**UNIVERSIDADE FEDERAL DE SANTA MARIA**
**CENTRO DE CIÊNCIAS RURAIS**
**PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA VETERINÁRIA**

**Paulo Roberto Antunes da Rosa**

# CARACTERIZAÇÃO DE UM NOVO MODELO DE MATURAÇÃO DE OÓCITO IN VITRO E PARTICIPAÇÃO DO MTOR NA OVULAÇÃO EM BOVINOS

Santa Maria, RS
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**Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Medicina Veterinária, Área de Concentração em Sanidade e Reprodução Animal, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de Doutor em Medicina Veterinária**

Orientador: Prof. Paulo Bayard Dias Gonçalves

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**Aprovado em 26 de fevereiro de 2016:**

_____
**Paulo Bayard Dias Gonçalves, PhD (UFSM)**
(Presidente/orientador)

_____
**Vilceu Bordignon, PhD (McGill)**

_____
**Fernando Silveira Mesquita, PhD (UNIPAMPA)**

_____
**Marcos Henrique Barreta, Dr (UFSC)**

_____
**Alfredo Quites Antoniazzi, Dr (UFSM)**

Santa Maria, RS
2016

# AGRADECIMENTOS

Primeiramente gostaria de fazer um agradecimento especial para meus pais, Paulo Roberto Mariani da Rosa e Ambrozina Antunes da Rosa que estão sempre presentes em todos os momentos da minha vida dando muito apoio, atenção, carinho e amor. Muito obrigado por saberem lidar com minha ausência durante os períodos que estive fora.

Aos demais membros da minha família, minha irmã Ana Cláudia, meu cunhado Augusto e meus sobrinhos Augusto Cesar e João Roberto, por todo apoio, carinho, amizade e compreensão.

Ao Professor João Francisco de Oliveira (*in memoriam*) que foi meu orientador durante o período inicial do doutorado, pelos ensinamentos e amizade ao longo dos anos que convivemos.

Ao meu orientador, Professor Paulo Bayard Dias Gonçalves, pela amizade, ensinamentos e pelo exemplo de dedicação à ciência.

Aos coorientadores, professores Vilceu Bordignon e Raj Duggavathi, pela amizade e por me acolherem e disponibilizarem seus laboratórios durante o período de estágio sanduíche no Canadá.

Aos amigos e ao Departamento de Ciências Animais da McGill University que me acolheram durante o período de estágio sanduíche.

A toda equipe do laboratório BioRep, professores, colegas de pós-graduação e estagiários, pela amizade, ajuda nos experimentos e convivência familiar durante os dias de trabalho.

Ao Frigorífico Silva, por disponibilizar a coleta dos ovários utilizados nos experimentos.

Ao CNPq e CAPES pelo apoio financeiro.

Enfim, a todos aqueles que colaboraram direta ou indiretamente para a realização deste trabalho.

## RESUMO

## CARACTERIZAÇÃO DE UM NOVO MODELO DE MATURAÇÃO DE OÓCITO IN VITRO E PARTICIPAÇÃO DO MTOR NA OVULAÇÃO EM BOVINOS

AUTOR: Paulo Roberto Antunes da Rosa
ORIENTADOR: Paulo Bayard Dias Gonçalves

O primeiro estudo caracterizou um modelo *in vitro* de bloqueio do reinício da meiose de oócitos bovinos. Em um primeiro momento, demonstramos que o uso de um inibidor dos EGFR (AG1478; 5µM) em um sistema de cultivo com metades foliculares (FHS) foi eficiente para manter 89,3% dos oócitos em vesícula germinativa durante 15 h. Esse efeito de bloqueio foi dependente das FHS uma vez que na sua ausência apenas 40% dos oócitos permanecem em vesícula germinativa. O sistema de bloqueio foi totalmente reversível, tendo em vista que os oócitos completaram a maturação após um período adicional de 18 e 20 h, e suportaram o desenvolvimento embrionário subsequente após fertilização *in vitro*. Quanto ao perfil molecular das células envolvidas no bloqueio, no oócito não foi verificado efeito do tratamento na expressão dos genes avaliados. Entretanto, nas células do cumulus, enquanto a expressão de EGR1, TNFAIP6 e HAS2 foi diminuída pelo tratamento com AG1478, a expressão de CX43 e IMPDH1 foi diminuída pela influência das FHS. Além disso, nas células da granulosa observamos uma diminuição nos níveis de expressão de PGR e ADAMTS1 pelo tratamento com AG1478. Os dados de Western blot nos mostraram que a abundância de p-ERK1/2 diminui em decorrência do tratamento com AG1478 associado as FHS. Posteriormente, verificamos que o efeito inibitório do AG1478 juntamente com as FHS não foi revertido pelo tratamento com AngII ou PGs. Em conclusão, este estudo propõem um modelo efetivo e reversível para o bloqueio do reinício da meiose de oócitos bovinos. Em um segundo estudo, investigamos o papel do sistema mTOR e sua relação com genes regulados pelo LH durante o período pré-ovulatório em bovinos. Utilizando um modelo *in vivo*, demonstramos que ocorre um aumento na atividade do mTOR em células da granulosa 3 e 6 h após indução da ovulação com GnRH. Em momentos similares (3 h após GnRH) ocorreu maior abundância proteica para p-ERK1/2, STAR e EGR1. Ao injetar rapamicina no ambiente intrafolicular *in vivo*, não foram observadas alterações nas taxas de ovulação. Entretanto, o uso da rapamicina em cultivo *in vitro* de células da granulosa inibiu a expressão de RNAm para EREG induzida pelo LH. Além disso, os dados de cultivo comprovaram o efeito da rapamicina em bloquear a atividade do mTOR, caracterizada pela abundância proteica de p-P70S6K, induzir um provável aumento na abundância de p-AKT e não alterar os níveis de p-ERK1/2 e EGR1. Esses resultados fornecem a primeira evidência em bovinos que o sistema mTOR é regulado positivamente pelo LH em momentos similares a p-ERK1/2, STAR e EGR1. Além disso, os dados de inibição do mTOR contribuem para sugerir uma outra rota para a ovulação controlada pela p-AKT, na qual ocorre ativação de ERK1/2 em uma via independente dos níveis de expressão de EREG, AREG e PTGS2.



**Palavras chave:** EGFR. Oócito. Granulosa. Ovulação. mTOR. Bovinos.

## ABSTRACT

### CHARACTERIZATION OF A NEW MODEL OF IN VITRO OOCYTE MATURATION AND PARTICIPATION OF MTOR IN OVULATION IN CATTLE

AUTHOR: Paulo Roberto Antunes da Rosa
ADVISOR: Paulo Bayard Dias Gonçalves

In the first study, we characterized an *in vitro* culture system able to delaying meiosis resumption of bovine oocytes. Firstly, we demonstrated that the use of an EGFR inhibitor (AG1478; 5μM) in a culture system with follicular hemisections (FHS) was effective to maintain 89.3% of the oocytes in germinal vesicle stage (GV) during 15 h. This blocking effect was dependent on the FHS, since in its absence only 40% of the oocytes remain in GV stage. The meiosis blockage was totally reversible, since the oocytes reached matured stages after an additional 18 and 20 h maturation period and were able to support the embryonic development after *in vitro* fertilization. Regarding the molecular profile of the cells involved in the blocking system, we did not observe treatment effect on mRNA expression of the genes evaluated in oocyte. However, in cumulus cells, whereas the expression of EGR-1, TNFAIP6 and HAS2 was inhibited by AG1478 treatment, the expression of CX43 and IMPDH1 was decreased by FHS influence. Moreover, in the granulosa cells we observed a downregulation in the expression levels of PGR and ADAMTS1 by AG1478 treatment. The Western blot data revealed that the treatment with AG1478 plus FHS induces a downregulation in p-ERK1/2 protein abundance. In the next experiment, we verified that the AngII or PGE2 and PGF2α did not reverse the inhibitory effect of AG1478 plus FHS on meiosis resumption. In conclusion, findings from this study revealed an effective and reversible system to prevent meiosis resumption of bovine oocytes. In the second study, we investigate the role of mTOR system and its relation with LH regulated genes during preovulatory period in cattle. Using an *in vivo* model, we demonstrated mTOR kinase activity in granulosa cells 3 and 6 h after induction of ovulation with GnRH. In the similar moments (3 h after GnRH), we observed an increase in p-ERK1/2, STAR and EGR1 protein abundance. The inhibition of mTOR kinase activity by intrafollicular injection of rapamycin did not alter the ovulation rate. However, the treatment of granulosa cells *in vitro* with rapamycin interrupted the LH-induced increase in *EREG* mRNA levels. Moreover, the effect of rapamycin in culture was proved by inhibiting the p-P70S6K protein levels. In the same Western blot analysis, we verified that rapamycin may be inducing AKT activity and did not alter Phospho-ERK1/2 status and EGR1 protein abundance. These results provided the first evidence in cattle that mTOR system is upregulated by LH at time points similar to p-ERK1/2, STAR and EGR1. In addition, the mTOR inhibition data contribute to suggest an AKT dependent pathway during ovulation process, in which occurs ERK1/2 activation in a pathway independent of EREG, AREG and PTGS2 mRNA levels.



**Keywords:** EGFR. Oocyte. Granulosa. Ovulation. mTOR. Bovine.

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# 1. INTRODUÇÃO

A produção *in vitro* de embriões (PIV) bovinos é uma biotecnologia da reprodução que vem sendo utilizada em larga escala, tanto em cunho comercial como na pesquisa básica e aplicada, entretanto, os resultados obtidos não ultrapassam 30 a 40% de blastocistos. O sucesso da PIV de embriões está intimamente ligado ao período de maturação o qual compreende modificações citoplasmáticas, moleculares e nucleares que ocorrem paralelas ao desenvolvimento folicular (HYTTEL et al., 1997; BREVINI GANDOLFI & GANDOLFI, 2001; LODDE et al., 2007). Dessa forma, os oócitos recuperados de folículos pequenos (3 mm), embora sejam competentes para reiniciar a meiose, os mesmos não acumulam RNAm e proteínas de origem materna suficientes para suportar o desenvolvimento embrionário subsequente. Isso se confirma pelas maiores taxas de blastocisto obtidas com a utilização de oócitos provenientes de folículos em fase final de desenvolvimento comparado aos obtidos de folículos em estádios iniciais de desenvolvimento (LONERGAN et al., 1994; ARLOTTO et al., 1996).

Com o intuito de prolongar o período de maturação *in vitro* disponibilizando tempo para a completa maturação dos oócitos, estudos prévios utilizaram metodologias de bloqueio do reinício da meiose *in vitro*. Dentre as metodologias utilizadas, o uso de inibidores do Fator Promotor da Maturação (MPF) tais como Roscovitini e Butyrolactone I se mostraram eficientes em manter oócitos bovino no estádio de vesícula germinativa por um período de até 24 h sem interferir na capacidade de desenvolvimento subsequente (MERMILLOD et al., 2000; LE BEUX et al., 2003). Entretanto, esse bloqueio meiótico induzido não se mostrou eficiente para aumentar as taxas de desenvolvimento embrionário e, além disso, não possibilita o estudo de fatores envolvidos na progressão meiótica, uma vez que, a atividade do MPF é a etapa final da cascata de eventos que regulam a meiose, não sendo possível reverter esse bloqueio durante o tratamento com as drogas supracitadas.

Participando da construção do conhecimento acerca dos fatores autócrinos e parácrinos envolvidos na progressão meiótica de oócitos bovinos, nosso laboratório vem utilizando ao longo dos anos um modelo de maturação *in vitro* com metades foliculares para bloqueio da progressão meiótica conforme descrito previamente (RICHARD & SIRARD, 1996a). Nesses estudos, foi demonstrado que a Angiotensina II (AngII) estimula a progressão meiótica revertendo o efeito inibitório das metades foliculares (GIOMETTI et al., 2005; STEFANELLO et al., 2006) e que a ação da AngII é mediada pela progesterona e Prostaglandinas E2 e F2α

(BARRETA et al., 2008; SIQUEIRA et al., 2012). Entretanto, ainda permanecem obscuras as rotas intracelulares ativadas em decorrência da ação da AngII durante a progressão meiótica.

A atividade do Epidermal Growth Fator Receptor (EGFR) é um importante modulador da ação gonadotrófica durante a maturação de complexos cumulus-oócito (COCs) de roedores e suínos. Sua inibição com o fármaco AG1478 impede a ocorrência do reinício da meiose e expansão das células do cumulus nessas espécies (PROCHAZKA et al., 2003; REIZEL et al., 2010a). Além disso, estudos em tecidos cardíacos revelam que a atividade do EGFR modula a ação da AngII no controle da pressão arterial (KAGIYAMA et al., 2002). Embora esses dados evidenciam uma participação do EGFR no controle da maturação nuclear de oócitos bovinos, esse mecanismo ainda não foi completamente entendido. Dessa forma, o objetivo deste primeiro estudo foi avaliar os efeitos da inibição dos EGFR no bloqueio da meiose *in vitro* em um sistema de cultivo com a presença ou ausência de metades foliculares, bem como compreender as implicações desse modelo na expressão gênica das células da granulosa, cumulus e oócito e sua aplicabilidade para o estudo de fatores envolvidos na progressão meiótica.

O segundo estudo descrito nesta tese descreve a caracterização do sistema mTOR (mammalian target of rapamycin) e sua relação com rotas intracelulares reguladas pelo LH durante o período pré-ovulatório em bovinos. Nesse estudo, utilizamos modelos experimentais *in vivo* pré-estabelecidos em nosso laboratório (FERREIRA et al., 2007; GASPERIN et al., 2012; TONELLOTTO DOS SANTOS et al., 2012). No primeiro modelo, a ovariectomia em momentos estratégicos, após a indução da ovulação, nos permite avaliar a atuação de diferentes fatores e rotas de sinalização nas células foliculares em diferentes momentos relacionados ao pico de LH. Aliado a este, o modelo de injeção intrafolicular e monitoramento ultrassonográfico do processo ovulatório nos permite manipular o ambiente folicular e identificar fatores essenciais ou não para a ocorrência da ovulação. Além dos modelos supracitados, este estudo contou também com um modelo *in vitro* de cultivo de células da granulosa previamente descrito, no qual fatores conhecidos, adicionados ao meio de cultivo, têm suas funções estabelecidas. Os modelos experimentais desenvolvidos em nosso laboratório nos permitem obter resultados fidedignos e representativos da fisiologia e, futuramente, para geração de tecnologia. Além disso, a associação do uso de modelos *in vivo* e *in vitro* se torna uma importante ferramenta para elucidar mecanismos fisiológicos de sistemas locais ovarianos em eventos reprodutivos.

Ao longo do processo de ovulação ocorrem alterações endócrinas que determinam ativação de diferentes rotas de sinalização intracelular e expressão de genes envolvidos nas alterações morfológicas e funcionais das células que compõem o ambiente folicular (PARK et

al., 2004). Eventos de diferenciação, proliferação e migração celular em diferentes tecidos serviram como base para a descoberta de mecanismos celulares atuantes no processo ovulatório. Um exemplo disso é o papel do sistema mTOR. Esse sistema é composto por dois complexos proteicos denominados mTOR Complex 1 (mTORC1) e mTOR complex 2 (mTORC2) cujas estruturas se diferem quanto as suas subunidades regulatórias que no mTORC1 denomina-se Raptor (regulatory associated protein of TOR) e no mTORC2 denomina-se Rictor (RPTOR independent companion of mTOR) (LOEWITH et al., 2002). O mTORC1 tem sua atividade marcada pela fosforilação da proteína P70S6K e, quando ativado, atua nos mecanismos proliferativos de diferentes tipos celulares através da aceleração do ciclo celular (EKIM et al., 2011), diferentemente do mTORC2, cuja função não está completamente estabelecida embora existam estudos que demonstram sua participação na regulação do citoesqueleto através da estimulação das fibras de actina (SARBASSOV et al., 2004).

O envolvimento do sistema mTOR na reprodução foi investigado inicialmente no ambiente uterino participando dos mecanismos de implantação embrionária (CHEN et al., 2009). Posteriormente, na tentativa de melhor compreender os eventos fisiológicos que ocorrem no processo ovulatório, estudos em roedores caracterizaram o papel do sistema mTOR como mediador da ação gonadotrófica nas células da granulosa, regulando a atividade proliferativa bem como a ocorrência do processo ovulatório (YU et al., 2011). Entretanto, até o momento não existem dados que mostrem o envolvimento do mTOR na ocorrência do processo ovulatório de espécies monovulatórias. Dessa forma, o objetivo deste trabalho foi caracterizar o sistema mTOR e sua relação com genes regulados pelo LH durante o período pré-ovulatório de bovinos.

# 2. REVISÃO BIBLIOGRÁFICA

## 2.1 Maturação oocitária

Ao longo do desenvolvimento folicular, os oócitos sofrem diversas modificações de ordem ultra estrutural, do citoesqueleto e bioquímicas. Tais eventos, que iniciam com a formação do folículo primordial e continuam até o momento da ovulação, são responsáveis por tornar o oócito competente para uma adequada fecundação e subsequente desenvolvimento embrionário (BREVINI GANDOLFI & GANDOLFI, 2001). Dessa forma, oócitos derivados de folículos grandes são mais capacitados para o desenvolvimento embrionário que oócitos oriundos de folículos pequenos (LONERGAN et al., 1994), uma vez que permaneceram mais tempo no ambiente folicular sofrendo os efeitos da interação com as células da granulosa (mural e cumulus) através de mecanismos de sinalização coordenados por fatores autócrinos e parácrinos e funcionalidade das *gap junctions* (GILCHRIST et al., 2004; LODDE et al., 2007; GILCHRIST, 2011).

Durante o processo de maturação molecular do oócito ocorre a síntese de RNAm materno e proteínas importantes para o desenvolvimento embrionário precoce anterior a ativação do genoma embrionário (SIRARD, 2001). No oócito, ao contrário do que ocorre com qualquer célula somática, o intervalo entre a síntese e a utilização do RNAm e moléculas protéicas pode ser de até várias semanas, ocorrendo o armazenamento dessas moléculas em uma forma quiescente para o seu emprego no tempo certo ao longo da maturação do oócito e desenvolvimento embrionário inicial (HYTTEL et al., 1997). Estudos recentes em roedores demonstraram que a capacidade de tradução e acúmulo de proteínas pelo oócito é dependente da ação do FSH o qual, por intermédio da ação do sistema EGF nas células somáticas do folículo, determinam a ativação da via phosphatidyl-inositol 3-phosphate/AKT no interior do oócito (FRANCIOSI et al., 2016). Provavelmente, a inibição desse mecanismo esteja envolvida na baixa competência para o desenvolvimento embrionário de oócitos bovinos incapacitados de promover a síntese completa de RNAm materno e de proteínas durante o seu crescimento (SIRARD, 2001).

