.*, 2015; Pereira *et al.*, 2019).

In cattle, P4 induces gene transcription and have an effect on oocyte competence by signaling through nuclear receptors (PGRs) and membrane P4 receptors (Aparicio *et al.,* 2011; O´Shea *et al.,* 2012). Bovine COCs express both nuclear (PGR-A, PGR-B) and membrane-bound P4 receptors (mPRa, mPRb, progesterone receptor membrane component 1 (PGRMC 1) and PGRMC2) (Luciano *et al.,* 2010; Aparicio *et al.,* 2011). Furthermore, protein expression of these receptors changes dynamically following *in vitro* maturation in response to LH, FSH or P4 (Aparicio *et al.,* 2011). Fair *et al.* (2012) confirmed the functional relevance of P4 and P4 receptor signalling during oocyte maturation to oocyte acquisition of developmental competence). Recently, Saad *et al.* (2019) reported that the presence of greater plasma P4 has a beneficial effect on oocyte recovery, oocyte quality, and early in vitro embryo production outcomes in Bos indicus dairy cows. Additionally, the activation of EGF network, in turn, has been implicated in the regulation of the meiotic maturation of oocytes (Richani *et al.,* 2018). The EGF system may contribute to the regulation of oocyte integrity via regulation of oocyte programming during the meiotic maturation phase (Tan *et al.,* 2009). The oocyte also is dependent on cumulus cell signals that are mediated via the EGF network to regulate mRNA translation during maturation(Richani *et al.,* 2018) . *In vitro* studies have demonstrated that exposure to amphiregulin (AREG) and/or epiregulin (EREG) enhances oocyte quality in murine, bovine and porcine species (Akaki *et al,* 2009; Prochazka *et al.,* 2011; Richani *et al.,* 2013; Richani *et al.,* 2014a; Richani *et al.,* 2014b; Sugimura *et al.,* 2014; Sugimura *et al.,* 2015). Moreover, FSH regulation of oocyte translation is mediated via EGFR (Franciosi *et al.,* 2016). Although the mechanisms involved in this link from the cumulus cells to the oocyte are still emerging, the EGF network control of maternal mRNA translation is mediated by PI(3)K-AKT mTOR signalling within the oocyte (Chen *et al.,* 2013; Franciosi *et al.,* 2016).

During meiotic maturation of bovine oocytes, the translation initiation factor 4E, *eIF4E* (the cap binding protein) and the substantial phosphorylation of this protein begins at

the time of germinal vesicle breakdown (GVBD) and continues to the metaphase II stage. The onset of *eIF4E* phosphorylation occurs in parallel with a significant increase in overall protein synthesis (Tomek *et al.*, 2002). Other enzymes are active, like the deadenylating enzyme *PARN*, that shorten the poly(A) tail originally acquired in the nucleus (Korner *et al.*, 1998; Richter *et al.*, 1999; Copeland *et al.*, 2001). Among the transcripts stored in the oocyte is oocyte-specific linker histone (*H1FOO*) that accumulates during oogenesis (Yan *et al.*, 2015), and its presence is maintained until the early stages embryo development (Tanaka *et al.*, 2001; Fu *et al*, 2003). Some studies have shown an important role of oocyte maturation factor Mos (*cMOS*), an kinase expressed exclusively in germ cells, and Cyclin B1 (*CCNB1*), regulatory unit of maturation-promoting factor (MPF) during oocyte maturation (Wu *et al.*, 1997). The *cMOS*-MAPK1/3 pathway is important for maintaining MII arrest (Phillips *et al.*, 2002). Besides these factors, the growth differentiation factor-9 (*GDF9*) is also involved in the process of oocyte maturation by regulating the function of cumulus cells of preovulatory follicles (Gui *et al.*, 2005) and may be secreted by oocyte and granulosa cells (Silva *et al.*, 2004; Spicer *et al.*, 2008) and promotes theca and granulosa cell proliferation, and stimulates the expression of genes in cumulus cellsElvin *et al.*, 2000).. In this sense, it is hypothesized that *in vitro* growth and prematuration of COCs of small and medium antral follicles in presence of EGF, P4 or both increase the levels of these mRNAs and have a positive impact on oocyte maturation *in vitro*.

The objectives of this study are to evaluate the influence of EGF, P4 or both on growth, meiosis progression and mRNA levels for *eIF4E*, *PARN*, *H1FOO*, *cMOS*, *GDF9* and *CCNB1* after *in vitro* growth and prematuration of oocytes derived from small and medium antral follicles.

2. Materials and methods

Unless indicated otherwise, culture media and other chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Experiment 1: Effects of EGF and P4 on oocyte maturation and expression of maturation-related transcripts in oocytes from small antral follicles (<2.0 mm)

Ovaries (n= 80) from mixed-breed cows were obtained from a local abattoir and transported within 1 h to the laboratory in saline solution (0.9% NaCl; 30 °C) containing 100 IU/mL penicillin and 50 µg/mL streptomycin sulfate. In the laboratory, the ovaries were washed in saline solution and COCs were aspirated from small antral follicles (<2.0 mm in diameter) using a 21 gauge needle connected to a sterile syringe. Under a stereomicroscope, COCs were classified according to the morphology of oocyte and cumulus cells. After morphological evaluation, COCs presenting compact cumulus cells and oocytes with no signs of cytoplasmic degeneration were considered normal and destined to *in vitro* culture.

For the growth of oocytes *in vitro*, the medium was TCM-199 supplemented with 4% polyvinylpyrrolidone (PVP), 1µg/mL estradiol, 4 mM hypoxanthine, 0.2 mM pyruvic acid, 2.2 mg/mL sodium bicarbonate, 5.0 µg/mL LH (Bioniche, Belleville, ON, Canada), 0.5 µg/mL FSH (Bioniche, Belleville, ON, Canada), 5% fetal bovine serum and 100 IU/mL penicillin and 50 µg/mL streptomycin sulfate. The COCs (n= 404) were cultured individually for 48 hours in growth medium alone or supplemented with EGF (10ng/mL); P4 (100µM) or both EGF (10ng/mL) and P4 (100µM) at 38.5^{o}C with 5% CO_2 in air. COCs were cultured individually in 48 well plates, each well containing 20 µL of culture medium. After the growth period, the morphology, oocyte diameters and meiotic progression were evaluated and the COCs were destined to *in vitro* prematuration.

The *in vitro* prematuration medium was TCM-199 containing Earle's salts and L-glutamine (Sigma) supplemented with 0.2 mM pyruvic acid, 5.0 mg/mL LH (Bioniche, Belleville, ON, Canada), 0.5 mg/mL FSH (Bioniche, Belleville, ON, Canada), 0.4% BSA, 10 μM of cilostamide and 100 IU/mL penicillin and 50 μg/mL streptomycin sulfate. The COCs (n= 240) were cultured individually for 20 hours in prematuration medium and at the end of this period, the COCs were evaluated for morphology, oocyte diameters and meiotic progression and subjected to *in vitro* maturation. In addition, four groups containing 10 oocytes were stored at -80 ° C for RNA extraction.

The IVM medium consists of TCM-199 containing Earle's salts and L-glutamine (Sigma) supplemented with 25 mM HEPES, 0.2 mM pyruvic acid, 2.2 mg/mL sodium bicarbonate, 5.0 mg/mL LH (Bioniche, Belleville, ON, Canada), 0.5 μg /mL FSH (Bioniche, Belleville, ON, Canada), 0.4% BSA and 100IU/mL penicillin and 50 μg /mL streptomycin sulfate. After culture, to evaluate meiotic progression, the cumulus cells were removed by vortexing and the oocytes were fixed in 4% paraformaldehyde for 15 min and transferred to 0.5% TritonX-100. The chromatin configuration during meiosis were assessed by 10 μg/mL bisbenzimide (Hoechst33342) and analyzed under an epi-fluorescent inverted microscope (Leica, DMI4000B, Wetzlar, Germany). Oocytes having nuclear membrane were considered at germinal vesicle (GV) stage, while those without nuclear membrane, i.e., that were at GVBD, metaphase I, anaphase I, telophase I, or metaphase II stages, were considered to have resumed meiosis. After the period of either prematuration or maturation, oocytes (n= 80) were stored in micro-centrifuge tubes at -80 °C, until RNA extraction.

Total RNA was extracted using the Trizol reagent (Invitrogen, São Paulo, Brazil). According to the manufacturer's instructions, 1 mL of Trizol solution was added to each frozen samples and the lysate was aspirated through a 20-gauge needle before centrifugation at 10,000 x g for 3 min at room temperature. Thereafter, all lysates were diluted 1:1 with 70%

ethanol and subjected to a mini-column. After binding of the RNA to the column, DNA digestion was performed using RNase-free DNase (340 K unitz units/mL) for 15 min at room temperature. After washing the column three times, the RNA was eluted with 30 mL RNAse-free water.