Provavelmente, o pico de LH ou a retirada do oócito do ambiente folicular seja um sinal comum para ativar o MPF (fator promotor da maturação) e a MAPK (mitogen-activated protein kinase) responsáveis pelo início da maturação nuclear do oócito, formação dos fusos meióticos e manutenção do segundo bloqueio meiótico na fase de metáfase II (COLLEDGE et al., 1994;

HASHIMOTO et al., 1994). O processo de reinício da meiose ocorre inicialmente com o fechamento das *gap junctions* e uma diminuição nos níveis de GMPc (Guanosine Monophosphate Cyclic) no interior do oócito. Esse sinal desencadeia a ativação da enzima Phosphodiesterase 3 (PDE3A) a qual atua degradando AMPc (cyclic adenosine monophosphate) (RICHARD et al., 2001; THOMAS et al., 2004). Essa diminuição nos níveis de AMPc determina a ocorrência do rompimento da vesícula germinativa (RVG) via desfosforilação da proteína CDK1 e consequente ativação do MPF (DUCKWORTH et al., 2002; OH et al., 2010). Eventos posteriores ao rompimento da vesícula germinativa tais como organização do material genético até atingir metáfase II, estão relacionados com a ativação da MAPK no interior do oócito (FAN & SUN, 2004). Em oócitos de mamíferos, estão presentes duas isoformas da MAPK conhecidas como extracellular regulated kinase [ERK1(p44)] e [ERK2(p42)] (FAN et al., 2002). A Ativação da MAPK é desencadeada pela fosforilação de resíduos de tirosina e treonina e é fundamental para a progressão da meiose de oócitos de diferentes espécies passando pelos estádios de metáfase I (MI), anáfase I (AI), telófase I (TI) e progredindo até a metáfase II (MII), na qual ocorre a segunda parada da meiose (SIRARD et al., 1989).

Além das modificações supracitadas, ocorrem também alterações citoplasmáticas no oócito ao longo do período de maturação tais como o rearranjo e aumento no número de organelas citoplasmáticas. Dentre essas alterações ocorrem aumento gradativo da quantidade de lipídeos, redução no tamanho do complexo de Golgi, compactação do nucléolo e alinhamento dos grânulos corticais em local próximo ao oolema (HYTTEL et al., 1997). As mitocôndrias desempenham função importante durante essa fase, uma vez que sintetizam o ATP necessário para a síntese de proteínas fundamentais para os processos de maturação e desenvolvimento embrionário subsequentes (STOJKOVIC et al., 2001).

A eficácia do processo de maturação depende também da participação de fatores autócrinos e parácrinos produzidos e secretados pelas células que compõem o ambiente folicular, os quais atuam isoladamente ou em associação com hormônios. Dentre esses, o IGF-1 (Insulin-like growth factor 1) adicionado ao meio de maturação *in vitro* acelera a progressão da meiose (SAKAGUCHI et al., 2002). Da mesma forma, estudos utilizando metades foliculares *in vitro* demonstraram um papel da AngII revertendo a inibição da meiose induzida pelas células foliculares (GIOMETTI et al., 2005; STEFANELLO et al., 2006; BARRETA et al., 2008). Com esse mesmo modelo, foi também comprovado que a ação da AngII ocorre via progesterona e prostaglandinas E2 e F2α (BARRETA et al., 2008; SIQUEIRA et al., 2012). Além desses, o sistema EGF (Epidermal Growth Factor) tem sido caracterizado como um

importante mediador da ação gonadotrófica nas células da granulosa e cumulus. O LH ao se ligar em seus receptores, predominantemente expressos nas células da granulosa (COTTERILL et al., 2012), induz a liberação dos peptídeos semelhantes ao EGF (amphiregulin (AREG), epiregulin (EREG) e betacellulin (BTC)) que ao se ligarem em seus receptores (EGFR), estimulam o reinício da meiose e expansão das células do cumulus (ESPEY & RICHARDS, 2002; SHIMADA et al., 2006). Estudos em roedores e suínos utilizando um inibidor dos EGFR (AG1478) durante o período de maturação *in vitro,* demonstraram um bloqueio no processo de reinicio da meiose e ausência de expansão das células do cumulus (PARK et al., 2004; REIZEL et al., 2010a; PROCHAZKA et al., 2012).

## 2.2 Ovulação

A ovulação nos mamíferos é similar a um processo inflamatório e ocorre em decorrência de um aumento na concentração de estradiol intrafolicular que induz o pico pré-ovulatório de LH originário da hipófise (KESNER et al., 1981; ROCHE et al., 1981). O volume folicular cresce rapidamente nas poucas horas que precedem a ovulação. Em fêmeas bos taurus, a capacidade ovulatória acontece quando o folículo atinge um diâmetro ≥12 mm (SARTORI et al., 2001). Existe uma correlação positiva entre o tamanho folicular, expressão de receptores de LH nas células da granulosa e a taxa de ovulação em fêmeas bovinas. Entretanto, nas células da teca, a abundância de receptores de LH não varia durante o desenvolvimento folicular da mesma forma que o observado nas células da granulosa (SIMOES et al., 2012). Isso contribui para explicar estudos passados os quais demonstraram que, embora exista uma grande densidade de receptores de LH nas células da teca, o processo ovulatório só inicia quando as células de granulosa adquirem receptores para LH (RICHARDS, 1980). Embora exista expressão de RNAm para receptores de LH nas células da granulosa de folículos antrais pequenos, os mesmos sofrem splicing's alternativos impedindo a formação da proteína funcional (ROBERT et al., 2003).

O LH ao se ligar em seus receptores associados à proteína G, estimula a adenil ciclase e ocorre um aumento nas concentrações do segundo mensageiro AMPc que ativam a proteína kinase A (PKA) (MCFARLAND et al., 1989). A ação da enzima phosphodiesterase PDE4D é requerida para manter os níveis de cAMP nas células da granulosa (TSAFRIRI et al., 1996). Posteriormente a isso, ocorre ativação de rotas intracelulares e fatores de transcrição que determinam uma intensa atividade de transcrição e tradução de diversos genes envolvidos nas modificações morfológicas e funcionais que as células foliculares passam até momentos antes

da ruptura folicular e liberação de um oócito no estádio de Metáfase II, apto a ser fertilizado (REEL & GORSKI, 1968; GILBERT et al., 2011). Dentre as alterações mencionadas, destacam-se alterações na esteroidogênese folicular aumentando os níveis de progesterona 4-5 vezes cerca de 1,5 horas após o pico de LH (FORTUNE et al., 2009) e diminuindo a secreção de estradiol a partir de 3 horas pós GnRH (TONELLOTTO DOS SANTOS et al., 2012). As alterações moleculares que acompanham essa mudança no padrão dos hormônios esteroides referem-se a ativação de reguladores da transcrição tais como o receptor da progesterona (PR), Early growth response protein 1 (EGR1) e CAAT enhancer binding protein (C/EBPb) os quais atuam coordenadamente na indução da expressão de genes envolvidos no processo ovulatório (CHRISTENSON et al., 1999; ROBKER et al., 2000; YOSHINO et al., 2002). Dentre os genes envolvidos no processo ovulatório destaca-se as proteases ADAMTS (A Disintegrin And Metalloproteinase with Thrombospondinmotifs), cujos níveis de RNAm são controlados pela ativação dos PR (FORTUNE et al., 2009), caracterizadas como as principais sheddases envolvidos na ativação e liberação de EGF-like growth factors (EGF-L – AREG, EREG e Betacelulina) extracelularmente a partir da membrana celular (SAHIN et al., 2004). Dentre as alterações estruturais, destaca-se o intenso remodelamento da matriz extracelular e indução da clivagem de proteínas da membrana celular, envolvendo proteases, colagenases e fatores vasoativos (ESPEY, 1980).

O estudo de mediadores do processo inflamatório e fatores angiogênicos em diferentes tecidos contribuíram para investigar suas funções durante o período ovulatório de mamíferos. Dentre esses, destacam-se as Prostaglandinas (PGs) cuja função está relacionada com a indução da vasodilatação tecidual e degradação da parede folicular antes da ovulação através da ativação de proteinases (SIROIS et al., 2004). As prostaglandinas E2 e F2α são derivadas do ácido araquidônico cuja conversão ocorre pela ação das enzimas Cicloxigenase 1 e 2 (COX1 e COX2) (HINZ & BRUNE, 2002). A inibição das cicloxigenases com o antiinflamatório indometacina, determina falhas na ocorrência do processo ovulatório (ESPEY et al., 1986; SENA & LIU, 2008). Como principal envolvida nos eventos coordenados pelo estímulo gonadotrófico, muitos modelos animais demonstraram que a expressão de COX2 é estimulada em células da granulosa tratadas com hCG (SIROIS, 1994; JOYCE et al., 2001; TSAI et al., 2008). Dentre as rotas intracelulares envolvidas na síntese de Prostaglandinas, estudos em células da granulosa de humanos demonstraram que a ativação do EGFR por seus ligantes AREG, EREG e BTC está envolvido no aumento de expressão de COX2 e produção de PGE2 pelo estímulo do LH. Além disso, esse estudo demonstrou também que os efeitos dos peptídeos semelhantes ao EGF na

indução de COX2 e produção de PGE2 é dependente da ativação de MAP kinase (FANG et al., 2013).

Com base nos efeitos da AngII no sistema cardiovascular controlando a pressão sanguínea e homeostase dos fluidos corporais via receptor do tipo 1 (AGTR1) (FYHRQUIST et al., 1995; DE LEEUW, 1999), estudos foram desenvolvidos com o intuito de investigar o papel da AngII nos eventos reprodutivos. Diferentemente do sistema circulatório, no ovário as ações da AngII são mediadas pelo receptor do tipo 2 (AGTR2) (YOSHIMURA et al., 1996; PORTELA et al., 2008). Em coelhas, sua atividade está relacionada à maturação do oócito, ovulação e esteroidogênese (YOSHIMURA et al., 1993; FERAL et al., 1995; HAYASHI et al., 2000). Em bovinos, a AngII participa da síntese de estradiol durante o desenvolvimento do folículo dominante e é indispensável nos momentos iniciais da cascata ovulatória induzida pelo LH (FERREIRA et al., 2007; FERREIRA et al., 2011). Os mecanismos intracelulares de ação da AngII no ovário ainda não são conhecidos, entretanto, estudos em tecidos cardíaco e renal utilizando células do músculo liso vascular, demonstraram uma diminuição do efeito da AngII com a utilização de um inibidor dos receptores de EGF (CARMINES et al., 2001; KAGIYAMA et al., 2002). Além disso, existem outras rotas intracelulares candidatas a possíveis alvos da AngII. Estudos em células do músculo liso de veias coronárias revelam que a atuação da AngII, em promover a síntese proteica, é inibida pela rapamicina, um potente inibidor da rota de sinalização mTORC1 (HAFIZI et al., 2004).

## 2.3 Mammalian Target of Rapamycin (mTOR)

### 2.3.1 Estrutura e funções

O mTOR (mammalian target of rapamycin) é uma serina/treonina quinase envolvida no controle do crescimento, proliferação e organização do citoesqueleto das células em resposta ao estímulo por fatores de crescimento, hormônios e nutrientes (MURAKAMI et al., 2004; LAPLANTE & SABATINI, 2012). Esse complexo proteico encontra-se subdividido no meio intracelular na forma de mTORC1 (complexo 1) e mTORC2 (complexo 2) com estruturas bastante semelhantes. O primeiro é composto pelas seguintes proteínas: raptor (regulatory associated protein of TOR), mLST8 (mTOR associated protein, LST8 homolog), e a proteína deptor (DEP dommain containing mTOR-interacting protein). O segundo, além de ter alguns dos componentes supracitados na sua estrutura, tais como, mTOR, mLST8 e deptor, é composto

também pelo rictor (RPTOR independent companion of mTOR), PRR5 (Proline-rich protein 5) (GUERTIN & SABATINI, 2007; WOO et al., 2007; PETERSON et al., 2009).

Quando a atividade do mTOR está acima do normal para a célula, ocorre aceleração no ciclo celular com consequente formação tumoral, dessa forma, algumas pesquisas investigam a utilização de inibidores do mTOR com o intuito de entender formas de tratamento contra o câncer (GUERTIN & SABATINI, 2007). A principal forma de atuação do mTOR no meio intracelular é através do controle da tradução proteica via fosforilação das proteínas p70S6K (*p70 S6 Kinase*) e 4E-BP1 (*eukaryotic translation initiation factor 4E-binding protein 1*). A 4E-BP1 hipofosforilada interfere na tradução proteica ao se ligar no fator de iniciação da tradução (*eukaryotic translation initiation factor 4E; eIF4E*) impedindo este de reconhecer o complexo de ligação cap (*cap binding complex*) presente na extremidade 5' do RNA mensageiro. Dessa forma, a fosforilação da 4E-BP1 pelo mTOR impede a ligação no fator de iniciação da tradução proporcionando a ocorrência do processo de tradução dependente de cap nas células eucarióticas (GINGRAS et al., 2001a; GINGRAS et al., 2001b).

A ativação da sinalização do mTOR é negativamente controlada pelo complexo protéico TSC1/2 (*tuberous sclerosis complex*), também conhecido como genes tumor-supressor, responsáveis pela tradução das proteínas Hamartin (TSC1) e tuberin (TSC2). Mutações nas proteínas traduzidas pelo TSC ou alterações nas rotas de sinalização intracelular que inibem a atividade do TSC levam a formação de tumores (GUERTIN & SABATINI, 2007; YANG & GUAN, 2007). Além disso, o aumento da expressão de TSC2 foi relacionado com a redução na fosforilação dos alvos do mTOR 4E-BP1 e p70S6K (TEE et al., 2002). Essa redução da atividade do mTOR, provocada pelo TSC, ocorre via inibição de uma enzima denominada *Ras-homolog enriched in brain* (Rheb), pertencente a classe das pequenas GTPases e positivamente relacionada com a atividade do mTOR (TEE et al., 2002; LONG et al., 2005). O knockout deste gene em camundongos é acompanhado pela ausência da fosforilação da proteína p70S6K e determina morte embrionária caracterizada pelo impedimento do desenvolvimento do sistema circulatório (GOORDEN et al., 2011).

### 2.3.2 Funções Reprodutivas do Sistema mTOR

Evidências da importância do mTOR na atividade ovariana foram obtidas inicialmente com a caracterização da sua expressão em células da granulosa e oócitos de ovelhas (ORTEGA et al., 2010), bem como da sua forma fosforilada e dos componentes Raptor e Rictor em células da granulosa de rato (YABA et al., 2008). Além da sua presença nas células que compõem o

ambiente folicular, estudos demonstraram a presença de mTOR no ambiente uterino inferindo seu envolvimento nos mecanismos de implantação embrionária uma vez que o número de locais de implantação foi diminuído com a injeção intrauterina do inibidor rapamicina (CHEN et al., 2009).

Participando da busca pelo conhecimento acerca do papel do sistema mTOR nos diferentes tipos celulares que compõem o ambiente folicular, estudos prévios demonstraram que a sinalização do mTOR em células da granulosa é positivamente relacionada com o crescimento e proliferação desse tipo celular, uma vez que sua inibição com o antagonista rapamicina resulta em falhas durante o processo de divisão celular (YU et al., 2011). Além disso, o sistema mTOR tem sido relacionado com mecanismos proliferativos de células da granulosa de ratos mediando a ação do FSH no aumento da expressão de RNAm da enzima ciclinaD2 (KAYAMPILLY & MENON, 2007). Em cultivo primário de células da teca, foi demonstrado um envolvimento do mTORC1 na síntese de andrógenos, uma vez que, o uso da rapamicina *in vitro* bloqueou a síntese de androstenediona estimulada pelo LH (PALANIAPPAN & MENON, 2012). Durante a meiose de oócitos de camundongos, foi demonstrado que a rapamicina interfere na formação das fibras do fuso, com anormalidades na separação dos cromossomos e progressão até o estádio de metáfase II (LEE et al., 2012). Em oócitos de bovinos, o uso de um inibidor inespecífico, que age tanto no mTORC1 quanto no mTORC2, durante o período de maturação, causou um bloqueio da progressão meiótica no estádio de metáfase I (MAYER et al., 2014).

Investigando o papel do mTOR na ovulação, diferentes metodologias para aumento ou diminuição da atividade do mTOR estão sendo aplicadas. Estudos utilizando camundongos knockout para o gene TSC (complexo proteico que regula negativamente o sistema mTOR) obtiveram um aumento no número de ovulações e acúmulo de corpo lúteo (HUANG et al., 2013). Outros estudos com o mesmo propósito, porém com o uso de modelos experimentais diferentes, demonstraram um impedimento da ocorrência do processo ovulatório em camundongos que receberam injeção intraperitoneal de rapamicina (YU et al., 2011).

**3. ARTIGO 1**

TRABALHO SUBMETIDO PARA PUBLICAÇÃO:

# Reversible meiotic arrest of bovine oocytes by EGFR inhibition and follicular hemisections

**Paulo Roberto Antunes da Rosa, Matheus Pedrotti de Cesaro, Andressa Minussi Pereira Dau, Raj Duggavathi, Vilceu Bordignon and Paulo Bayard Dias Gonçalves.**

**REPRODUCTION, 2016**

# Reversible meiotic arrest of bovine oocytes by EGFR inhibition and follicular hemisections

Paulo Roberto Antunes da Rosa[1,2], Matheus Pedrotti De Cesaro[1], Andressa Minussi Pereira Dau[1], Raj Duggavathi[2], Vilceu Bordignon[2*] and Paulo Bayard Gonçalves[1*]

[1]Laboratory of Biotechnology and Animal Reproduction - BioRep, Federal University of Santa Maria, Santa Maria, RS, Brazil.

[2]Department of Animal Science, McGill University, Sainte-Anne-de-Bellevue, QC, Canada.

*Co-corresponding authors:

Vilceu Bordignon: Department of Animal Science, McGill University, 21111, Lakeshore road, Room MS1-089, Sainte-Anne-de-Bellevue, QC, Canada, H9X3V9. Email: vilceu.bordignon@mcgill.ca

Paulo B. D. Gonçalves: BioRep, Federal University of Santa Maria, Room 16, 1000 Roraima AV, Veterinary Hospital, Santa Maria, RS, Brazil. Email: bayard@ufsm.br

**Short title:** Bovine oocytes and EGFR inhibition

**Abstract**

The objective of this study was to investigate the effects of inhibiting the epidermal growth factor receptor (EGFR) pathway on meiosis blockage and resumption, gene expression and development of bovine oocytes. Bovine cumulus-oocyte complexes (COCs) were cultured for 15 h in the presence of the EGFR inhibitor (AG1478) and follicular hemisections (FHS). Most of the oocytes (89.3%) remained at the germinal vesicle (GV) stage when cultured in the presence of FHS and 5 µM AG1478. The inhibitory effect was reversible since most oocytes (84%) were able to resume and complete meiosis after additional 20 h of maturation in standard oocyte maturation medium. Embryo development to the blastocyst stage after in vitro fertilization was similar in oocytes that were cultured for 15 h with AG1478 plus FHS and then matured for 20 h in standard medium (39.3%) compared to control oocytes matured in standard conditions (41.1%; $P>0.05$). Transcript levels for *EGR1*, *TNFAIP6* and *HAS2* genes and p-ERK1/2 protein in cumulus cells were lower in samples treated with AG1478 plus FHS compared to FHS alone ($P<0.05$). In granulosa cells of FHS, AG1478 treatment reduced mRNA levels for *PGR* and *ADAMTS1* ($P<0.05$). The inhibitory effect of AG1478 was not reverted by treatment with angiotensin II (ANG2) or prostaglandins ($PGF_{2\alpha}$ or $PGE_2$). Findings from this study revealed that inhibition of EGFR in the presence of FHS is a reliable approach to promote reversible arrest of bovine oocytes at the GV stage.