The RNA concentration was estimated by reading the absorbance at 260 nm and was checked for purity at 280 nm in a spectrophotometer (Amersham, Biosciences Cambridge, England). Before the reverse transcription reaction, samples of RNA were incubated for 5 min at 70°C and then cooled on ice. Reverse transcription was performed in a total volume of 20 μL composed of 10 μL of sample containing 2 mg of RNA, 4 μL reverse transcriptase buffer (Invitrogen, São Paulo, Brazil), 8 units RNAsin, 150 units of reverse transcriptase Superscript III, 0.036U random primers, 10 mM DTT and 0.5 mM of each dNTP (Invitrogen, São Paulo, Brazil). The mixture was incubated at 42°C for 1 h, subsequently at 80°C for 5 min, and finally stored at -20 °C. The negative control was prepared under the same conditions, but without addition of reverse transcriptase.

Quantification of the mRNA for *GDF9*, *CCNB1*, *H1FOO*, *cMOS*, *PARN* and *eIF4E* was performed by using SYBR Green. Each reaction in real time (20 μL) contained 10 μL of SYBR Green Master Mix (Applied Biosystems, Warrington, UK), 7.3 μL of ultrapure water, 1 μL of cDNA and 5 mM of each primer. Real time PCR was performed in at thermocycler (Mastercyclers ep Realplex, Eppendorf, Germany). The primers designed to perform amplification of mRNA for *CCNB1*, *H1FOO*, *GDF9*, *cMOS*, *PARN* and *eIF4E* are shown in Table 1. This table also shows glyceraldehyde3-phosphatedehydrogenase (*GAPDH*), which was used as endogenous controls for normalization of messenger RNA expression. The specificity of each primer pair was confirmed by melting curve analysis of PCR products. The thermal cycling profile for the first round of PCR was initial denaturation and activation of the polymerase for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30s at 58°C, and 30 s

at 72°C. The final extension was for 10 min at 72°C. Primer efficiency was determined using serial dilutions of the target cDNA. All reactions were performed in triplicate in a real time PCR Mastercyclers Realplex (Eppendorf Germany). The negative control was prepared under the same conditions but without addition of cDNA. The delta–delta-CT method was used to transform CT values in to normalized relative messenger RNA expression levels (Livak *et al.*, 2001).

The percentages of GV in the different treatments were evaluated using Mann Whitney test. The data of expression of mRNAs for *GDF9, CCNB1, H1FOO, cMOS, PARN* and *eIF4E* in oocytes from small antral follicles (<2.0 mm) were analyzed by the Kruskal-Wallis test, followed by the post hoc non-parametric Dunn's Multiple Comparison Test. Differences were considered significant when $P<0.05$.

Experiment 2: Effects of EGF and P4 on oocyte maturation and expression of maturation-related transcripts in oocytes from medium antral follicles (3.0 – 6.0 mm)

Cow ovaries (n= 80) were obtained from a local abattoir and transported within 1 h to the laboratory as described previously. In the laboratory, the ovaries were washed in saline solution and COCs were aspirated from medium antral follicles (3.0 to 6.0 mm in diameter) using a 21 gauge needle connected to a sterile syringe. Under a stereomicroscope, COCs were classified according to the morphology of oocyte and cumulus cells. After morphological evaluation, COCs presenting compact cumulus cells and oocytes with no signs of cytoplasmic degeneration were considered normal and destined to *in vitro* culture. Oocytes from this category were not submitted to the *in vitro* growth period.

For prematuration and maturation *in vitro*, those COCs classified as grade 1 (with multilayered compact cumulus and granulated oocyte cytoplasm), and grade 2 (with one to

three layers of cumulus cells and granulated oocyte cytoplasm) were used (Mikkelsen and Lindenberg, 2001). These COCs (n=320) were selected for culture and randomly distributed into 200 µL of IVM medium with or without follicular hemi-sections and cultured in an incubator at 38.5°C in a saturated humidity atmosphere containing 5% CO2 and 95% of air.

The *in vitro* prematuration medium was TCM-199 containing Earle's salts and L-glutamine (Sigma) supplemented with 0.2 mM pyruvic acid, 5.0 mg/mL LH (Bioniche, Belleville, ON, Canada), 0.5 mg/mL FSH (Bioniche, Belleville, ON, Canada), 0.4% BSA, 100 IU/mL penicillin and 50 µg/mL streptomycin sulfate added of cilostamide (10µM) and hemisections follicular. To evaluate the effects of EGF and P4 on meiosis resumption, COCs (n=320) were subjected to pre-IVM for 12h in pre-IVM medium alone or supplemented with EGF (10ng/mL); P4 (100µM) or both EGF (10ng/mL) and P4 (100µM). In addition, four groups containing 20 oocytes were stored at -80°C for RNA extraction, performed as described in experiment 1.

To obtain follicular hemi-sections, follicles were isolated from the ovaries and dissected free of stromal tissue and transparent follicles measuring 3.0 to 6.0 mm in diameter were selected (Richard *et al.*, 1996a). The follicles were sectioned into equal halves with a scalpel and the oocytes were removed and discarded. Then, the follicular hemi-sections were washed in TCM-199 containing 0.4% bovine serum albumin (BSA) and randomly distributed into 4-well culture plates (Nunc, Roskilde, Denmark) containing culture medium. Two follicular hemi-sections were each added to 50 µL of medium, and then cultured for 2 h before incubation with COCs (De Cesaro *et al.*, 2013).

After the pre-IVM period, the COCs (n=160) were submitted to the *in vitro* maturation period (IVM) and evaluated as described previously. The percentages of GV in the different treatments were evaluated using Mann Whitney test. The data of expression of mRNAs for *GDF9*, *CCNB1*, *H1FOO*, *cMOS*, *PARN* and *eIF4E* in oocytes from medium antral follicles

(3.0 – 6.0 mm) were analyzed by the Kruskal-Wallis test, followed of the post hoc nonparametric Dunn's Multiple Comparison Test. Differences were considered significant when $P<0.05$.

3. Results

3.1 Experiment 1: Effects of EGF and P4 on oocyte maturation and expression of maturation-related transcripts in oocytes from small antral follicles (<2.0 mm)

When compared to time 0, cultured oocytes had their diameters increased, but no effects of EGF, P4 or both were observed after growth and prematuration periods (Table 2). After prematuration, COCs from small antral follicles cultured in presence of both EGF and P4 showed no significant difference of oocytes at GV or resumption of meiosis stages when compared to those COCs cultured in TCM-199 alone (Table 3).

Figure 1 shows that mRNA levels for *cMOS* (Figure 1A), *H1FOO* (Figure 1B) and *CCNB1* (Figure 1C) in oocytes from small antral follicles cultured with P4 were higher than those cultured in the control medium ($P<0.05$). The mRNA levels for *eIF4E* (Figure 1D) in oocytes from small antral follicles cultured with both EGF and P4 showed a significant increase when compared to those cultured with EGF alone. On the other hand, mRNA levels for *GDF9* (Figure 1E) did not show significant difference between the treatments. Despite this, mRNA levels for *PARN* (Figure 1F) in oocytes from small antral follicles cultured with both EGF and P4 had a significant increase when compared to those cultured with P4 alone.

3.2 Experiment 2: Effects of EGF and P4 on oocyte maturation and expression of maturation-related transcripts in oocytes from medium antral follicles (3.0 – 6.0 mm)

In vitro prematuration of COCs in the presence of EGF did not influence oocyte growth from time zero until the end of prematuration. On the other hand, P4 alone and associated with EGF promoted an increase in oocyte diameter when compared to time zero (Table 4). Table 5 shows that the presence of EGF, P4 or EGF and P4 in prematuration medium did not influence the rates of resumption of meiosis.

Figure 2A shows that mRNA levels for *cMOS* in oocytes from medium antral follicles cultured in the presence of EGF or P4 were higher than those pre-matured in the control medium ($P<0.05$). The mRNA levels of *H1FOO* (Figure 2B) in oocytes cultured with both EGF and P4 showed a significant increase when compared to those cultured in presence of EGF alone ($P<0.05$). On the other hand, mRNA levels for *CCNB1* (Figure 2C) in oocytes cultured with P4 were higher than those cultured in presence of only EGF ($P<0.05$). Besides that, the levels of mRNA for *EIF4E* (Figure 2D) showed a significant reduction in oocytes cultured with P4, while mRNA levels for *GDF9* (Figure 2E) showed a significant reduction when cultured with EGF in comparison with those cultured in control medium. The levels of mRNA for *PARN* (Figure 2F) did not present a significant difference between the treatments. Figure 3 summarizes the changes in the levels of transcripts in oocytes from small and medium antral follicles after culture in medium supplemented with EGF, P4 or both EGF and P4.