## Introduction

Oocyte maturation in mammals involves cell signaling cascades that occur simultaneously with follicular development. Inhibitory factors produced by follicular somatic cells keep oocytes arrested at prophase I or germinal vesicle (GV) stage. Oocytes resume meiosis after the preovulatory LH surge *in vivo* or after they are removed from the follicular environment (Pincus & Enzmann 1935, Sirard *et al.* 1989). Epidermal growth factor receptor (EGFR) is an important mediator of LH signaling. The preovulatory LH surge induces a cascade of cellular events that starts in granulosa cells with secretion of the EGF-like peptides amphiregulin (AREG), epiregulin (EREG) and betacellulin (BTC) (Shimada *et al.* 2006). Binding of these peptides to EGFR in granulosa and cumulus cells activates downstream effectors, including the extracellular regulated kinase (ERK1/2) and the phosphatidylinositol 3-kinase/AKT (PI(3)K-AKT), which are involved in cumulus expansion and oocyte maturation by modulating genes controlling hyaluronic acid synthesis and closure of intercellular gap junctions (Nagyova 2012, Prochazka *et al.* 2012).

Meiosis resumption involves a cascade of events that culminate in the closure of gap junctions and thus termination of bidirectional communication between cumulus cells and the oocyte. This induces a decrease in cyclic guanosine monophosphate (cGMP) levels in the oocyte and activation of cyclic adenosine monophosphate (cAMP) phosphodiesterase (PDE3A), which decreases cAMP levels, thus culminating in germinal vesicle breakdown (GVBD) (Richard *et al.* 2001, Thomas *et al.* 2004). A decrease in cAMP levels in the oocyte results in activation of the meiosis promoter factor (MPF) complex, which is formed by CDK/Cyclin B proteins. Lower cAMP levels in the oocyte leads to CDK dephosphorylation and MPF activation, which causes meiosis resumption (Duckworth *et al.* 2002, Oh *et al.* 2010). The ERK1/2 kinase, also known as mitogen-activated protein kinase (MAPK), is also involved in the control of meiotic cell cycle progression in oocytes. In bovine oocytes, MAPK

activation is triggered by dual phosphorylation on threonine and tyrosine residues. MAPK activity increases at the GVBD stage, around 6 h of in vitro maturation (IVM), reaches it maximum at the metaphase I (MI) stage, after around 15 h of IVM, and remains elevated until pronuclear formation (Fissore *et al.* 1996).

Oocytes are known to acquire meiotic and developmental competence during follicular growth (Hyttel *et al.* 1997). Since meiotic competence is reached earlier than developmental competence, when oocytes are removed from large antral follicles, a higher proportion of them can resume and complete meiotic maturation to metaphase II (MII) stage, which prematurely interrupt accumulation of transcripts and proteins required for developmental competence (Fair *et al.* 1995, Macaulay *et al.* 2014). This is likely the main reason explaining the lower developmental competence of IVM oocytes compared to those matured in vivo (Rizos *et al.* 2002, Wrenzycki *et al.* 2007, Nivet *et al.* 2012).

Delaying of meiotic resumption and extension of the maturation period has been proposed as an alternative to improve developmental competence of IVM oocytes. In this regard, a number of approaches have been tested, including inhibitors of MPF (Lonergan *et al.* 2000, Mermillod *et al.* 2000) and phosphodiesterase (Albuz *et al.* 2010, Guimaraes *et al.* 2015). Culture in the presence of follicular hemisections was also shown to delay meiosis progression of bovine oocytes (Richard & Sirard 1996b, Barreta *et al.* 2008), but oocytes were not effectively arrested at the GV stage. On the other hand, MPF inhibitors such as Roscovitine and Butyrolactone I can successfully hold cattle oocytes at the GV stage for up to 24 h (Lonergan *et al.* 2000, Mermillod *et al.* 2000, Quetglas *et al.* 2009, Guemra *et al.* 2014). Nonetheless, oocyte developmental capacity has not been significantly improved after extended *in vitro* maturation using those inhibitors (Leal *et al.* 2012). One possibility is that by acting at the end of the meiosis resumption cascade, MPF inhibitors would not prevent early cellular events necessary to improve oocyte developmental competence.

The EGFR signaling is an initial step in the cascade of cellular events triggered by LH and its inhibition was shown to prevent cumulus expansion and delay meiosis progression (Prochazka *et al.* 2003, Reizel *et al.* 2010a). In light of these previous findings, we hypothesized that EGFR inhibition in association with follicular hemisections would create an *in vitro* system to maintain oocytes at the GV stage that is more similar to the *in vivo* follicular environment. The objectives of the present study were to: i) evaluate the effect of EGFR inhibition and FHS on meiosis arrest, resumption and embryo development; ii) investigate the effects of this treatment on gene expression in oocytes, cumulus cells and granulosa cells of the FHS; and iii) determine if meiosis arrest maintained by EGFR inhibition and FHS would be reverted by ANG2, $PGF_{2\alpha}$ or $PGE_2$.

## Material and Methods

*Cumulus oocyte-complexes (COCs) collection and maturation*

Bovine ovaries were collected from a local abattoir and transported to the laboratory in a thermal box at 30°C in saline solution (0.9% NaCl) containing 100 IU/ml penicillin and 50 µg/ml streptomycin. In the laboratory, follicles measuring 2 to 8mm in diameter were aspirated with a vacuum pump and grade 1 and 2 COCs were selected, randomly distributed in groups of 25-30 and matured in 4-well culture plates (Nunc®, Roskilde, Denmark) containing 200 µl maturation medium at 39°C and 5% CO2. The maturation medium consisted of TCM199 containing Earle's salts and L-glutamine (Life Technologies, São Paulo, Brazil) supplemented with 25 mM HEPES, 0.2 mM pyruvic acid, 2.2 mg/ml sodium bicarbonate, 0.5 µg/ml FSH (Bioniche, Belleville, ON, Canada), 0.4% fatty acid-free BSA, 100 IU/ml penicillin, and 50 µg/ml streptomycin sulfate.

*Preparation of Follicular Hemisections*

Ovaries were collected from a local abattoir and transported to laboratory at 5°C in saline solution (NaCl 0.9%) containing penicillin (100 UI/ml) and streptomycin (50 µg/ml). Follicles measuring 2–5 mm in diameter were isolated from the ovaries and dissected free of stromal tissue (Richard & Sirard 1996a). Follicles were then sectioned into equal halves with a scalpel, washed in TCM 199 containing 0.4% BSA and incubated in the maturation medium for 2 h before adding the COCs. The number of hemisections (8 per 200 µL of maturation medium) was based on previous studies by our group (Giometti *et al.* 2005, Stefanello *et al.* 2006, Barreta *et al.* 2008, De Cesaro *et al.* 2013).

*In Vitro fertilization (IVF) and embryo culture*

Oocytes were inseminated with previously tested frozen semen that was fractionated on discontinuous Percoll (Amersham Bioscience AB, Uppsala, Sweden) gradients (Parrish *et al.* 1986). The concentration of sperm used was 2 x $10^6$ sperm/ml diluted in Fert-TALP medium containing 10 µg/ml heparin, 30 µg/ml penicilinamine, 15 µM hypotaurine and 1 µM epinephrine (Parrish *et al.* 1988). In vitro fertilization was carried out by co-culture of sperm and oocytes in 400 µl drops of IVF medium for 18 h in the same atmospheric conditions used for maturation. After gamete co-incubation, cumulus cells were removed by vortexing. Presumptive zygotes and embryos were washed thrice and then cultured at 39°C in a saturated humidity atmosphere of 5% CO2, 5% O2 and 90% N2 for 9 days in 400 µl of synthetic oviduct fluid (SOF) medium.

*Oocyte, cumulus and granulosa cells collection for molecular analysis*

At the end of the maturation period, cumulus cells were separated from the oocytes by vortexing in TCM 199 medium. Denuded oocytes were collected, washed five times in PBS, and then transferred in groups of 30-40 to a tube containing 100 µL of Trizol (Invitrogen, São

Paulo, SP, Brazil) for RNA extraction. Cumulus cells were collected by centrifuging the remaining vortex medium for 2 minutes. The supernatant was removed and 100 µL of Trizol was added to each tube with cumulus cells. Granulosa cells were mechanically removed from follicular hemisections by repeated pipetting in maturation medium, followed by centrifugation, removal of supernatant, and addition of 100 µL Trizol. All samples were stored at −80°C until RNA and/or protein extraction.

*RNA extraction and quantitative reverse transcriptase PCR (qRT-PCR)*

Total RNA was extracted using Trizol and according to the manufacturer's instructions. The reminiscent Trizol phase was stocked at -80°C for further protein extraction. Total RNA was quantified by absorbance at 260 nm using a NanoDrop 1000 spectrophotometer (Thermo Scientific). Purity was measured by absorption rate based on OD260/OD280 ratios and samples with ratios <1.7 were discarded. To generate the complementary DNA (cDNA), 700 ng total RNA was reverse-transcribed using the iScript cDNA Synthesis Kit (Bio-Rad).

All qPCR reactions were run in a CFX384 real-time PCR detection system (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad), 500 nM of primers and 2 µl of cDNA. Primers (Table 1) were designed using Primer-Blast, and specificity was confirmed using BLAST (NCBI). Standard two-step qPCR was performed to amplify each transcript with an initial denaturation at 95°C for 5 minutes followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 58°C for 30 seconds. Melting-curve analysis was performed to verify the specificity of reaction products. Samples were run in duplicates, standard curve method was used to determine the abundance of mRNA for each gene, and expression was normalized to the mean abundance of internal control genes actin beta

(*ACTB*), cyclophilin, ribosomal protein S18 and *GAPDH*. All reactions used for quantification had efficiency between 90–110%, R2 ≥ 0.98 and slope values from −3.6 to −3.1.

*Immunoblot analyses*

The reminiscent Trizol phase obtained during RNA extraction was used for protein extraction from cumulus and granulosa cells according to the manufacturer's instructions (Invitrogen, São Paulo, SP, Brazil). After extraction, samples were diluted in Laemmli buffer (Bio-Rad) and boiled at 95°C for 10 minutes. Total lysate was resolved on a 10% SDS gel and electrotransferred onto nitrocellulose membranes (Bio-Rad). After blocking for 2 h with 5% skim milk in Tris-buffered saline (TBS) + 0.1% Tween, pH 7.6, membranes were incubated overnight at 4°C with ERK1/2 (1:1000; #4695; Cell Signaling), phopho-ERK1/2 (1:1000; #4376; Cell Signaling), EGR1 (1:5000; sc-189; Santa Cruz Biotechnology), PGR (1:500; sc-7208; Santa Cruz Biotechnology), or ACTB (1:10 000; ab8227; Abcam Inc.) primary antibodies. Subsequently, membranes were washed three times (10 minutes each) with TBS-T, and then incubated with anti-rabbit (ab6721; Abcam) secondary antibody diluted 1:10,000 for 1.5 hour at room temperature, followed by three washes of 5 minutes each with TBS-T. Immunoreactivity was detected with Immun-Star Western Chemiluminescence Kit (Bio-Rad, CA, USA), according to the manufacturer's instructions and images were captured using a ChemiDoc system (Bio-Rad). Images were analyzed and bands were quantified using the Image Lab 3.0 software (Bio-Rad).

*Experimental design*

The first experiment was performed to evaluate whether the treatment of COCs with AG1478 in a culture system with follicular hemisections prevents meiosis resumption of bovine oocytes. A total of 312 COCs were used for this experiment. The COCs were cultured for 15 h

188 in maturation medium alone (control group), in the presence of 8 follicular hemisections (FHS
189 group) or with FHS plus 0.05, 0.5, 5 or 50 µM of AG1478. The AG1478 inhibitor was diluted
190 as recommended by the manufacturer and maintained at -80°C in aliquots of 10 mM stock
191 solution for use throughout the entire study. After 15 h of culture, oocytes from each treatment
192 were striped from their cumulus cells by vortexing, fixed in 4% paraformaldehyde for 15
193 minutes, and then transferred to PBS containing 0.5% Triton X-100. Oocytes were stained with
194 10 µg/ml bisbenzimide (Hoechst 33342) and analyzed under a fluorescence microscope to
195 determine the stage of meiosis.

196 The second experiment aimed to verify the effectiveness of 5µM AG1478 with or
197 without FHS in maintaining oocytes at GV stage. A total of 206 COCs in three different
198 replicates were used. After 15 h of culture, the meiotic maturation status was evaluated as
199 described above.

200 The third experiment was conducted to test if the meiotic arrest induced by FHS plus 5
201 µM AG1478 was reversible. A total of 194 COCs in three different replicates were used for
202 this experiment. COCs that were cultured for 15 h in the presence of FHS plus 5 µM AG1478
203 were transferred to maturation medium and matured for an additional period of 16, 18 and 20
204 h without the meiotic inhibitors. The maturation medium consisted of TCM199 containing
205 Earle's salts and L-glutamine (Gibco Labs) supplemented with 25 mM HEPES, 0.2 mM
206 pyruvic acid, 2.2 mg/ml sodium bicarbonate, 0.5 µg/ml FSH (Bioniche), 5.0 mg/ml LH
207 (Lutropin-V, Bioniche), 100 IU/ml penicillin, 50 µg/ml streptomycin sulfate, and 10% (v/v)
208 bovine calf serum (FBS; GibcoLabs). At the end of the maturation, the oocytes were denuded
209 from cumulus cells, fixed, stained with Hoechst 33342, and analyzed under a fluorescence
210 microscope to evaluate their meiotic maturation.

211 The fourth experiment was conducted to evaluate cleavage and development to the
212 blastocyst stage after IVF. Control COCs (n=73) cultured for 24h in maturation medium were

compared to COCs that were maintained for 15 h in the presence of FHS plus 5 µM AG1478 followed by 20h in maturation medium (n=61). Cleavage (at day 2) and blastocyst (at day 7) rates were calculated based on the total number of oocytes used in each treatment.

The experiment 5 was performed to evaluate the effect of meiotic arrest on gene expression. In this experiment, COCs were cultured for 15 h in the presence of FHS, FHS plus 5 µM AG1478 or absence of treatment (control group). A total of 360 COCs, 30/group/replicate, were used in this experiment. This experiment was repeated four times.

The experiments 6 and 7 were performed to determine if the meiotic arrest induced by treatment with FHS plus 5 µM AG1478 would be reversed by ANG2) or prostaglandins. In the experiment 6, COCs were cultured for 15 h in one the following treatments: a) control (maturation medium alone); b) FHS; c) FHS plus ANG2 ($10^{-9}$M); d) FHS plus ANG2 plus saralasine ($10^{-5}$M; an inhibitor of angiotensin receptor II); and e) FHS plus ANG2 plus AG1478 (5 µM). A total of 182 COCs were used for this experiment. In the experiment 7, COCs were matured for 15 h in one of the following treatments: a) FHS; b) FHS plus AG1478 (5 µM); c) FHS plus AG1478 plus $PGF_{2\alpha}$ (1 µM); and d) FHS plus AG1478 plus $PGE_2$ (1 µM). A total of 203 COCs were used for this experiment. After 15 h of maturation, the oocytes from each treatment were striped from cumulus cells, fixed in 4% paraformaldehyde, stained with Hoechst 33342, and then evaluate in a fluorescent microscope to determine their meiotic stage.

*Statistical analysis*

Percentages of germinal vesicle stage, cleavage and blastocysts were analyzed by chi-squared test, using PROC CATMOD. Multi-comparisons among different groups were performed by means of contrasts using the SAS statistical package (SAS Institute Inc, Cary, NC, USA). Data from mRNA and protein experiments were tested for normality using the

Shapiro-Wilk test, normalized when necessary according to each distribution, and submitted to ANOVA using the JMP software (SAS Institute Inc., Cary, NC, USA). Multiple comparison tests were run for each significant result using the least-squares means (LSMEANS). Results are presented as mean ± standard error of the mean. Differences were considered statistically significant if $P < 0.05$.

## Results

*Effect of EGFR inhibition and follicular hemisections on oocyte meiotic resumption*

In the first experiment we evaluated if meiotic resumption could be prevented for 15 h by treatment with AG1478, in the presence of FHS. Although FHS alone failed to arrest meiotic resumption, most of the oocytes remained at GV stage when cultured for 15 h in the presence of FHS and 5 (89.3%) or 50 µM (90.6%) AG1478 (Figure 1A; $P<0.05$). However, in the absence of FHS 5 µM AG1478 resulted in only 40% of the oocytes remaining in GV stage after 15 h of culture (Figure 1B; $P<0.05$).

In order to evaluate if this meiotic arrest was reversible, COCs were cultured for 15 h in the presence of FHS and 5 µM AG1478, and then transferred and cultured for 16, 18 or 20 h in standard IVM medium. The meiotic blockage was reversible, since most (84%) of the oocytes progressed to the metaphase II (MII) stage after 20 h of maturation in standard IVM medium (Figure 2A). The proportion of oocytes that reached MII stage was higher ($P<0.001$) in the groups matured for 20 h (84%) and 18 h (77.6%) compared to 16 h (38.1%), which indicated that oocytes required an additional period of maturation of at least 18 h (Figure 2A).

*Development of meiotic arrested oocytes*

In this experiment, embryo development to the blastocyst stage after IVF was compared between control oocytes matured for 24 h in standard IVM medium and those

matured for 15 h in the presence of FHS plus 5 µM AG1478 followed by 20 h in standard IVM medium. Embryo cleavage (71.2% vs 59.0%) and blastocyst (41.1% vs 39.3%) rates were not statistically different (P>0.05) between control and meiotic arrested oocytes, respectively (Figure 2B).