4. Discussion

This study shows for the first time the influence of EGF and P4 on oocyte growth and expression of maturation-related genes in oocytes from small and medium antral follicles. Culture COCs of small antral follicles for 48 h increases their diameters in vitro. Yamamoto

et al. (1999) also reported the in vitro growth of oocytes from small antral follicles and emphasized the importance of hypoxanthine to maintain cAMP levels within the oocyte by its inhibitory action on cAMP-phosphodiesterase and to maintain meiotic arrest (Downs *et al.,* 1993). This substance also promotes coupling between oocytes and surrounding granulosa cells (Eppig *et al.,* 1989), via heterologous gap junctions through which small molecules, such as energy substrates, nucleotides and amino acids, are transferred into the oocytes (Heller *et al.,* 1981). Despite we have not seen an effect of EGF or P4 on oocyte growth, previous studies have shown that the effects of EGF on oocytes depends on stimulation of cumulus or granulosa cells, and that EGF signaling improves the ability of oocytes to support embryonic development (Conti *et al.,* 2018). In relation to P4, its effects are mediated by its interaction with progesterone receptors (PRs), PR type A (PR-A) and PR type B (PR-B) (Aparicio *et al.,* 2011) and the expression of PRs in bovine COCs after IVM suggest important local actions of P4 during oocyte maturation (Aparicio *et al.,* 2011). The regulation of PRs during bovine IVM is dependent on cell (oocyte or cumulus) and receptor-specific, potentially indicating a different role for each receptor during bovine oocyte maturation (Yamashita *et al.,* 2003; Montano *et al.,* 2009).

Although inhibition of expression of the PR-A and PR-B proteins has been shown to inhibit meiotic resumption and cumulus cell expansion in pigs (Shimada *et al.,* 2004), our study showed that P4 alone or assotiated with EGF did not influence meiosis resumption of bovine oocytes from small antral follicles. It is well described the switch from estradiol to P4 dominance in the follicular fluid of preovulatory follicles in the period between the LH surge and ovulation (Dieleman *et al.,* 1983), coincident with resumption of meiosis and maturation of the oocyte (Aparicio *et al.,* 2011). Other studies have shown that P4 do not affect the rate of IVM of bovine oocytes (Karlach *et al.,* 1986; Carter *et al.,* 2010; Nagyiva *et al.,* 2014). Regarding EGF, studies indicate that the promotion of EGFR signaling in cumulus cells

seems to be a key factor in developmental oocyte acquisition competence (Richani *et al.*, 2018). In pigs, it was shown that COCs from small antral follicles possess less competence for cumulus expansion in response to EGF or EGF-like peptides, partly because of immature EGFR signaling in cumulus cells (Sugimura *et al.,* 2015). In bovine species, oocytes from such small antral follicles have low developmental competence for many reasons, not only because of insufficient EGFR signaling in cumulus cells (Ritter *et al.,* 2015).

This study shows that P4 alone or associated with EGF promotes *in vitro* growth of oocytes from medium antral follicles. In fact, Aparicio et al. (2011) showed the presence of P4 receptors in granulosa, cumulus and oocyte cells, and that this signalling patways control cycle, oocyte maturation and probably bovine oocyte growth during prematuration. Previous studies have demonstrated that progestin concentrations in the folicular fluid increase in follicles with 3 to 9 mm diameter (Ireland *et al.*, 1979). Additionally, *in vivo* studies demonstrated that dairy cows with follicles growing with high P4 levels were related to greater fertility cows (Bisinotto *et al.,* 2010) that beef cows with low P4 levels (Nasser *et al.,* 2011).

This study showed that P4 increases mRNA expression of *cMOS*, *H1FOO* and *CCNB1* in oocytes derived from small antral follicles. In this same category, EGF increased mRNA levels for *CCNB1*. On the other hand, in oocytes from medium antral follicles, only EGF and P4 increased *cMOS* mRNA levels. The *cMOS* proto-oncogene, encoding a putative serine/threonine protein kinase (Maxwell *et al.,* 1985) and it is expressed at high levels in the germ cells of vertebrates (Keshet *et al.*, 1988). Studies indicate that synthesis of the endogenous MOS protein is stimulated during progesterone-induced oocyte maturation and is required for both activation of MPF and GVBD (Sagata *et al.,* 1988; Sagata *et al.,* 1989). In addition, De Moor and Richter (1997) showed that P4 binds to its receptor in the oocyte membrane and activates *MOS* mRNA polyadenylation and translation. MOS then activates

MAP kinases and cdc2 kinase, which induces GVBD and leads to the completion of meiosis. EGF and P4, in its turn, together promoted an increase in *H1FOO* mRNA levels when compared to EGF alone. In mammals, *H1FOO* is transcribed specifically in oocytes (Tanaka *et al.,* 2001) and is localized to the nucleus of germinal vesicle (GV) stage oocytes and binds to chromosomes in the subsequent stages (Tanaka *et al.,* 2001). Interestingly, a recent study revealed that *H1FOO* plays a role in forming the loose structure of chromatin in single cell embryos (Funaya *et al.,* 2018). Based on our data, it is possible that a threshold level of *H1FOO* expression is required for oocyte maturation, such that only those oocytes that possess more than the threshold limit will be able to have a higher chance to continue for maturation. This can explain why individual GV oocytes have different fates (arrested at GV, MI or matured to MII) after *in vitro* maturation. The *in vitro* collected GV-stage oocytes come from different follicles or different ovaries with diverse conditions, thus accumulating variable levels of *H1FOO*; which decide oocyte fate (Yun *et al.,* 2015). *CCNB1*, as well as its cytoplasmic displacement to the nucleus, along with dephosphorylation of the catalytic subunit are required for meiosis progression (Mermillod *et al.,* 2000). In our study, in oocytes from small antral follicles, EGF increased mRNA expression levels for *CCNB1*. A study with canine oocytes demonstrated the influence of EGF on cellular signaling pathways, promoting changes in CCNB1 cytoplasmic localization, and influencing the acquisition of oocyte competence (Pereira *et al.,* 2019) In bovine oocytes, CCNB1 was detected throughout the cytoplasm with granular appearance, without large variations between immature or mature oocytes (Quetglas *et al.,* 2009). According to De Vantery et al. (1996), cyclin is preferably stored as mRNA in immature oocytes and is only translated with meiosis resumption.

In conclusion, P4 alone or associated with EGF promotes the growth of oocytes from medium antral follicles, but not of oocytes from small antral follicles. P4 or EGF increases the expression of mRNAs that are stored in the oocyte, such as *cMOS*. Future studies are still

needed to evaluate the relationship between RNA expression, oocyte competences and/or embryonic development

Conflict of interest statement

There was no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Figures and Tables