*Effect of meiotic arrest treatment on transcripts profile in oocyte, cumulus and granulosa cells*

There was not difference in the mRNA abundance of *PDE3A*, *CCNB1*, *MAPK1*, *STC1*, *ANXA1* and *SERPINE1* genes between oocytes that were matured for 15 h in the presence of FHS or in the presence of FHS plus 5 µM AG1478 (data not shown). In cumulus cells, mRNA levels for *EGR1*, *TNFAIP6* and *HAS2* were significantly higher (P<0.05) and those of *CX43* and *IMPDH1* were lower (P<0.05) in COCs matured with FHS compared to control COCs matured in standard IVM medium (Figure 3). However, when COCs were matured in the presence of FHS plus 5 µM AG1478, the transcript levels for *EGR1*, *TNFAIP6* and *CX43* were similar and *HAS2* was lower compared to cells from control COCs matured in standard IVM medium. There was no effect of treatments on the transcript levels of *HSP90*, *WASL*, *PTGS2*, *SPRY2*, *PGR* and *IMPDH2* genes in cumulus cells (P>0.05; Figure 3).

The transcript levels of *PGR* and *ADAMTS1* were lower in granulosa cells of FHS treated with compared to those without 5 µM AG1478 (P<0.05; Figure 4). There was no difference in mRNA levels for *PGRMC1*, *PTGER2*, *PTGER4*, *PTGS2*, *CTSV*, *CEBPB*, *EGR1*, *SPRY2*, *IMPDH1* and *IMPDH2* between AG1478 treated and non-treated cells (Figure 4).

*Protein abundance in cumulus and granulosa cells*

Based on the results observed in the qPCR analyses, the protein abundance of total and phosphorylated ERK1/2, EGR1 and PGR were assessed in cumulus and granulosa cells of control and treated groups. P-ERK1/2 abundance was significantly increased (P<0.05) in

cumulus cells and tended to be higher (P=0.09) in granulosa cells from the FHS group compared the AG1478 group (Figure 5). There was no statistical difference in the protein levels of EGR1 between groups in either cumulus or granulosa cells, despite of numerically lower levels observed in both cell types from the AG1478 treated group (Figure 5). Protein levels for PGR were also numerically lower but not statistically different in granulosa cells of AG1478 treated versus non-treated groups (Figure 5).

*Effects of angiotensin 2, prostaglandin E2 and prostaglandin F2α on meiotic arrest induced by EGFR inhibition*

The meiotic maturation status of oocytes was evaluated to determine if the treatment with ANG2, $PGF_{2\alpha}$ or $PGE_2$ could induce meiotic resumption in oocytes treated with FHS and 5 µM AG1478. Treatment with ANG2 $10^{-9}$M accelerated meiosis resumption in COCs matured in the presence of FHS (Figure 6A). This effect was inhibited by a treatment with $10^{-5}$M saralasin, a competitive antagonist of AngII (Figure 6A). However, ANG2 was unable to induce meiosis resumption in COCs matured in the presence of FHS plus 5 µM AG1478 (Figure 6A). Similarly, treatment with either $PGF_{2\alpha}$ or $PGE_2$ did not stimulate meiosis resumption in COCs cultured with FHS plus 5 µM AG1478 (Figure 6B).

## Discussion

The present study investigated if an effective system to maintain bovine oocytes arrested at the GV stage during in vitro culture could be established by inhibiting EGFR in the presence of FHS. Findings from this study revealed that culture in the presence of 5 µM of the EGFR inhibitor AG1478 and FHS can maintain bovine oocytes arrested at GV stage for at least 15 h. More importantly, we observed that this treatment was completely reversible since

312 normal maturation to MII stage and blastocyst development were obtained after an additional
313 20 h period of IVM, and then fertilization and culture under standard conditions.

314 Inhibition of meiosis resumption to extend the period of maturation has been proposed
315 as an alternative to increase developmental competence of oocytes matured in vitro. In mice,
316 successful arrest of oocytes at GV stage is obtained by preserving intracellular cAMP levels
317 with phosphodiesterase inhibitors or adenylate cyclase activators (Eppig *et al.* 1985,
318 Vanhoutte *et al.* 2008). However, this approach is not effective in preventing GVBD in
319 bovine oocytes (Thomas *et al.* 2002, Sasseville *et al.* 2009). On the other hand, MPF
320 inhibitors can prevent meiosis resumption of bovine and porcine oocytes without apparent
321 detrimental effects on oocyte development (Lonergan *et al.* 2000, Mermillod *et al.* 2000, Le
322 Beux *et al.* 2003). However, protocols for improving oocyte competence based on meiosis
323 arrest induced by MPF inhibitors remain to be established. Findings from this study open a
324 new avenue for research on oocyte competence since EGFR inhibition acts upstream to cAMP
325 and MPF to prevent meiosis resumption. Although we have observed that meiosis arrest using
326 this protocol is reversible at both meiotic and developmental levels, further studies should
327 determine if this approach can be used to mature oocytes with improved developmental
328 competence.

329 It is possible that a protocol based on EGFR inhibition and FHS provides an in vitro
330 system that more closely resembles the follicular environment that oocytes develop in vivo.
331 Indeed, we observed that the effect of EGFR inhibition to promote meiosis arrest depends on
332 interactions with somatic cells present in the hemisections of follicular walls. In this regard,
333 bovine oocytes seem to differ from mouse and pig oocytes since EGFR inhibition can prevent
334 meiosis resumption in absence of follicular cells in those species (Park *et al.* 2004, Reizel *et*
335 *al.* 2010b, Prochazka *et al.* 2012).

It is known that in the follicular environment, molecular crosstalk between the oocyte, cumulus and granulosa cells regulate follicular and oocyte development. To investigate if EGFR inhibition affects the molecular crosstalk between the oocyte and somatic follicular cells in vitro, transcripts and proteins were quantified in the oocytes, cumulus and granulosa cells after 15 h of in vitro culture. We observed that mRNA levels of *PDE3A*, *CCNB1*, *MAPK1*, *STC1*, *ANXA1* and *SERPINE1* genes in oocytes were not affected by EGFR inhibition, which suggests that oocyte transcription or degradation have not been affected by treatment. However, differences were observed in both transcript and protein levels in cumulus and granulosa cells, indicating that both COCs and follicular wall compartments were affected by EGFR inhibition. In cumulus cells, transcript levels of *EGR1*, *TNFAIP6* and *HAS2* genes, which are involved in cell expansion, were lower in the COCs matured in the presence of the EGFR inhibitor and FHS compared to FHS alone. Although both treatments prevented cumulus cells expansion compared to control COCs matured in standard IVM medium, the lower levels of these transcripts in the cumulus cells indicate that EGFR inhibition more effectively prevented the activation of molecular events associated with cumulus expansion. Previous studies have reported that EGFR inhibition had also downregulated *TNFAIP6*, *PTGS2* and *HAS2* genes in porcine COCs (Prochazka *et al.* 2012). Since control COCs cultured in standard IVM medium had cumulus expansion after 15 h of maturation, we believe that lower mRNA levels of *TNFAIP6*, *PTGS2* and *HAS2* genes observed in this group compared to the FHS group was due to mRNA translation or degradation. In support to this, previous studies have shown that mRNA levels of *HAS2* and *PTGS2* increase at the beginning of maturation and then decrease after cell expansion at later stage of oocyte IVM (Adriaenssens *et al.* 2011). Transcript levels of *CX43* and *IMPDH1*, which are involved in cGMP production and transport to the oocyte (Luciano *et al.* 2011, Wigglesworth *et al.* 2013), were lower in cumulus cells of COCs treated with FHS compared

to control. This indicates that these genes were more affected by the presence of FHS than EGFR inhibition. At the protein level, we observed that EGFR inhibition significantly reduced p-ERK1/2 in cumulus cells and had a similar trend effect on granulosa cells, which confirmed the inhibitory effect of AG1478 treatment on ERK1/2 activation in both cell layers.

Findings from this study also revealed that EGFR inhibition reduced transcript levels of *PGR* and *ADAMTS1* in granulosa cells of FHS. There was also a trend for reduction of *PGR* mRNA in cumulus and protein in granulosa cells in response to EGFR inhibition. Progesterone signaling was shown to be involved in meiosis resumption in cattle oocytes. It was shown that meiosis resumption in vivo after the LH surge was inhibited by intrafollicular injection of a progesterone antagonist (Siqueira *et al.* 2012). Moreover, *ADAMTS1* is a downstream gene regulated by PGR activation (Robker *et al.* 2000). This suggests that the PGR may be involved in the process of meiotic arrest induced by EGFR inhibition.

This study also evaluated if the inhibitory effect of EGFR inhibition and FHS on meiotic resumption would be affected by ANG2, $PGF_{2\alpha}$ or $PGE_2$. Previous studies from our group demonstrated that ANG2 reverted the inhibitory effect of FHS on meiotic progression through $PGE_2$ and $PGF_{2\alpha}$ (Giometti *et al.* 2005, Stefanello *et al.* 2006, Barreta *et al.* 2008). Other studies have shown that EGFR and MAPK are downstream effectors of ANG2 signaling in a rat model of cardiac hypertrophy and hypertension (Eguchi *et al.* 1998, Kagiyama *et al.* 2002). EGFR activation is also required for $PGE_2$ effects on migration and invasion of colon adenocarcinoma cell line (Buchanan *et al.* 2003). However, we have observed that treatment with ANG2, $PGE_2$ or $PGF_{2\alpha}$ did not revert the inhibitory effect of EGFR inhibition on meiotic resumption of bovine oocytes. This suggests that EGFR inhibition blocked downstream pathways induced by ANG2 and prostaglandins to induce meiosis progress.

In conclusion, findings from this study revealed that the inhibition of the EGFR pathway in the presence of FHS can effectively arrest bovine oocytes at GV stage during 15 h of culture in vitro without compromising their meiotic and developmental capacity. Molecular changes observed in cumulus cells and in granulosa cells, including lower transcript levels for *HAS2*, *PGR* and p-ERK1/2 protein, as well as absence of cumulus expansion indicates that this protocol prevents meiosis resumption by mimicking the in vivo follicular environment. This system provides a new paradigm for the study of molecular events regulating meiosis resumption and may have important implications for developing new culture protocols to increase the developmental competence of in vitro matured oocytes.

**Declaration of Interest**

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

**Funding**

This study was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES-Brazil), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq-Brazil) and the Natural Sciences and Engineering Research Council (NSERC-Canada).

**Acknowledgements**

The authors would like to thanks Frigorífico Silva for the donation of bovine ovaries used in this research and McGill University facilities for providing laboratory space.

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552 **Figure legends**

553 **Figure 1:** Effect of treatment with follicular hemisections (FHS) in association with EGFR
554 inhibitor (AG1478) on meiosis resumption of bovine oocytes. a) Rates of germinal vesicle
555 (GV) stage oocytes after 15 h of maturation with FHS and different concentrations (0.05, 0.5,
556 5 or 50 µM) AG1478. b) Rates of GV stage oocytes matured for 15 h with 5µM AG1478 or
557 FHS alone or its combination. The experiments were performed in triplicates using at least 15
558 COCs per treatment and replicate for a total of 312 (Figure 1A) and 206 (Figure 1B) COCs.
559 Different letters indicate statistical differences ($P \leq 0.05$) between groups.

560 **Figure 2:** Meiotic stage of oocytes matured for 15 h in FHS plus 5µM AG1478 (15 h), or
561 after an additional period of 16 (+16 h), 18 (+18 h) or 20 (+20 h) h in standard IVM medium
562 (A). In vitro development of embryos produced from control and meiotic arrested oocytes.
563 Cleavage (day 2) and blastocyst (day 7) rates after fertilization of control oocytes matured for
564 20 h in standard IVM medium (control; dark bars) or oocytes matured for 15 h with follicular
565 hemisections plus 5 µM AG1478 followed by 20 h in IVM medium (FHS + AG1478; gray
566 bars) (B).

567 **Figure 3:** Gene transcripts in cumulus cells retrieved from COCs cultured for 15 h in standard
568 IVM medium (CT; dark bar), in the presence of follicular hemisections (FHS; gray bar) or
569 both FHS plus 5 µM AG1478 (FHS + AG1478; white bar). The mRNA abundance was
570 calculated relative to the reference genes ACTB, Cyclophilin, RP18S and GAPDH. Data are
571 from 360 COCs. Different letters indicate significant differences between groups ($P < 0.05$).

572 **Figure 4:** Gene transcripts in granulosa cells retrieved from follicular hemisections (FHS)
573 cultured for 15 h with COCs in absence (FHS; gray bars) or presence of 5 µM AG1478 (FHS
574 + AG1478; white bars). The mRNA abundance was calculated relative to the reference genes
575 ACTB, Cyclophilin, RP18S and GAPDH. Different letters indicate significant differences
576 between groups ($P<0.05$).

**Figure 5:** Phosphorylated ERK1/2 and EGR1 protein abundance in cumulus cells (a) and Phosphorylated ERK1/2, EGR1 and PGR protein abundance in granulosa cells (b). Cumulus cells were collected from COCs and granulosa cells were collected from follicular hemisections. Dark bar represent COCs cultured for 15 h in maturation medium without cells (CT); Gray bar represent COCs cultured in the presence of follicular hemisections (FH); and white bar represent COCs cultured in the presence of follicular hemisections and treated with 5 µM AG1478 (AG1478). Different letters indicate statistical difference between groups ($P < 0.05$).

**Figure 6:** Role of EGFR in the control of oocyte meiosis progression induced by Angiotensin 2 and prostaglandins F2α and E2. Metaphase rates of 182 COCs cultured for 15h in maturation medium alone; in the presence of follicular hemisections (FHS); FHS plus ANG2 (10-9M); FHS plus ANG2 plus saralasine (10-5M; an inhibitor of angiotensin receptor II); and FHS plus ANG2 plus AG1478 (5 µM) (A). A total of 203 COCs were cultured for 15 h in maturation medium or in coculture with follicular hemisections (FHS) plus 5 µM AG1478, FHS plus AG1478 plus PGF2α (1 µM) and FHS plus AG1478 plus PGE2 (1 µM) (B). For both experiments the COCs were denuded and fixed for further evaluation of the nuclear status and classification according the percentage of oocytes reaching Metaphase I stage. Different letters indicate statistical difference between groups ($P < 0.05$).

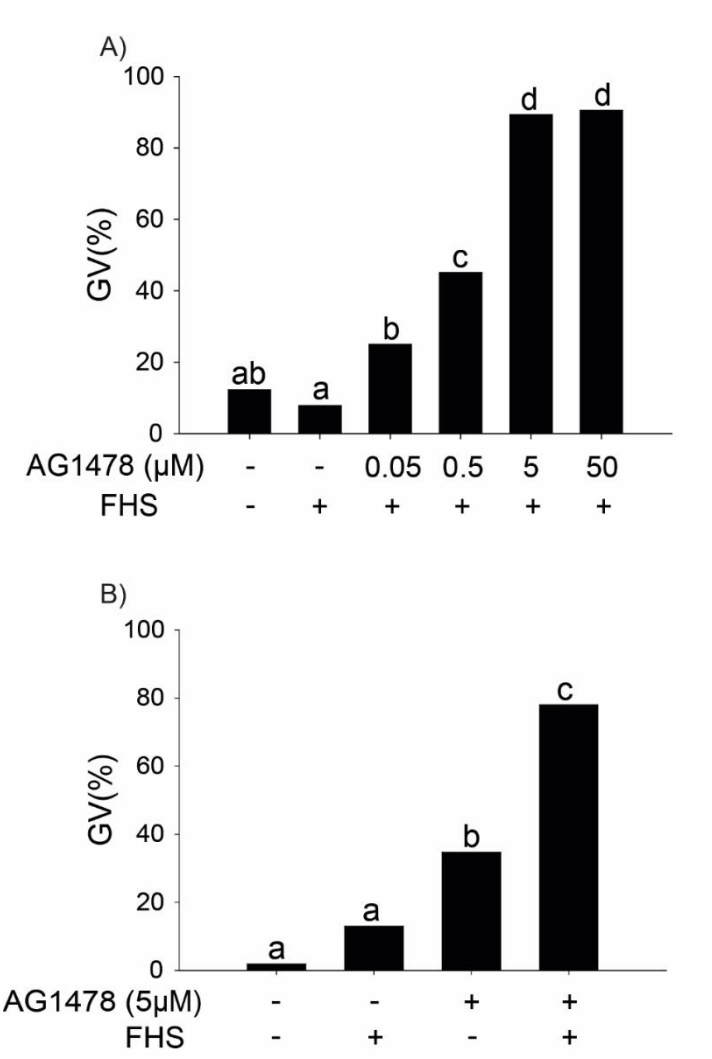
595 **Table 1.** Primers used for quantitative real-time PCR.