List of figures and tables

Figure 1

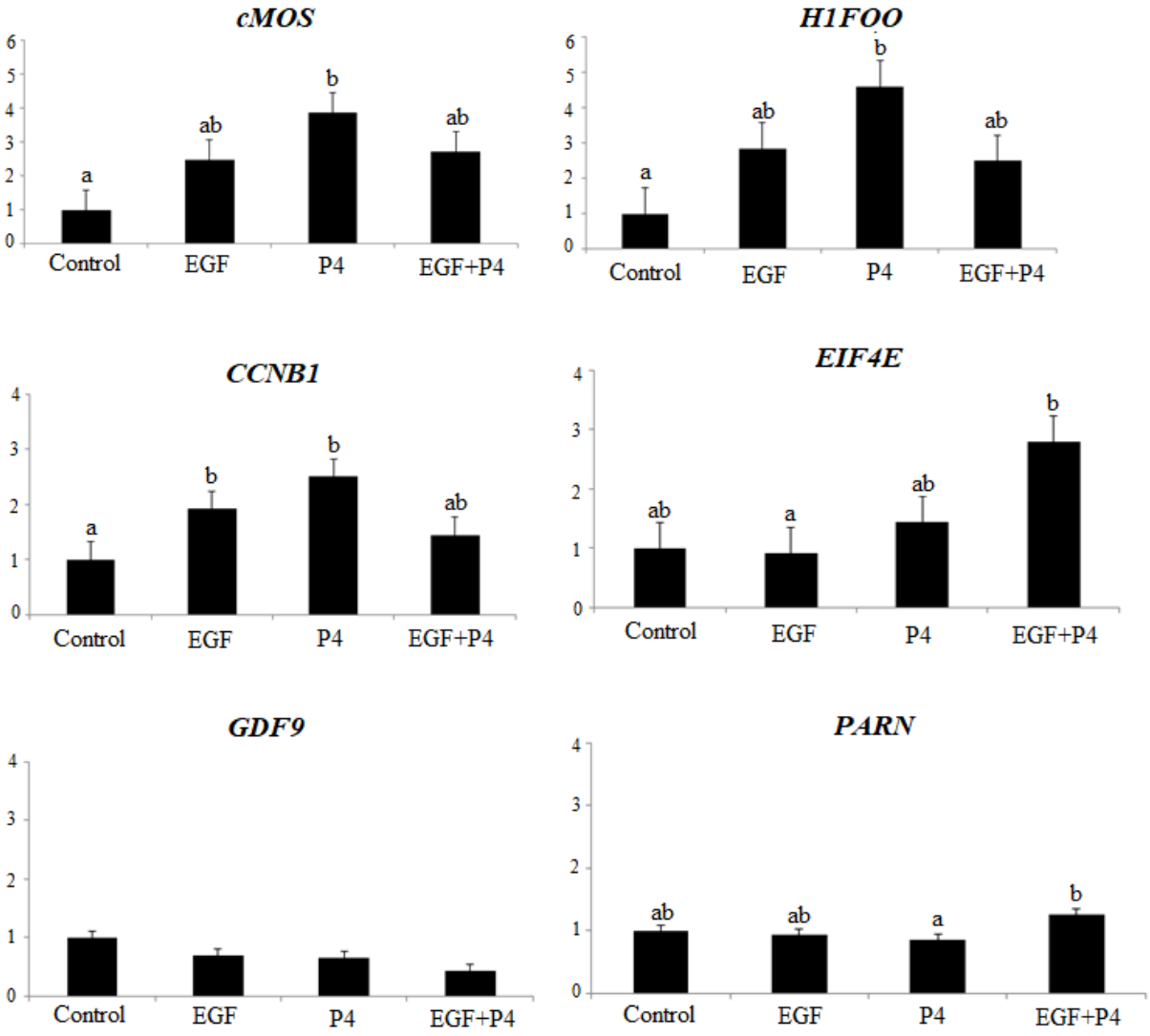

Figure 2

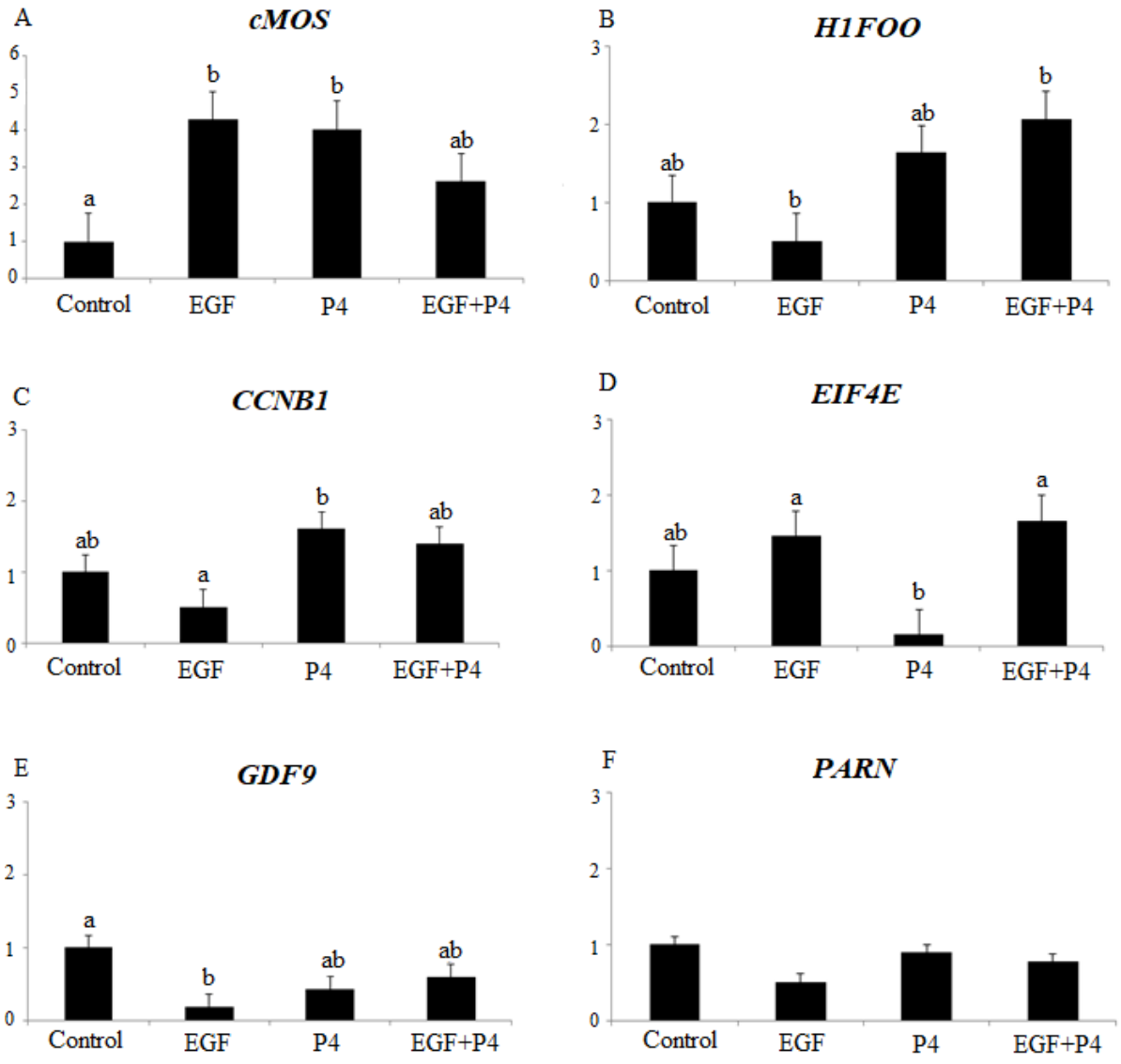
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Table 2

	Day 0	After 48 h	After pré-IVM
TCM-199	97.8 ± 1.2µm^{a,A}	101.1 ± 0.7µm^{a,b,A}	102.2 ± 0.5µm^{b,A}
EGF	98.6 ± 1.1µm^{a,A}	102.9 ± 1.0µm^{b,A}	103.9 ± 0.7µm^{b,A}
P4	98.0 ± 1.3µm^{a,A}	101.4 ± 0.8µm^{a,b,A}	102.5 ± 1.0^{b,A}
EGF+P4	98.2 ± 1.5µm^{a,A}	103.4 ± 1.2µm^{b,A}	104.5 ± 1.3µm^{b,A}

a,b represents a significant difference between the culture time. A,B represents a significant difference between the treatments.

Table 3

	Germinal vesicle (GV)		Resumption of meiosis	
	Pre-Maturation (%)	IVM (%)	Pre-Maturation (%)	IVM (%)
TCM-199	78.0 (32/41)^{A}	22.5 (9/40)^{A}	19.5 (8/41)^{A}	77.5 (31/40) ^{A}
EGF	85.4 (35/41) ^{A}	23.1(9/39)^{A}	14.6 (6/41)^{A}	76.9 (30/39)^{A}
P4	88.1 (37/42)^{A}	26.8 (11/41)^{A}	11.9 (5/42)^{A}	73.2 (30/41)^{A}
EGF+P4	90.0 (36/40)^{A}	27.5 (11/40)^{A}	10.0 (4/40)^{A}	72.5 (29/40) ^{A}

A,B represents a significant difference between the treatments.

Table 4

	Day 0	After pre-IVM
TCM-199	107.1 ± 0.9µm^{a,A}	111.4 ± 0.9µm^{b,A}
EGF	107.3 ± 1.1µm^{aA}	110.8 ± 1.8µm^{a,A}
P4	110.3 ± 1.0µm^{a,A}	115.2 ± 0.5µm^{b,B}
EGF+P4	110.5 ± 1.0µm^{a,A}	115.3 ± 1.2µm^{b,B}

a,b represents a significant difference between the culture time.

A,B represents a significant difference between the treatments.

Table 5

	Day 0	After pre-IVM
TCM-199	107.1 ± 0.9µm^{a,A}	111.4 ± 0.9µm^{b,A}
EGF	107.3 ± 1.1µm^{aA}	110.8 ± 1.8µm^{a,A}
P4	110.3 ± 1.0µm^{a,A}	115.2 ± 0.5µm^{b,B}
EGF+P4	110.5 ± 1.0µm^{a,A}	115.3 ± 1.2µm^{b,B}

a,b represents a significant difference between the culture time.

A,B represents a significant difference between the treatments.

8 CONCLUSÕES GERAIS

- O crescimento de folículos secundários até os estágios de folículos antrais pequenos, médios e grandes é acompanhando de um aumento nos níveis de RNAm para H1FOO, GDF9 e PARN em seus oócitos, .

- O cultivo *in vitro* de folículos secundários, associado com a pré-maturação e a maturação dos oócitos dos folículos antrais aumentam a expressão de RNAm para eIF4E, PARN, H1FOO, cMOS, GDF9 e CCNB1.

- P4 sozinho ou P4 associado ao EGF promove o crescimento de oócitos derivados de folículos antrais médios, mas não influencia o crescimento de oócitos derivados de folículos antrais pequenos.

- P4 sozinho ou P4 associado ao EGF aumentam a expressão de RNAm armazenados no oócito, tais como cMOS, CCNB1 e H1FOO.

9 PERSPECTIVAS

O conhecimento dos níveis de expressão de RNAm que são estocados nos oócitos, bem como, dos efeitos de EGF e P4 no crescimento e pré-maturação de oocitos e sua influência no nível de expressão de genes relacionados ao desenvolvimento oocitário, abre novas perspectivas para a elucidação do papel das diversas substâncias que atuam na regulação da meiose oocitária. Além disso, podem contribuir para o aumento da taxa de competência oocitária após o cultivo *in vitro* de folículos secundários, bem como, o crescimento e pré-maturação de oócitos derivados de pequenos e médios folículos antrais aptos a assegurar o desenvolvimento embrionário inicial.

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