596

| Gene | Forward Primer (5'→ 3') | Reverse Primer (5'→ 3') | Accession Number |
|---|---|---|---|
| *PDE3A* | CTAATGCTTTCCTGGTTGCCACG | GCCGCATGATGATTCTCCAAG | XM_010805441.1 |
| *CCNB1* | CAAACTTTCCCCCGAGCCTA | GTTTGGATCCGCTCCGTCTT | NM_001045872.1 |
| *STC1* | GTGACACAGATGGGATGTACGAC | CGAATGGCCAGGAAGACC | NM_176669.3 |
| *ANXA1* | ATGGTATCTGAATTCCTCAAGCAG | TGCAAGGCCTCAACATCC | NM_175784.3 |
| *SERPINE1* | CAGGCGGACTTCTCCAGTT | CATTCGGGCTGAGACTACAAG | NM_174137.2 |
| *MAPK1* | TATTCGAGCACCGACCATCG | TGGAAGGTTTGAGGTCACGG | NM_175793.2 |
| *HSP90* | GAGGAAACACTCTCGGACGG | TCGGTCTTGCTGCTCCATAC | NM_174700.2 |
| *WASL* | GTGCAGTGGTGTTGCTTGTC | TGTCGTCGTCCCAACAAGTC | NM_174219.2 |
| *TNFAIP6* | GCTCACGGATGGGGATTCAA | CGTGCTTCCCTGTGGTAGAC | NM_001007813.2 |
| *HAS2* | GCATGTCACCCAGTTGGTCT | TGGGTCAAGCATGGTGTCTG | NM_174079.2 |
| *CX43* | GGGTGACTGGAGTGCCTTAG | GTCCCCAGTAGCAGGATTCG | NM_174068.2 |
| *SPRY2* | GTGAAGAGTTAAGCCAGGTGGG | CCAAGAGGAAGGAGACGGTT | NM_001076147.1 |
| *EGR1* | TCCCCTGTTCACAATGGTTT | TGGGAGAAAAGGTGGTTGTC | NM_001045875.1 |
| *PTGS2* | CCCTTCTGCCTGACGTCTTT | GGAAGATTCCTACCGCCAGC | NM_174445.2 |
| *PGR* | CAGAGCCCACAGTACAGCTT | ACTTTCGGCCTCCAAGAACC | NM_001205356.1 |
| *IMPDH1* | TCTGTCCTGCGGTCCATGAT | AGCCGCTTCTCGTAAGAGTG | NM_001077841.2 |
| *IMPDH2* | CATCACTCAGGAAGTGCTGGC | CGTCAGCAATGACAGGAACAC | NM_001034416.1 |
| *PGRMC1* | ACCAAAGGCCGCAAGTTCTA | AGGAGTGAGGTCGGAAAGGT | NM_001075133.1 |
| *ADAMTS1* | CTGGCAGAAACAGCACAACC | TCCGCCATGCCAAGAGTATC | NM_001101080.1 |
| *PTGER2* | GAAAGGGTATCCATGGCGGA | GAGCTTGGAGGTCCCACTTT | NM_174588.2 |
| *PTGER4* | CCTCTCTGGTGGTGCTCATC | ATGACTGGCTCCAGTTGTGG | NM_174589.2 |
| *CTSV* | GGCCTGGACTCAGAGGAATC | GAGGGATGTCAACGAAGCCA | NM_174032.2 |
| *CEBPB* | TAGCATCGGAGAGCACGAG | TAGTCGTCGGAGAAGAGGTC | NM_176788.1 |

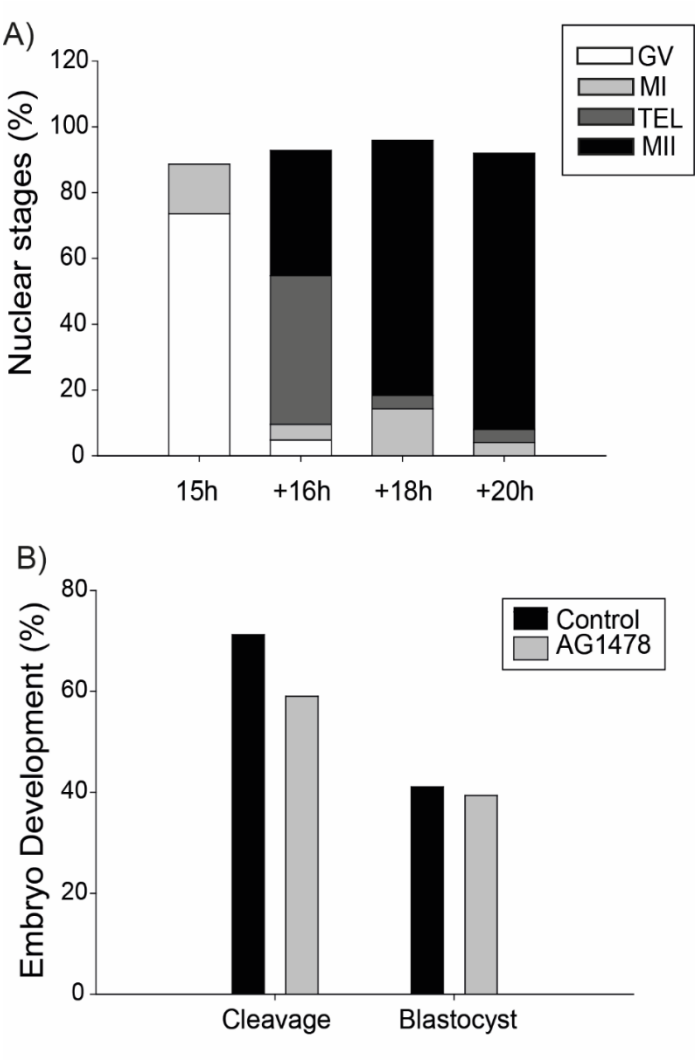
597

598 **Figure 1**



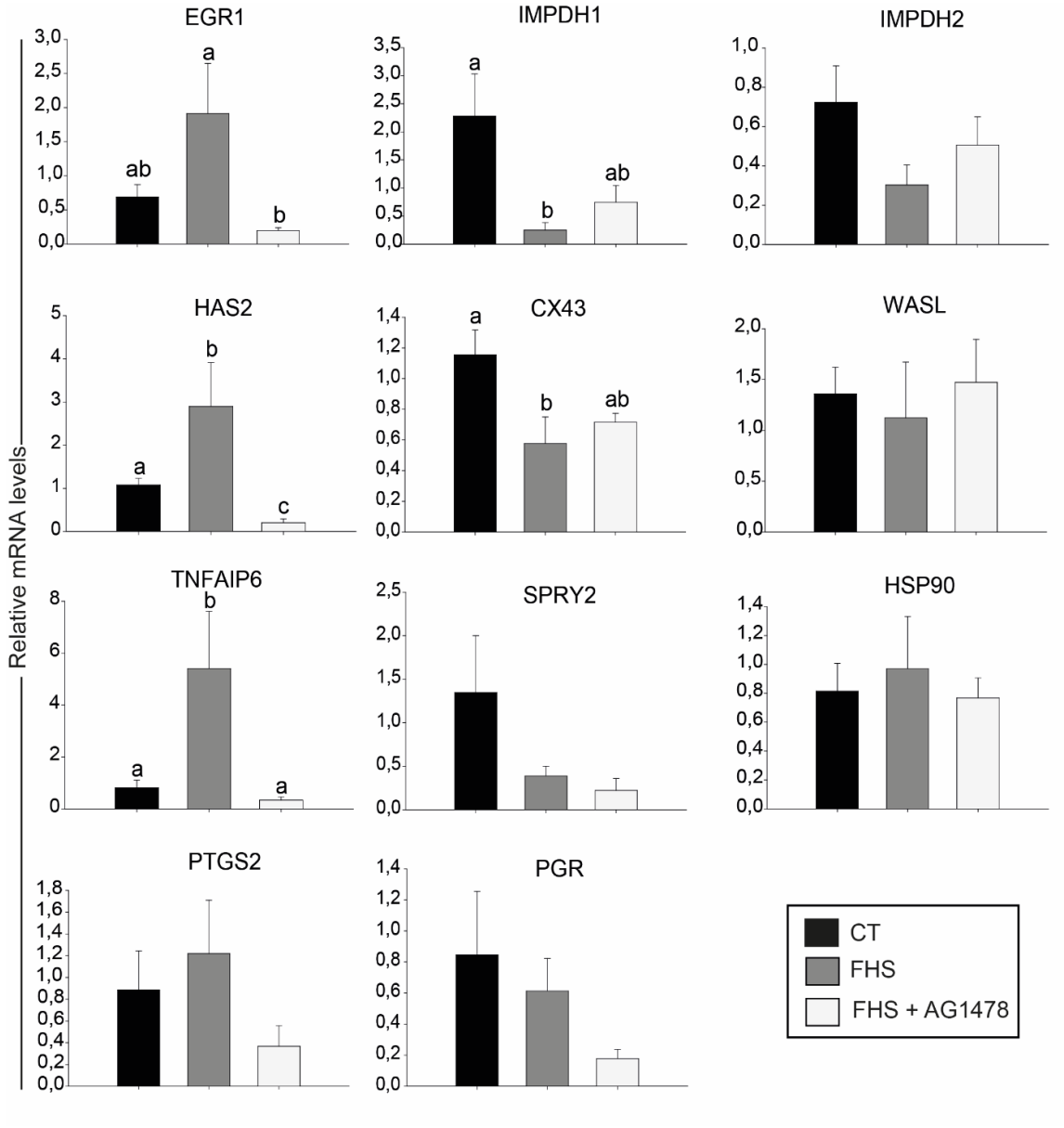

599

600 **Figure 2**



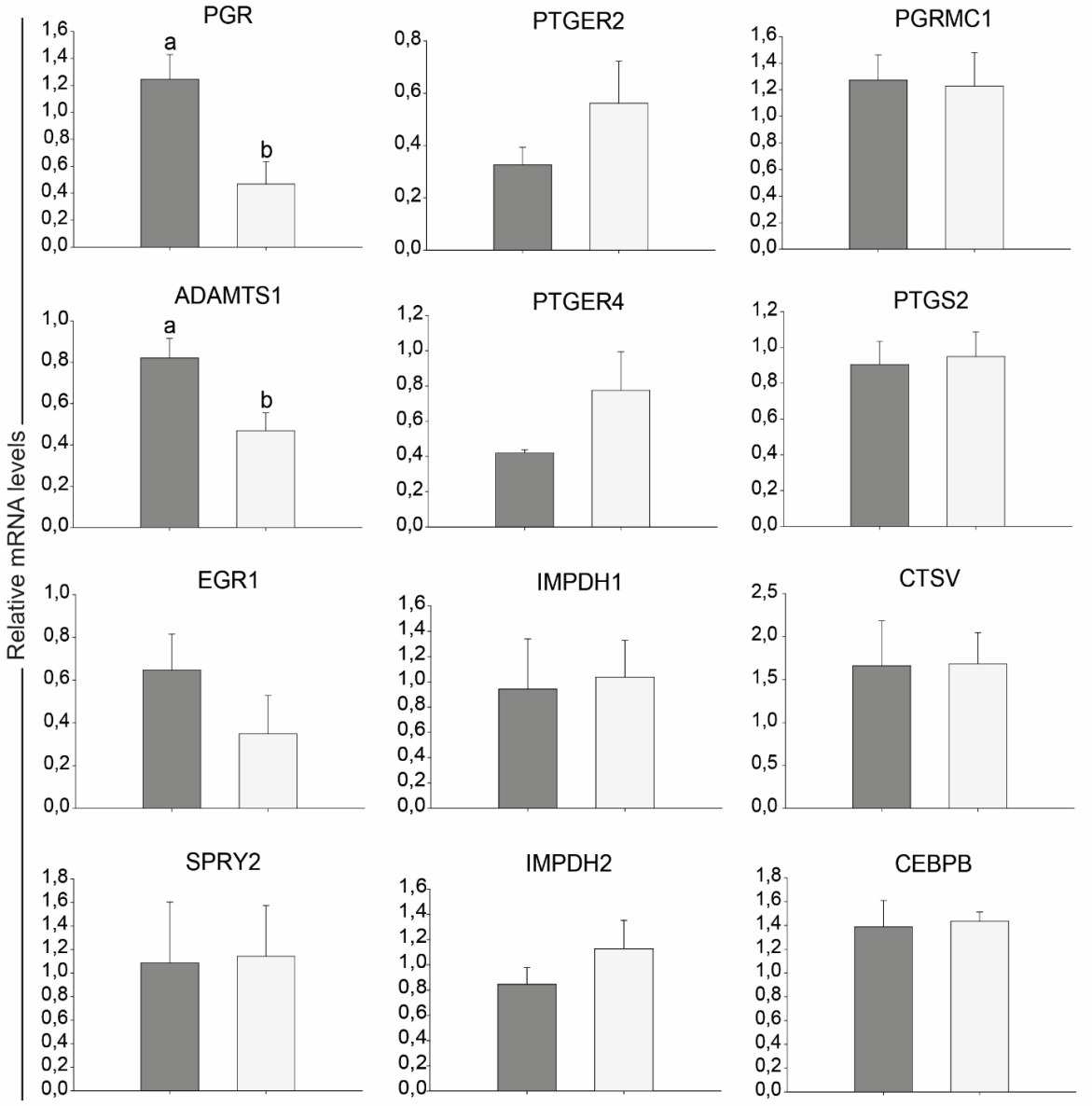

601

602 **Figure 3**




603

604 **Figure 4**

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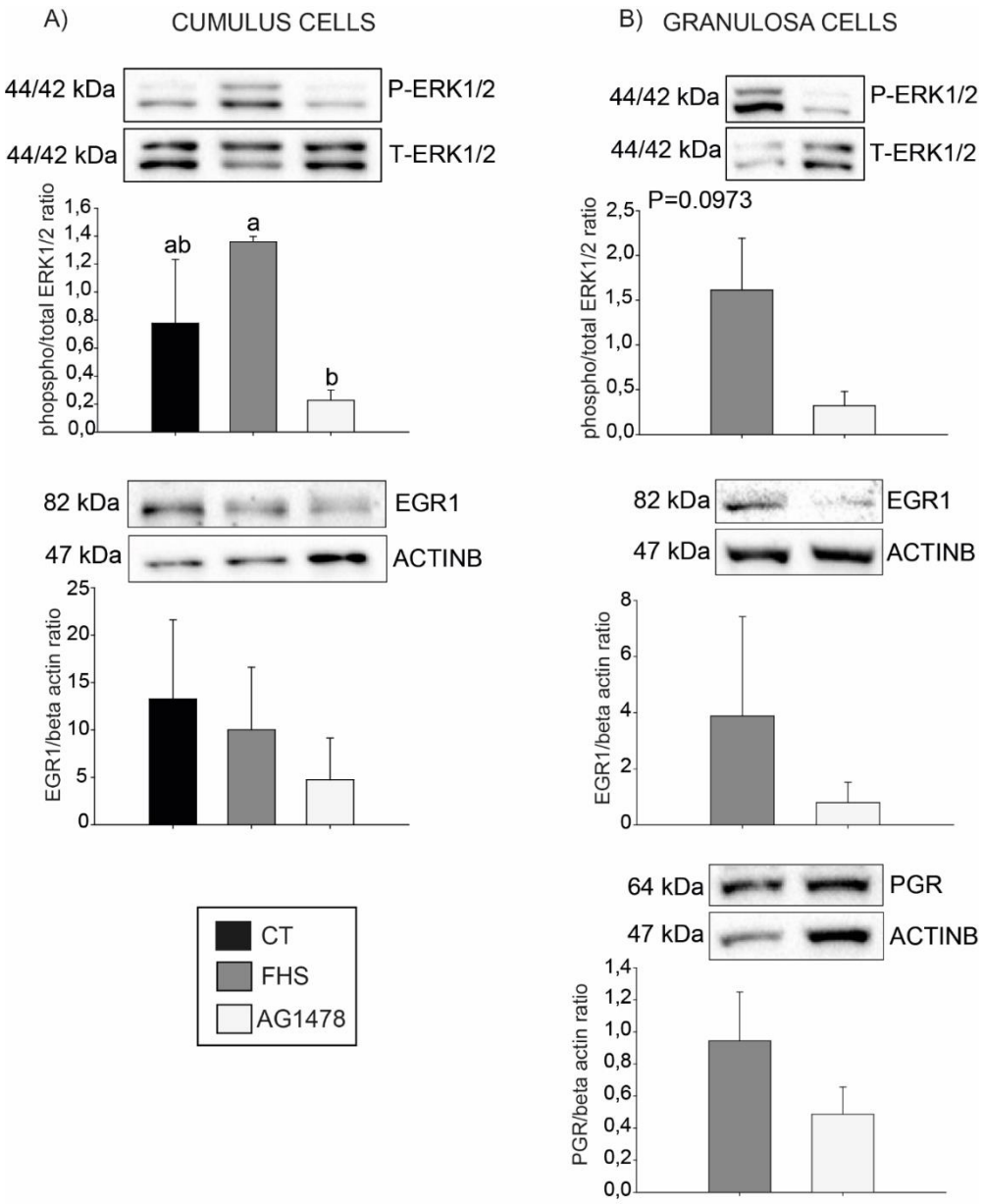
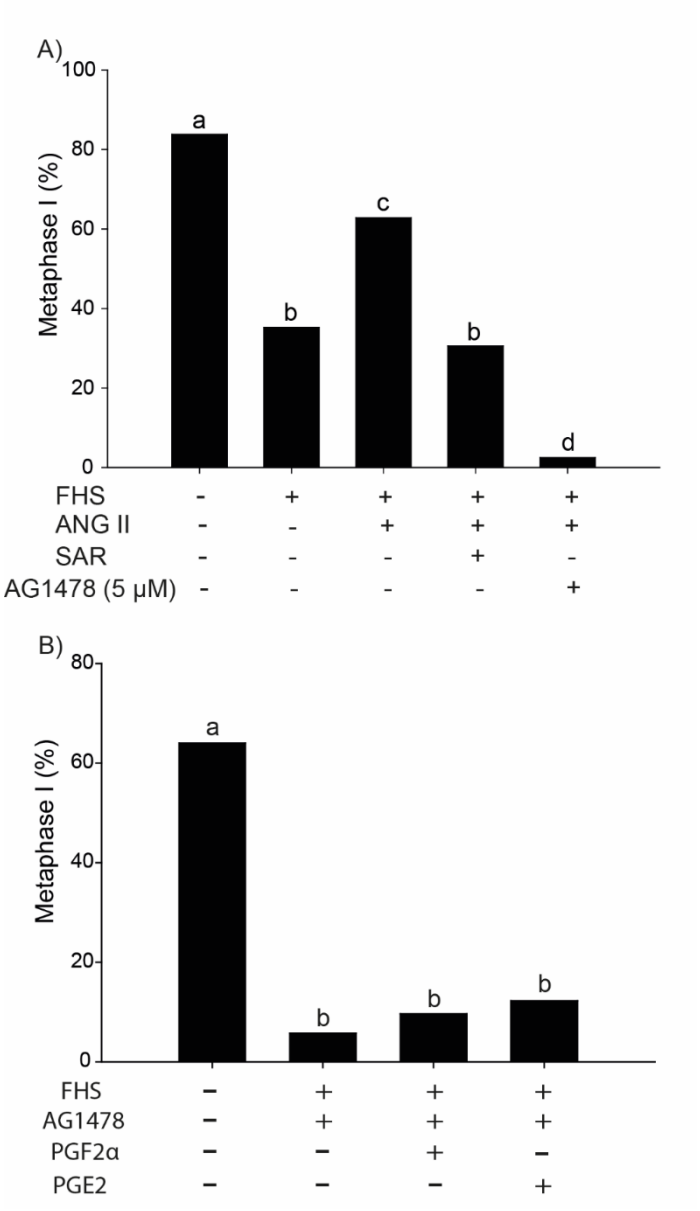
606 **Figure 5**




607

608 **Figure 6**

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610

## 4. ARTIGO 2

TRABALHO SUBMETIDO PARA PUBLICAÇÃO:

# Mechanistic target of rapamycin (mTOR) is activated in bovine granulosa cells after LH surge but is not essential for ovulation

**Paulo Roberto Antunes da Rosa, Andressa Minussi Pereira Dau, Matheus Pedrotti De Cesaro, Joabel Tonellotto dos Santos, Bernardo Garziera Gasperin, Raj Duggavathi, Vilceu Bordignon and Paulo Bayard Dias Gonçalves.**

**ANIMAL REPRODUCTION SCIENCE, 2016**

# Mechanistic target of rapamycin (mTOR) is activated in bovine granulosa cells after LH surge but is not essential for ovulation

Paulo Roberto Antunes da Rosa[a], Andressa Minussi Pereira Dau[a], Matheus Pedrotti De Cesaro[a], Joabel Tonellotto dos Santos[a], Bernardo Garziera Gasperin[b], Raj Duggavathi[c], Vilceu Bordignon[c] and Paulo Bayard Dias Gonçalves[a, *]

[a]Laboratory of Biotechnology and Animal Reproduction – BioRep, Federal University of Santa Maria, Santa Maria, RS, Brazil

[b]Laboratory of Animal Reproduction – ReproPEL, Federal University of Pelotas, Capão do Leão, RS, Brazil

[c]Department of Animal Science, McGill University, Sainte-Anne-de-Bellevue, QC, Canada.

*** Corresponding authors:**

Vilceu Bordignon: Department of Animal Science, McGill University, 21111, Lakeshore road, Room MS1-089, Sainte-Anne-de-Bellevue, QC, Canada, H9X3V9. Email: vilceu.bordignon@mcgill.ca

Paulo B. D. Gonçalves: BioRep, Federal University of Santa Maria, Room 16, 1000 Roraima AV, Veterinary Hospital, Santa Maria, RS, Brazil. Email: bayard@ufsm.br

**Abstract**

The preovulatory period is characterized by functional and structural changes in granulosa cells. Mechanistic target of rapamycin (mTOR) has emerged as an integrator of signaling pathways that mediate gonadotrophic stimuli. We hypothesized that mTOR kinase activity integrate and modulate molecular pathways induced by LH in granulosa cells during the preovulatory period. Cows were ovariectomized at different time points (0, 3, 6, 12, and 24 h) after GnRH injection and granulosa cells were collected for molecular analysis. Whereas *RHEB* mRNA levels increased at 3 and 6 h after GnRH treatment, returning to basal levels at 12 and 24 h, *RHOA* mRNA levels increased at 6 h and remained high thereafter. Western blot analyses revealed an increase in P70S6K activity at 3 and 6 h after GnRH injection. Similarly, ERK1/2, STAR and EGR1 were upregulated 3 h after GnRH treatment. Rapamycin treatment blockaded mTOR activity and induced AKT phosphorylation but did not alter phospho-ERK1/2 status and EGR1 protein abundance in cultured granulosa cells. Moreover, the use of rapamycin *in vitro* inhibited the LH-induced increase in *EREG* mRNA levels. The inhibition of mTOR kinase activity by intrafollicular injection of rapamycin did not suppress ovulation. These findings suggest that mTOR is involved in the control of *EREG* expression in cattle, which may be triggered by LH surge stimulating *RHEB* and P70S6K activity. Moreover, the protein abundance of p-AKT, p-ERK1/2 and EGR1 in rapamycin treated granulosa cells *in vitro*, suggest that this signaling may be coordinating the normal ovulation rates in rapamycin injected follicles *in vivo*.

**Keywords**: granulosa cells, ovulation, mTOR, rapamycin, cattle.

## Introduction

The LH surge induces functional and morphological modifications in granulosa cells that culminate with the rupture of the ovulatory stigma, release of the oocyte and luteinization. The classical mechanism by which LH induces ovulation involves the cAMP- protein kinase A (PKA) pathway in granulosa cells (Marsh, 1976; Richards, 1994). The LH signal in granulosa cells, mediated through growth factors, stimulates important pathways that modulate transcription, translation and phosphorylation during the ovulatory process, including phosphatidylinositide 3 kinase (PI3K)/AKT and extracellular signal regulated kinases 1/2 (ERK1/2) pathways (Fan et al., 2008; Fan et al., 2009). In mice, LH surge induces the release of epidermal growth factor (EGF)-like growth factors, which activate their receptors in granulosa and cumulus cells and modulate the expression of genes associated with cumulus expansion, oocyte maturation and ovulation through ERK1/2 intracellular pathway (Park et al., 2004; Panigone et al., 2008; Andric et al., 2010). However, this complex mechanism involves genes and intracellular pathways whose function remains unclear. Among this pathways, mechanistic target of rapamycin (mTOR), which mediates nutrients and growth factors signaling in different cell types (Sengupta et al., 2010), seems to be also involved in the regulation of ovulatory events (Huang et al., 2013).

The mTOR complex is a ser/thr kinase comprising two distinct complexes, mTORC1 and mTORC2. Their structure are distinct and while mTORC1 have the cofactor Raptor (regulatory associated protein of TOR), the mTORC2 have the cofactor Rictor (RPTOR independent companion of mTOR). mTORC1 signaling roles in reproductive events have been characterized (Yu et al., 2011; Huang et al., 2013). The cascade of signal that converges toward mTOR kinase activity is controlled initially by PI3K, which allows Akt to be activated through its phosphorylation on Thr-308 and Ser-473. Activated AKT inhibited tuberous sclerosis complex (TSC) by its phosphorylation. When TCS complex is inhibited by AKT, a small G-

protein RHEB becomes activated and induces P70S6K phosphorylation (Huang and Manning, 2009). The phosphorylation of P70S6K at thr389 is a main marker of mTORC1 activation. In addition, phospho-P70S6K triggers a negative feedback on PI3K. This is evident in cells lacking the TSC1-TSC2 complex where an interruption in growth factors mediating PI3K activation occurs (Harrington et al., 2004).

The mTOR pathway is known to participate in the process of cell growth and proliferation by controlling mRNA translation, autophagy and metabolism (Guertin and Sabatini, 2005; Wullschleger et al., 2006). In the same way, mTOR has been characterized as a potent regulator of cell cycle regulatory proteins (Fingar et al., 2004; Kayampilly and Menon, 2007). In the ovary, there is few data regarding mTOR involvement on ovulation in mammals and some of them are controversial. Inhibiting mTOR activation using rapamycin reduced granulosa cell proliferation arresting the cells at G1 phase and decreased the ovulation rate in mice (Yu et al., 2011). In another study, rapamycin treatment did not alter the occurrence of ovulation in hCG stimulated mice but resulted in downregulation of P70S6K (Siddappa et al., 2014). In cattle, mTOR inhibition prevents meiosis progression (Mayer et al., 2014), but its effect on the ovulatory process has not been investigated.

The present study was based on the hypothesis that mTOR pathway participates in the intracellular signal process triggered by LH in granulosa cells during the preovulatory period. The objectives were to: 1) investigate if mTOR kinase is activated in granulosa cells of bovine preovulatory follicles after LH surge; 2) assess the effect of mTOR inhibition on the regulation of genes induced by LH in granulosa cells cultured in vitro; and 3) determine if mTOR activity is required for ovulation in cattle.

## Materials and methods

*Animals, ovariectomy and granulosa cells isolation*

A total of 40 beef cows (Bos taurus taurus), with body condition score between 3 and 4 (1 = thin, 5 = fat) were used in this study in accordance with procedures approved by the Ethics and Animal Welfare Committee of the Federal University of Santa Maria (n°: 23081.004717/2010-53 - CCR / UFSM). Cows were submitted to a synchronization protocol by inserting an intravaginal device with 1 g progesterone (DIB®, Intervet/Schering-Plough, Brazil), and injecting 2 mg oestradiol benzoate, and 500 µg sodium cloprostenol (PGF) intramuscularly (i.m.) on Day 0. On day 9, the intravaginal device was removed and the ovaries were examined daily by transretal ultrasonography using an 8 MHz linear-array transducer (AquilaVet scanner, Pie Medical, Netherlands). Cows having follicles with diameter >12 mm were ovariectomized by colpotomy at 0, 3, 6, 12 or 24 h (n=5 animal in each time point) after 100 µg gonadorelin acetate (Profertil®, Tortuga, Brazil) i.m. treatment. Immediately after ovariectomy, follicular fluid was recovered and granulosa cells were isolated as previously described (Buratini et al., 2007). Isolated cells were immediately frozen and stored in liquid nitrogen. Total protein and RNA were extracted using the AllPrep® DNA/RNA/Protein kit (Qiagen). Follicular fluid was stored frozen for hormonal analysis.

*Cell culture*

To obtain granulosa cells, pairs of bovine ovaries without corpus luteum were collected in a local abattoir and transported to the laboratory in a saline solution (0.9% NaCl), containing 100 IU/ml penicillin and 50 µg/ml streptomycin at 30°C stored in a thermal box. In the laboratory, follicular status was determined by the presence of corpus luteum and on a high estradiol:progesterone (E:P) ratio in the follicular fluid measured by chemioluminescence. Then, granulosa cells were isolated from healthy preovulatory follicles with diameter >12 mm by flushing with PBS. Cells were pooled and washed twice by centrifugation at 200 g for 10

117 min. After isolation, cells were suspended in 2 ml of DMEM-F12 medium supplemented with
118 bovine serum albumin (BSA; 0.1%), penicillin (100 IU/ml), streptomycin (100 µg/ml),
119 amphotericin (50 ng/ml), androstenedione (10-7 M), follicle-stimulating hormone (FSH; 1
120 ng/ml) and insulin (10 ng/ml). The number of viable cells was estimated with 0.4% trypan blue
121 stain. Separated experiments were conducted for mRNA and protein analyses. For mRNA
122 analysis, cells were seeded into 96 well tissue culture plates at a density of $5x10^4$ viable cells
123 per well. For protein analysis, cells were seeded into 24-well tissue culture plates at a density
124 of $5x10^5$ viable cells per well. In both experiments, cells were stimulated with LH (100 ng/ml)
125 or LH with 1 or 10 µM rapamycin and cultured at 39°C, in an atmosphere of 5% CO2 in air for
126 6h.
127
128 *RNA extraction and RT-PCR*
129 Total RNA was extracted from granulosa cells using a silica-based protocol (in vivo
130 experiment; Qiagen, Mississauga, ON, Canada) or Trizol (in vitro experiment; Invitrogen)
131 according to the manufacturer's instructions and quantified by absorbance at 260 nm using a
132 spectrophotometer (NanoDrop, Thermo Fischer Scientific Inc., Waltham, MA). Total RNA was
133 first treated with 0.2 U DNase (Promega, Madison, WI) at 37ºC for 30 min, followed by heating
134 at 65ºC for 3 min. RNA (1µg and 800 ng for *in vivo* and *in vitro* experiments respectively) was
135 reverse transcribed (RT) in the presence of 1 µM oligo (dT) primer, 4 U Omniscript RTase
136 (Omniscript RT Kit; Qiagen), 0.5 µM dideoxynucleotide triphosphate (dNTP) mix, and 10 U
137 RNase inhibitor (Invitrogen) in a volume of 20 µL at 37ºC for 1 h. The reaction was terminated
138 by incubation at 93ºC for 5 min.
139 The relative gene expression was assessed by real-time PCR (RT-PCR) using the
140 StepOnePlus™ RT-PCR system (Applied Biosystems, Foster City, CA) with Platinum SYBR
141 Green qRT-PCR SuperMix (Invitrogen) and bovine-specific primers (table 1). The reaction was

carried out as following: 50ºC for 2 min, 95ºC for 10 min, 40 cycles at 95ºC for 15 sec and 60ºC for 1 min. Melting curve analyses were performed to verify product identity. Samples were run in duplicate and the variability in the amount of mRNA was corrected by amplification of cyclophilin housekeeping gene, and relative expression was performed as recommended by (Pfaffl, 2001). Bovine-specific primers were taken from the literature or designed using Primer Express Software version 3.0 (Applied Biosystems) and synthesized by Invitrogen.

*Western Blot Analysis*

Protein samples of granulosa cells obtained from *in vivo* experiment were extracted using AllPrep kit (Qiagen), and those obtained from *in vitro* culture experiment were extracted using Ripa Buffer (Sigma-Aldrich). After protein extraction, samples were diluted in Laemmli buffer (Bio-Rad) and boiled at 95°C for 10 minutes. Total lysate was subjected to 10% SDS gel and electrotransferred onto nitrocellulose membranes (Bio-Rad). After blocking for 2 h with 5% skim milk powder in Tris-buffered saline (TBS) + 0.1% of Tween, pH 7.6, membranes were incubated overnight at 4°C with the following primary antibodies: monoclonal anti total-p70S6 kinase (1:1000; #2708; cell signaling), polyclonal anti Phospho-p70S6 kinase (1:1000; #9205; Thr 389; cell signaling), monoclonal anti total-ERK1/2 (1:1000; #4695; Cell Signaling), monoclonal anti Phospho-ERK1/2 (1:1000; #4376; Thr202/Tyr204; Cell Signaling), polyclonal anti total-AKT (1:1000; #9272; cell signaling), polyclonal anti Phospho-AKT (1:1000; #9271; Ser473; cell signaling), polyclonal anti STAR (1:500; sc-25806; Santa Cruz Biotechnology), polyclonal anti EGR1 (1:5000; sc-189; Santa Cruz Biotechnology) or polyclonal anti β-Actin (1:10,000; ab8227; Abcam). Membranes were then washed three times for 10 minutes each with TBS-T and then incubated with anti-rabbit (ab6721; Abcam) secondary antibody diluted 1:10000 for 1.5 h at room temperature, followed by three washes for 5 minutes each with TBS-T. The immunoblotted proteins were detected using Immun-Star Western C Chemi luminescent

Kit (Bio-Rad) and images were captured using Chemidoc Analyzer (BioRad). For detection of multiple proteins, membranes were incubated with stripping buffer (62.5 mM Tris-HCl, 2% SDS, 0.8% 2-mercaptoethanol, pH 6.8) and re-blotted with another primary antibody. Images were analyzed and bands were quantified using the Image Lab 3.0 software (Bio-Rad). Levels of phosphorylated isoforms were normalized to their total isoforms for quantitative analyses.

*Intrafollicular injection of Rapamycin*

This experiment was performed to study the role of the mTOR pathway in the ovulatory process in bovine. Cyclic beef cows (bos Taurus Taurus) were submitted to a follicular wave induction protocol (Tonellotto dos Santos et al., 2012). After intravaginal device removal, the follicular diameter was monitored by transrectal ultrasonography, using a 8 MHz linear-array transducer (AquilaVet scanner, Pie Medical, Netherlands). Cows that had gonadotropin-releasing hormone (GnRH)-responsive preovulatory follicles (≥12 mm) received an intrafollicular injection of vehicle (0.9% saline solution) or 50 µM rapamycin. The intrafollicular injection method was validated in our laboratory (Ferreira et al., 2007; Ferreira et al., 2011). After the intrafollicular injections, cows were challenged with GnRH agonist (100 µg gonadorelin acetate, i.m.). A total of 17 cows responded to the synchronization protocol and were successfully injected. The volume to be injected was calculated based on the volume of follicular fluid as described by (Ferreira et al., 2007) to obtain a final intrafollicular concentration of 50 µM rapamycin. These procedures were approved by the Ethics and Animal Welfare Committee of the Federal University of Santa Maria (n°: 115/2014 - CCR / UFSM).

*Statistical Analysis*

Data of mRNA and protein abundance were tested for normality by the Shapiro-Wilk test, normalized when necessary according to each distribution and submitted to ANOVA using

192 the JMP software (SAS Institute Inc., Cary, NC, USA). Multiple comparison tests were run for
193 each significant result using the Least Squares Means Student's t-test. Results are presented as
194 mean ± standard error of the mean. Differences were considered statistically significant if $P \leq$
195 0.05.
196
197 **Results**
198 *Validation of the experimental model*
199 The *in vivo* ovulatory model was validated based on follicular diameter and estradiol
200 levels as previously documented (Tonellotto dos Santos et al., 2012). Briefly, the follicular
201 diameter did not change at different time-points after GnRH challenge and the concentration of
202 estradiol was elevated at 3 h after treatment with GnRH and gradually decreased until hour 24.
203
204 *RHEB and RHOA mRNA expression during preovulatory period*
205 In this experiment, we quantified the transcript levels of two small GTPases in granulosa
206 cells collected from live animals at different time points after GnRH treatment. *RHEB* mRNA
207 levels were increased at 3 h, reached maximum levels at 6 h, and returned to basal levels at 12
208 h and 24 h after GnRH injection (Figure 1a). *RHOA* mRNA levels also reached maximum
209 values at 6h and remained higher than before GnRH treatment (Figure 1b).
210
211 *Changes in P70S6K, ERK1/2, STAR and EGR1 protein levels during the preovulatory period*
212 Total and phospho-p70S6K proteins were examined by Western blot in granulosa cells
213 at different time points after GnRH injection. The relative abundance of p-p70S6K was elevated
214 at 3 h (around the time of LH surge) and 6 h after GnRH treatment, then decreased at 12 h and
215 re-increased at 24 h post-GnRH. The relative abundance of p-ERK1/2 protein was increased at
216 3 h, but decreased at 6 and 12h post-GnRH injection. Both STAR and EGR1 protein abundance

increased at 3 h and decline at 6 h post-GnRH. While EGR1 protein levels remain low after 3 h, STAR protein levels re-increased at 24 h post-GNRH (Figure 2).

*Effect of rapamycin on MTOR kinase signaling in cultured granulosa cells*

To investigate the role of mTOR signaling and its relation with other pathways, we evaluated the effect of different doses of rapamycin (0.1, 1 and 10 μM) in granulosa cells cultured in the presence of LH for 6 h. Rapamycin in all concentrations prevented p-P70S6K ($P<0.05$), which confirms that mTOR activity was effectively inhibited (Figure 3a). Total AKT protein was not affected by mTOR inhibition. However, p-AKT tended to increase in cell exposed to the higher concentrations of rapamycin, but the increase was not significantly different from cells treated with LH (Figure 3b). Rapamycin treatment tend to increase both total and phosphorylated ERK1/2, but the ration between the total and phosphorylated forms was not affected by mTOR inhibition (Figure 3c). EGR1 protein abundance was not affected by rapamycin treatment (Figure 3d).

*Effect of rapamycin treatment on gene expression in cultured granulosa cells*

Transcript levels of *AREG*, *EREG*, *PTGS2*, *LHCGR*, *BAX* and *BCL2* were quantified in cultured granulosa cells after 6 h from treatment with 1 or 10 μM rapamycin. Transcript levels of *AREG*, *EREG* and *PTGS2* were significantly increased by LH ($P<0.05$). Both concentrations of rapamycin inhibited *EREG* mRNA increase induced by LH ($P<0.05$). A similar trend was observed for *AREG* and *PTGS2* mRNA abundance, despite of not been statistically significant (Figure 4a). Rapamycin treatment had no significant affect ($P>0.05$) on mRNA levels of *LHCGR* and *BCL2*, but *BAX* mRNA levels were lower in samples treated with either LH alone or combined with rapamycin (Figure 4b).

*Effect of intrafollicular injection of rapamycin on ovulation*

In this experiment, we investigated if the inhibition of MTOR in preovulatory follicles would affect GnRH-induced ovulation. The ovulation rate of follicles injected with 50 µM of rapamycin (88.8%) was not statistically different (P>0.05) than those injected with vehicle alone (100%; Figure 5).

**Discussion**

The signaling pathways involved in the regulation of the ovulatory process in response to the LH surge have not been completely elucidated. In the present study, the expression profile of genes involved in ovulatory process was investigated at both mRNA and protein levels in granulosa cells collected from live cows at different time-points after GnRH injection and in cultured granulosa cells treated with LH. Findings from this study revealed significant changes at mRNA (*RHEB*, *RHOA*), protein (STAR, EGR1), and post-translational modifications (p-P70S6K, p-ERK1/2) induced by GnRH treatment. This study confirmed rapamycin effect on mTOR activity (p-P70S6K) inhibition and revealed that rapamycin inhibited transcripts induced by LH (*EREG*) in cultured granulosa cells, but did not prevent ovulation after injection into preovulatory follicles.

To investigate the role of the mTOR kinase in the ovulatory process of nonovulatory species, we have first quantified changes in mRNA and proteins regulated by mTOR in granulosa cells recovered from live cows at different time-points after GnRH injection. The mTOR pathway has been implicated in many cellular processes including protein translation and signaling from hormones, nutrients and growth factors (Kim et al., 2008; Huang and Manning, 2009). In this study we observed that GnRH treatment increases mTOR activity in granulosa cells as revealed by the increase in p-P70S6K (Siddappa et al., 2014) and *RHEB* (Inoki et al., 2003; Guertin and Sabatini, 2009) mRNA levels. This indicates that mTOR activity

267 is inducted by gonadotropins in granulosa cells from preovulatory follicle of monovulatory
268 species as previously observed in polyovulatory species (Yu et al., 2011; Siddappa et al., 2014).
269 In this study, it was observed that the expression profile of p-P70S6K protein and *RHEB*
270 mRNA after GnRH treatment was very similar to the changes observed in protein levels of
271 STAR, EGR1 and p-ERK1/2, which suggest a potential link between these protein and mTOR
272 activity. On the other hand, mRNA levels of *RHOA*, which encodes a protein involved in the
273 extracellular matrix remodeling (Kakinuma et al., 2008) and likely more important for later
274 events of ovulatory cascade (Ny et al., 2002; Joseph et al., 2012), had a different expression
275 profile compared to p-P70S6K and *RHEB* mRNA.
276 It is known that ERK1/2 is induced by LH surge (Fan et al., 2009; Sayasith and Sirois,
277 2015), and is a key regulator of granulosa cell differentiation and proliferation (Mebratu and
278 Tesfaigzi, 2009), and is required for ovulation in polyovulatory species (Fan et al., 2009).
279 Nevertheless, the role of ERK1/2 during the ovulatory process has not been fully characterized
280 in monovulatory species. In this study, we observed that p-ERK1/2 and p-P70S6K proteins
281 were similarly induced in granulosa cells of live cows after GnRH treatment. Although the
282 molecular link between mTOR and ERK1/2 has not been well-characterized, it has been
283 proposed that mTOR kinase inhibition with rapamycin can upregulate ERK1/2 (Dai et al.,
284 2014). However, we observed that neither total nor p-ERK1/2 protein was significantly
285 increased in cultured granulosa cells after rapamycin treatment, which suggests that ERK1/2
286 activity may not be affected by mTOR in bovine granulosa cells.
287 The LH surge is known to induce dynamic changes in granulosa cell function by
288 stimulating the expression of several genes (Carletti and Christenson, 2009). This includes a
289 shift in follicular steroidogenesis from estradiol to progesterone (Komar et al., 2001), which
290 involves changes in the levels of the STAR protein. Indeed, we observed that STAR protein
291 has a biphasic regulation that includes a peak at 3 h after GnRH injection, which coincides with

292 the highest levels of estradiol secretion (Tonellotto dos Santos et al., 2012), and a second peak
293 at 24 h, which correlates with the increase in progesterone secretion (Fortune et al., 2009).
294 EGR1 is also induced by LH signaling in granulosa cells and participates in the ovulatory
295 process as a downstream effector of Mapk3/1 (Siddappa et al., 2015). In this study, we observed
296 maximum levels of EGR1protein at 3h after GnRH treatment. Even though the protein profile
297 of both STAR and EGR1at the different time points after GnRH injection mimic those of p-
298 P70S6K, our findings using cultured cells did not support a correlation between mTOR activity
299 and EGR1 protein expression in bovine granulosa cells.

300 This study has also revealed that inhibition of mTOR activity with rapamycin prevents
301 the increase in *EREG* mRNA induced by LH treatment. This suggests the mTOR may
302 participate in the regulation of *EREG* expression after LH surge. However, previous study in
303 mice observed that rapamycin injection could not prevent the upregulation of *EREG* mRNA
304 induced by hCG treatment (Siddappa et al., 2014). This suggests that mTOR effect on *EREG*
305 regulation may differ in nonovulatory and polyovulatory species. On the other hand, our
306 findings with cultured granulosa cells agree with the study in mice by showing the *AREG* and
307 *PTGS2* mRNA levels were not affected by rapamycin treatment. Moreover, our findings reveal
308 that rapamycin treatment increased p-AKT levels in granulosa cells cultured with LH compared
309 to control cells. This suggests that AKT is involved in mTOR activation induced by LH in
310 bovine granulosa cells. Indeed, studies conducted using different cell have shown that AKT is
311 an upstream effector for mTOR activation (Manning et al., 2002; Huang and Manning, 2009).

312 To evaluate if mTOR is required for ovulation in nonovulatory species, rapamycin was
313 injected into pre-ovulatory follicles (with diameter > 12 mm) of cows that were simultaneously
314 treated with GnRH. It was observed that rapamycin injection did not prevent ovulation in cattle,
315 which suggests that mTOR activity is not essential for the final stages of follicular growth and
316 rupture in cattle. Although previous studies have reported that rapamycin treatment decrease

317 the ovulation rate in mice (Yu et al., 2011), other studies in mice have not observed alteration
318 in the ovulation rate of mice that were intraperitoneally injected with rapamycin (Siddappa et
319 al., 2014).

320 In conclusion, findings from this study revealed that: i) mTOR activity is induced by
321 GnRH in bovine granulosa cells of preovulatory follicles; ii) the profile of mTOR activity
322 mimics those of other important factors regulated by LH that are involved in the ovulatory
323 process including ERK1/2, STAR and EGR1; and iii) mTOR activity is not essential for
324 ovulation in cattle.

325

326 **Acknowledgements**

327 The authors would like to thank the Leão and Guassupi ranches for providing the
328 animals used in this work. We are grateful to Silva abattoir for the donation of bovine ovaries
329 used in this research and McGill University facilities for providing laboratory space. This study
330 was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq),
331 Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and the Natural
332 Sciences and Engineering Research Council (NSERC-Canada).

333

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**Figure Legends:**

**Figure 1:** Relative mRNA abundance for *RHEB* (a) and *RHOA* (b) in granulosa cells obtained 0, 3, 6, 12 and 24 h after GnRH injection. n=5 animal in each time-point. Different letters indicate statistical difference between groups ($P < 0.05$).

**Figure 2:** Protein levels of P70S6K (a), ERK1/2 (b), STAR (c) and EGR1 (d) in granulosa cells collected at 0, 3, 6, 12 and 24 h after GnRH injection. The abundance of phosphorylated proteins was normalized to the corresponding total protein level. Total protein levels were normalized to the loading control b-actin. Different letters indicate statistical difference between groups ($P < 0.05$). n=5 animal in each time-point.

**Figure 3:** Protein levels of P70S6K (a), AKT (b), ERK1/2 (c) and EGR1 (d) in granulosa cells *in vitro* cultured for 6 h in the presence of LH and rapamycin. The abundance of phosphorylated proteins was normalized to the corresponding total protein level. Total protein levels were normalized to the loading control b-actin. Data represent the mean ± SEM for three independent replicates. Different letters indicate statistical difference between groups ($P < 0.05$).

**Figure 4:** Relative mRNA levels for *AREG*, *EREG* and *PTGS2* (a), and *LHCGR*, *BAX* and *BCL2* in granulosa cells cultured *in vitro* for 6 h in the presence of LH and rapamycin. Data represent the mean ± SEM for three independent replicates. Different letters indicate statistical difference between groups ($P < 0.05$).

**Figure 5:** Ovulation rate following ultrasound-mediated intrafollicular injection of 50 µM rapamycin. Follicles with 12 mm or more in diameter were injected with vehicle (n=8; white bar) and rapamycin (n=9; dark bar) and the cows received GnRH agonist (100 µg gonadorelin acetate, i.m.) to induce ovulation.

462 **Table 1** Primers used in the expression analysis of candidate genes. Primer sequences and
463 concentrations used to amplify each product are described

| Gene | Primer sequence | Conc. (nM) | Reference or accession n° |
|---|---|---|---|
| *RHEB* | F: CGCAGCGTTTTTGGAATCAT | 200 | NM_001031764.1 |
| | R: TCACCGAGCAGGAAGACTTTC | 200 | |
| *RHOA* | F: TCGAAACGACGAGCACACA | 200 | NM_176645.3 |
| | R: CCATGTACCCAAAAGCACCAA | 200 | |
| *AREG* | F: CCATTTTCTTGTCGAAGTTTCTTTC | 200 | (Li et al., 2009) |
| | R: TGTTTTTATTACAATCCTGCTTCGAA | 200 | |
| *EREG* | F: ACTGCACAGCATTAGTTCAAACTGA | 200 | XM_010806226.1 |
| | R: TGTCCATGCAAACAGTAGCCATT | 200 | |
| *PTGS2* | F: TTTGACCCAGAGCTGCTTTT | 200 | NM_174445.2 |
| | R: GAAAGACGTCAGGCAGAAGG | 200 | |
| *LHCGR* | F: GCACAGCAAGGAGACCAAATAA | 200 | NM_174381.1 |
| | R: TTGGGTAAGCAGAAACCATAGTCA | 200 | |
| *BAX* | F: GACATTGGACTTCCTTCGAGA | 200 | (Mani et al., 2010) |
| | R: AGCACTCCAGCCACAAAGAT | 200 | |
| *BCL2* | F: GTGGATGACCGAGTACCTGAAC | 200 | (Mani et al., 2010) |
| | R: AGACAGCCAGGAGAAATCAAAC | 200 | |

464 F, Forward primer; R, Reverse primer; Conc., primer concentration used for gene
465 amplification.
466

**Figure 1**

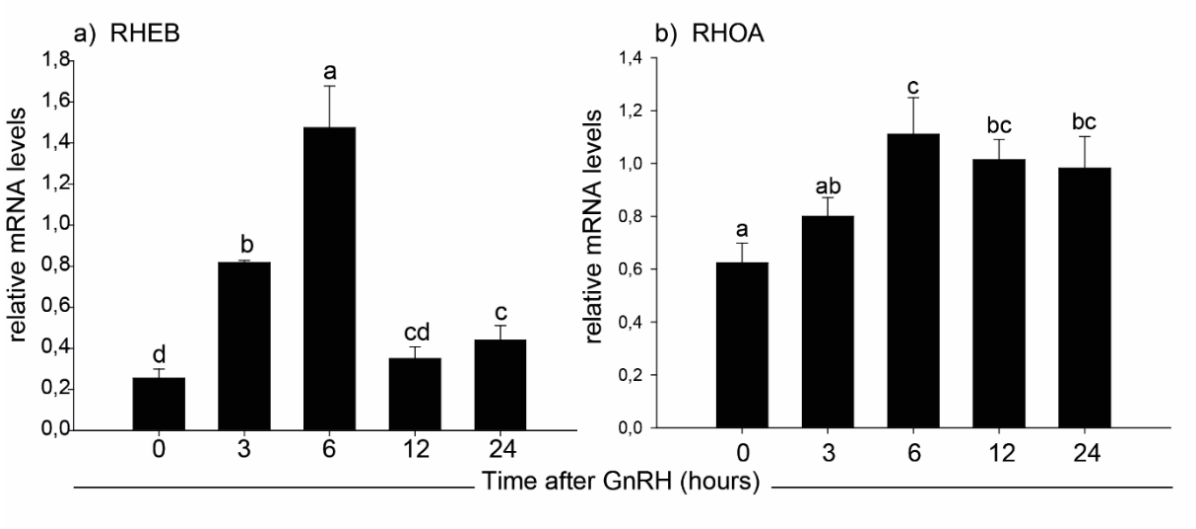
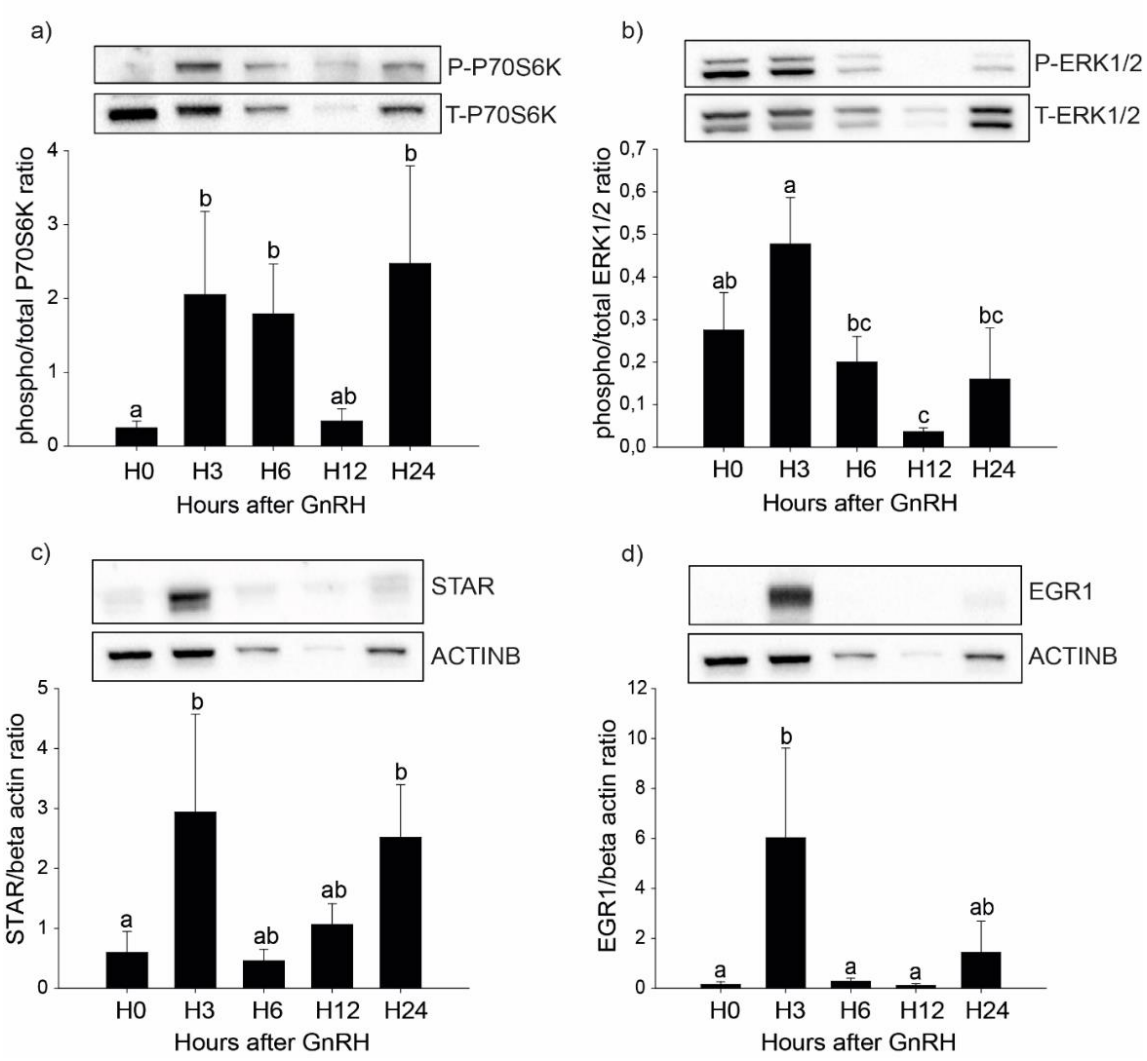
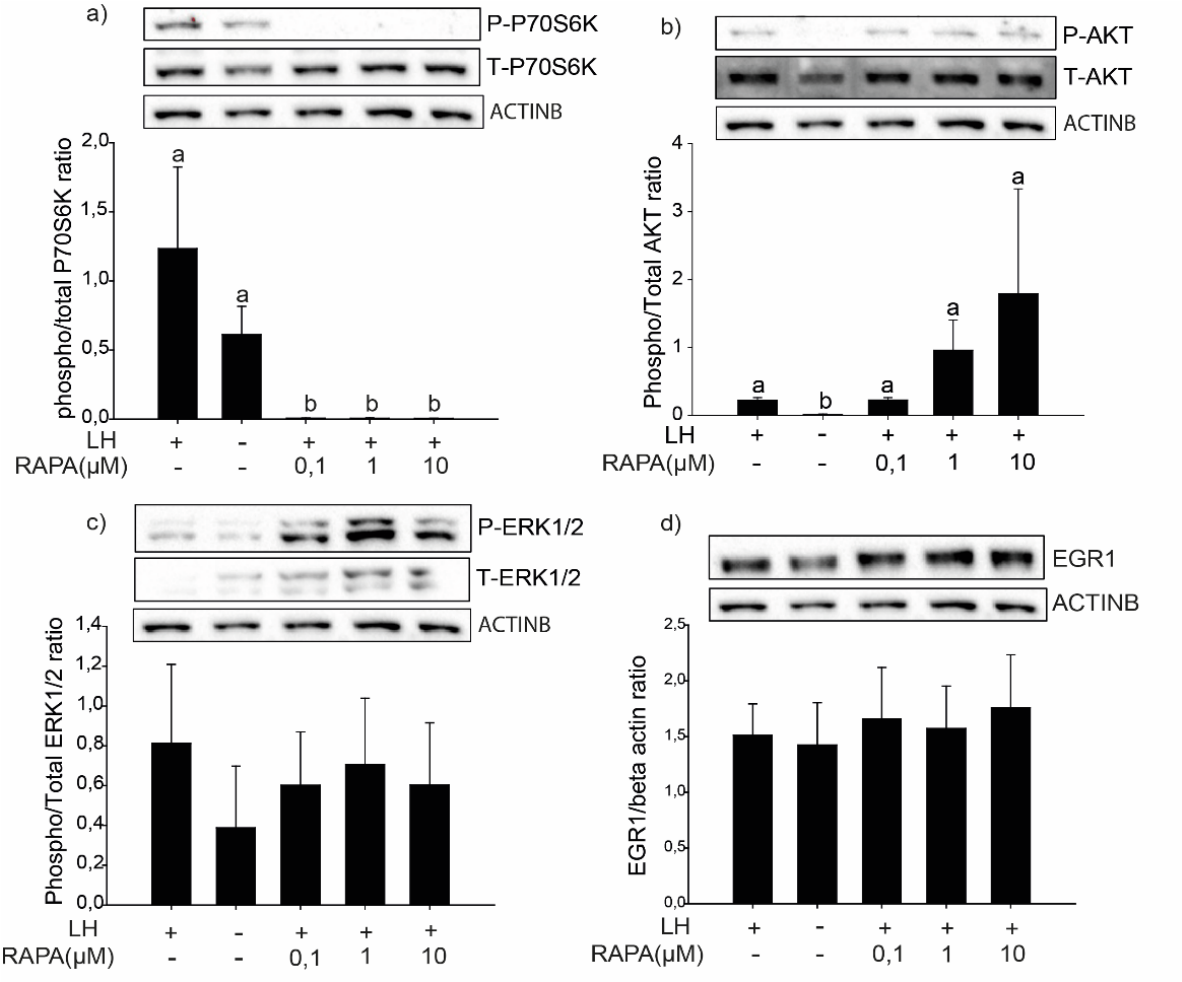

a) RHEB
relative mRNA levels
1,8
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
d
b
a
cd
c
0
3
6
12
24
b) RHOA
relative mRNA levels
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
a
ab
c
bc
bc
0
3
6
12
24
Time after GnRH (hours)

**Figure 2**

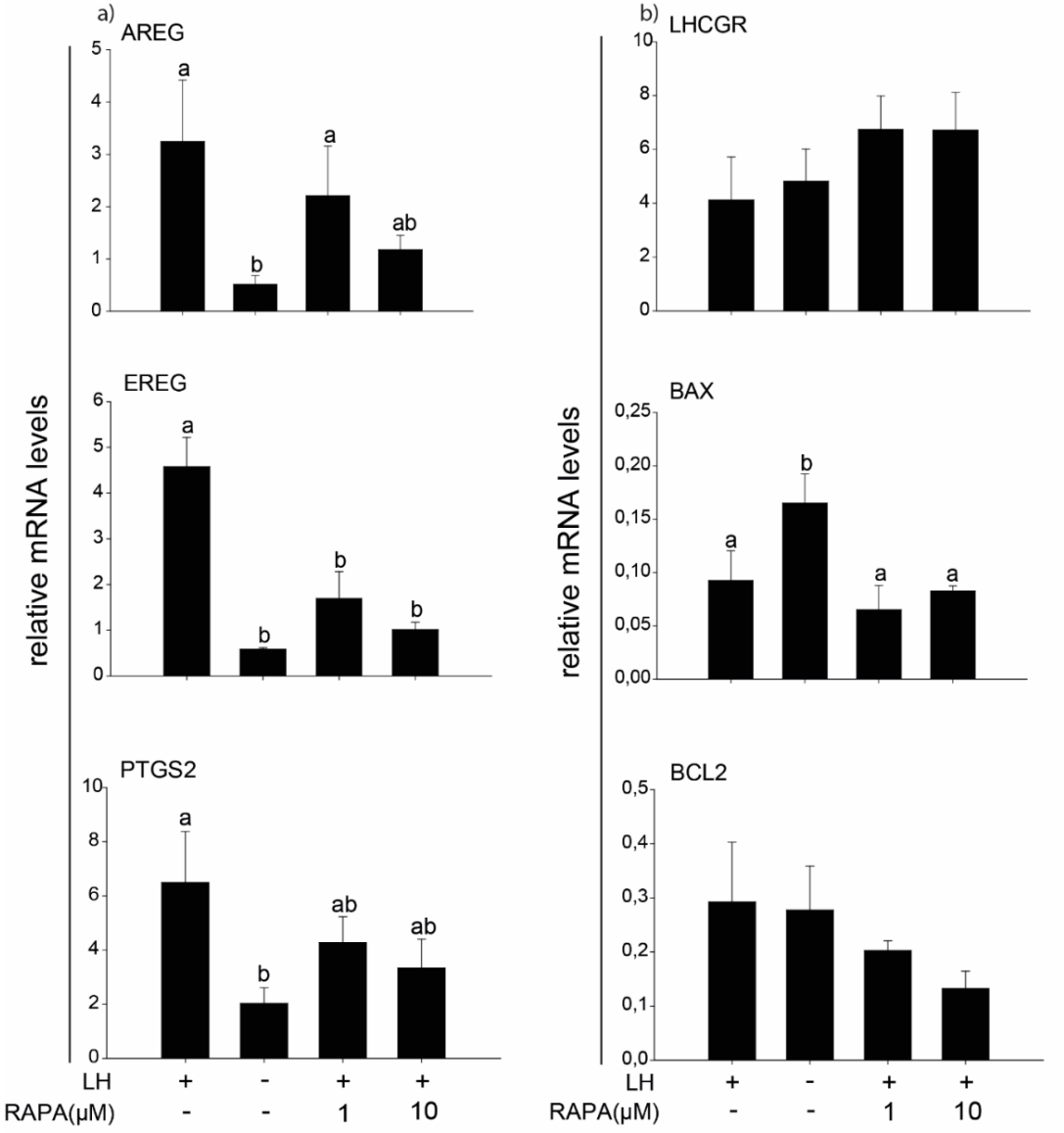

a)
P-P70S6K
T-P70S6K
phospho/total P70S6K ratio
0
1
2
3
4
a
b
b
ab
b
H0
H3
H6
H12
H24
Hours after GnRH
b)
P-ERK1/2
T-ERK1/2
phospho/total ERK1/2 ratio
0,0
0,1
0,2
0,3
0,4
0,5
0,6
0,7
ab
a
bc
c
bc
H0
H3
H6
H12
H24
Hours after GnRH
c)
STAR
ACTINB
STAR/beta actin ratio
0
1
2
3
4
5
a
b
ab
ab
b
H0
H3
H6
H12
H24
Hours after GnRH
d)
EGR1
ACTINB
EGR1/beta actin ratio
0
2
4
6
8
10
12
a
b
a
a
ab
H0
H3
H6
H12
H24
Hours after GnRH

473 **Figure 3**




474

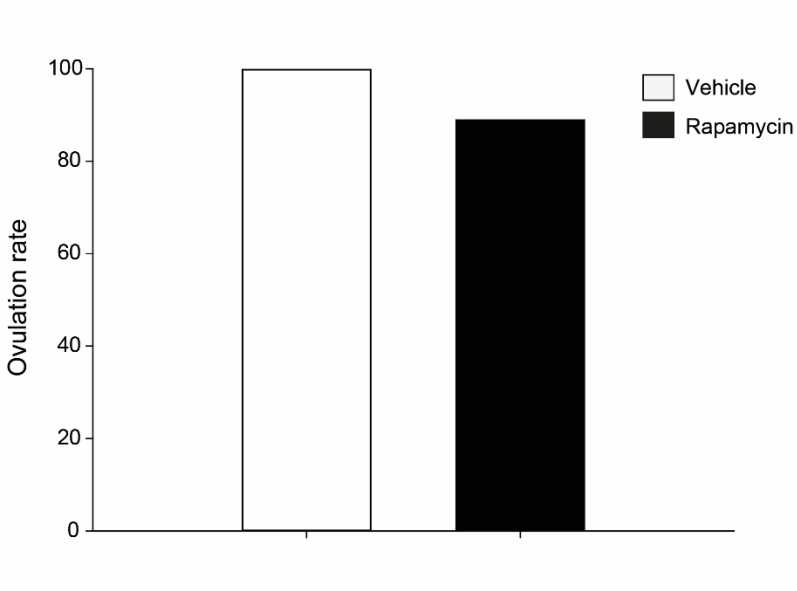
475 **Figure 4**




476

477

**Figure 5**



# 5. DISCUSSÃO

Os processos de maturação nuclear do oócito e ovulação são eventos celulares distintos, mas que acontecem em decorrência do pico pré-ovulatório de LH. Participando da busca pelo conhecimento desses eventos celulares, nosso grupo iniciou uma série de estudos conduzidos em modelos experimentais *in vitro* e *in vivo* os quais nos permitiram compreender ao longo dos anos a participação de diversos fatores envolvidos tanto na maturação nuclear do oócito (GIOMETTI et al., 2005; STEFANELLO et al., 2006; BARRETA et al., 2008; SIQUEIRA et al., 2012) como na funcionalidade das células da granulosa ao longo do período pré-ovulatório (FERREIRA et al., 2007; TONELLOTTO DOS SANTOS et al., 2012; SIQUEIRA et al., 2013). O entendimento desses mecanismos envolvidos na maturação e ovulação serve como base para melhor explorar o potencial reprodutivo de fêmeas e/ou elaborar técnicas contraceptivas com menos efeitos colaterais.

Fatores produzidos pelas células foliculares ao longo do desenvolvimento folicular, atuam no COC promovendo a completa capacitação do oócito para que, ao ser fertilizado, tenha condições de suportar o desenvolvimento embrionário (LODDE et al., 2007; GILCHRIST, 2011). Em procedimentos de maturação e fecundação *in vitro*, a coleta de oócitos de folículos em diferentes fases de desenvolvimento determina uma variabilidade no grau de capacitação desses oócitos, o que contribui para a baixa eficiência da Produção In Vitro (PIV) de embriões bovinos (LONERGAN et al., 1994; NIVET et al., 2012). Com o intuito de proporcionar essa capacitação *in vitro* em um ambiente capaz de preservar a comunicação entre o COC e as células foliculares, o primeiro estudo descrito nesta tese conta com a caracterização de um modelo de bloqueio do reinicio da meiose através da utilização de um inibidor dos EGFR em um sistema de cultivo com metades foliculares.

Até o momento, estudos que objetivaram prolongar o período de maturação *in vitro* inibindo o reinicio da meiose utilizaram produtos farmacológicos com ação direta na atividade do MPF (MERMILLOD et al., 2000; LE BEUX et al., 2003). Uma vez que a atividade de MPF é o mecanismo final na cascata de eventos que culmina na maturação nuclear, essa metodologia não impede a ocorrência dos eventos anteriores a sua inibição. Isso pode determinar que os oócitos progridam nos outros eventos da maturação, interferindo negativamente na qualidade do oócito após o período de bloqueio meiótico. No entanto, apesar da existência dos modelos com metades foliculares, os quais atrasam a progressão meiótica mas não impedem o rompimento da vesícula germinativa (RICHARD & SIRARD, 1996b; DE CESARO et al., 2013), este é o primeiro estudo que caracterizou um modelo de bloqueio do reinicio da meiose

*in vitro*, reversível e que não altera a capacidade de desenvolvimento dos oócitos, utilizando um sistema de cultivo similar ao ambiente folicular *in vivo*, no qual é preservada a comunicação entre o oócito e as células somáticas.

O estímulo *in vitro* para o reinício da meiose oocitária se dá ao retirar o oócito do ambiente folicular, fato este que simula a ocorrência do pico pré-ovulatório de LH *in vivo*. A ativação dos EGFR parece ser o principal mecanismo responsável por propagar a sinalização do LH a partir das células somáticas até o gameta feminino (REIZEL et al., 2010b). A inibição dos EGFR aqui proposta foi baseada em modelos de maturação *in vitro* de oócitos de roedores e suínos nos quais a utilização do AG1478 bloqueou o reinicio da meiose e expansão das células do cumulus (PARK et al., 2004; REIZEL et al., 2010b; PROCHAZKA et al., 2012). Entretanto, ainda não eram conhecidos os efeitos da inibição dos EGFR em COCs de bovinos, e os resultados aqui apresentados demonstram que, diferentemente de roedores e suínos, a presença das células foliculares é fundamental para a inibição do reinicio da meiose induzida pelo AG1478 em bovinos. Além disso, o bloqueio parcial do reinicio da meiose induzido pelo AG1478 em meio sem metades foliculares, nos sugere que, em bovinos, enquanto o estímulo *in vivo* para o reinicio da meiose parece ser dependente da ativação dos EGFR, *in vitro* esse mecanismo parece não ser o único envolvido.

A inibição dos EGFR ao longo do período de interação entre o CCO e as células foliculares determinou alterações moleculares que podem explicar alguns mecanismos pelos quais ocorre o bloqueio meiótico e a inibição da expansão das células do cumulus. Provavelmente, a diminuição da forma fosforilada da proteína ERK1/2 nas células do cumulus seja um sinal comum que determinou a diminuição na expressão dos genes de expansão (TNFAIP6 e HAS2), bem como de genes envolvidos na maturação nuclear (IMPDH1 e CX43) os quais modulam a síntese e passagem de fatores inibidores do reinício da meiose para o interior do oócito (LUCIANO et al., 2011; WIGGLESWORTH et al., 2013). Além disso, a diminuição nos níveis de RNAm para os receptores de progesterona (PGR) pela ação do AG1478 nas células da granulosa oriundas das metades foliculares, nos sugere que os efeitos da progesterona em estimular a progressão meiótica de oócitos bovinos (SIQUEIRA et al., 2012) pode ser dependente da ativação dos EGFR.

O papel da AngII e PGE2/PGF2α como mediadores da maturação nuclear de oócitos em ruminantes induzida por gonadotrofinas já havia sido demonstrado (BARRETA et al., 2008). No entanto, até o momento, não existiam dados a respeito das vias intracelulares envolvidas. A ausência de reversão do bloqueio meiótico induzido pelas FHS e AG1478 revela que é necessária a ativação dos EGFR no mecanismo de ação da AngII e PGs.

Na busca pelo conhecimento das demais rotas de sinalização diferentemente ativas nas células da granulosa de folículos em processo ovulatório, o segundo artigo desta tese caracterizou a atividade do sistema mTOR em função do estímulo gonadotrófico ao longo do período pré-ovulatório bem como seu papel tanto como mediador da ação do LH na expressão de genes e ativação de outras vias intracelulares, como ocorrência do processo ovulatório. Apesar de estudos recentes terem relatado um envolvimento do mTOR na progressão meiótica de oócitos bovinos (MAYER et al., 2014), até então não eram conhecidos dados do envolvimento desta rota no processo ovulatório de bovinos.

Inicialmente, os estudos de caracterização foram realizados com um modelo experimental *in vivo* já descrito em outros estudos conduzidos pela nossa equipe (TONELLOTTO DOS SANTOS et al., 2012; SIQUEIRA et al., 2013) no qual as células da granulosa foram obtidas de folículos ≥12mm em diferentes momentos após a indução da ovulação pela administração de GnRH. Baseado nos dados de expressão de RNAm da enzima RHEB e abundância proteica de p-P70S6K, demonstramos que ocorre atividade do mTOR em resposta ao estímulo gonadotrófico *in vivo* similarmente ao que ocorre em roedores (YU et al., 2011; SIDDAPPA et al., 2014). Como validação do modelo, foram demonstrados também os níveis de expressão das proteínas p-ERK1/2, cuja regulação pelo LH já foi descrita em roedores (FAN et al., 2009), STAR, responsável pela esteroidogênese nas células da granulosa, e cujos picos de expressão nos momentos 3 h e 24 h confere com os momentos de síntese de estradiol e progesterona respectivamente (FORTUNE et al., 2009; TONELLOTTO DOS SANTOS et al., 2011) e EGR1, cuja regulação pelo LH já foi descrita em bovinos (SAYASITH et al., 2006).

Como forma de entender prováveis mecanismos secundários a atividade do mTOR, foram conduzidos experimentos *in vitro* com o uso da rapamicina em cultivo de células da granulosa. A atividade do mTOR marcada pela fosforilação da proteína P70S6K, determina um feedback negativo na Phosphoinositide 3-kinase (PI3K), entretanto, na presença da rapamicina esse feedback negativo deixa de existir, ocorrendo ativação da AKT (HUANG & MANNING, 2009). Nossos resultados de abundância proteica revelaram que, além da rapamicina ter induzido um provável aumento nos níveis de p-AKT, rota esta, induzida pela PI3K ativada, foi observado também que a rapamicina não alterou os níveis de p-ERK1/2 e EGR1. Além disso, os resultados demonstraram também que a rapamicina interferiu negativamente no estímulo do LH em induzir a expressão de AREG, EREG e PTGS2.

Embora o mTOR seja ativado em resposta ao estímulo gonadotrófico durante o período pré-ovulatório, seu real envolvimento na ovulação ainda é controverso. Estudos em roedores com modelos de injeção intraperitoneal do inibidor rapamicina obtiveram resultados opostos quanto a inibição ou não da ocorrência da ovulação (YU et al., 2011; SIDDAPPA et al., 2014). O experimento de injeção intrafolicular da rapamicina conduzido neste estudo não inibiu a ovulação, entretanto os dados obtidos *in vitro* nos auxiliam a compreender a existência de uma rota compensatória para a ocorrência da ovulação, na qual o estímulo gonadotrófico mantém a atividade de ERK1/2 e tradução do gene EGR1 em uma rota coordenada pela PI3K-AKT. Esse provável mecanismo está de acordo com dados prévios, os quais enfatizam que a sinalização via PI3K nas células da granulosa funciona sinergicamente com ERK1/2 mediando a ação gonadotrófica no controle do desenvolvimento folicular final até a ovulação (HUNZICKER-DUNN & MAIZELS, 2006; FAN & RICHARDS, 2010).

# 6. CONCLUSÃO

Os dados gerados no primeiro estudo nos possibilitaram desenvolver um modelo para maturação *in vitro* no qual o bloqueio do reinicio da meiose durante 15 h, em condições similares ao ambiente folicular *in vivo*, não compromete a capacidade de desenvolvimento dos oócitos. Através das análises moleculares relatamos que a inibição dos EGFR, durante o período de interação entre o CCO e as células foliculares, impede os mecanismos de expansão das células do cumulus e reinício da meiose oocitária através da regulação nos genes EGR1, TNFAIP6, HAS2, IMPDH1 e CX43 nas células do cumulus e PGR e ADAMTS1 nas células da granulosa. Provavelmente, a ativação de ERK1/2 seja um sinal comum que modula os efeitos da atividade dos EGFR nas células somáticas envolvidas no sistema proposto. Com o nosso modelo sugerimos um mecanismo no qual a inibição dos EGFR determina um bloqueio na fosforilação de ERK1/2 nas células do cumulus, isso determina que as gap junctions se mantenham abertas ocorrendo a entrada de GMPc para dentro do oócito e manutenção do oócito no estádio de vesícula germinativa. Esse sistema de maturação *in vitro* tem potencial para contribuir com as pesquisas aplicada e básica através de estratégias futuras que visam incrementar a capacidade de desenvolvimento de oócitos *in vitro*, bem como estudar mecanismos celulares envolvidos no reinício da meiose em bovinos.

Com relação ao envolvimento do mTOR nos mecanismos iniciais da cascata de ovulação, observamos que, embora o mTOR não seja essencial para a ocorrência da ovulação, sua atividade é induzida em função do estímulo gonadotrófico e está envolvido na expressão de genes regulados pelo LH. O uso da rapamicina *in vivo* e *in vitro* sugerem uma rota alternativa para a ovulação na qual o LH induz a atividade de ERK1/2 em uma via coordenada pela PI3K/AKT independentemente de uma regulação na expressão de RNAm dos genes AREG, EREG e PTGS2. Portanto, o estímulo desses genes não é a única via envolvida na ativação de ERK1/2 e desencadeamento do processo ovulatório em bovinos.

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