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INSTITUTO DE BIOCIÊNCIAS

EFEITOS DOS FATORES SECRETADOS PELO OÓCITO (OSF) SOBRE A DIFERENCIAÇÃO DAS CÉLULAS DO CUMULUS DURANTE A MATURAÇÃO IN VITRO (MIV) EM BOVINOS

PAULA FERNANDA DE LIMA

Tese de doutorado

Botucatu - SP

Novembro de 2016

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Tese de Doutorado Apresentada ao Programa de Pós-Graduação em Farmacologia e Biotecnologia do Instituto de Biociências da Universidade Estadual Paulista-Unesp, para Obtenção do Grau de Doutor em Farmacologia e Biotecnologia.

Orientador: Prof. Dr. José Buratini Junio

Coorientadora: Profa Dra. Fernanda da Cruz Landim

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PAULA FERNADA DE LIMA

Efeitos dos fatores secretados pelo oócito (OSF) sobre a diferenciação das células do cumulus durante a maturação in vitro (MIV) em bovinos

Dissertação apresentada ao Programa de Pós-Graduação em Farmacologia e Biotecnologia do Instituto de Biociências da Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de Botucatu, para obtenção do título de Doutora

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Botucatu, 04 de novembro de 2016

Dedico esse trabalho aos meus pais Maria Isabel e Paulo

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“Descobrir consiste em olhar para o que todo mundo está vendo e pensar uma coisa diferente”.
(Roger Von Oech)

RESUMO

DE LIMA, P.F. Efeitos dos fatores secretados pelo oócito (OSF) sobre a diferenciação das células do cumulus durante a maturação in vitro (MIV) em bovinos. Botucatu, 2016. Tese (Doutorado – Farmacologia e Biotecnologia) – Instituto de Biociências, IB, Universidade Estadual Paulista –UNESP.

O oócito é responsável por regular os mecanismos da maturação do complexo cumulus oócito (COC) principalmente via secreção de fatores. parácrinos.Dentre os fatores secretados pelo oócito (OSF) desta-se o fator de crescimento dos fibroblastos 10 (FGF10), e dois fatores TGFβ mais estudados a proteína morfogenética óssea 15 (BMP15) e fator de crescimento de diferenciação 9 (GDF9) e estão associados com o desenvolvimento da competência oocitária. O presente trabalho investigou a regulação do oócito sobre as células do cumulus em dois cenários atuais da maturação *in vitro*, na presença de hormônio folículo estimulante (FSH) ou ampirregulina (AREG). A ausência do oócito afeta a expansão das células do cumulus e também o consumo de glicose e a produção de lactato e a adição de oócitos desnudos reverte o efeito da oocectomia na expansão mas não no consumo de glicose nem na produção de lactato. A oocectomia afeta também a expressão gênica reduzindo a abundância de RNAm de hialurona sintetase 2 (*HAS2)* e elevando a expressão de prostaglandina sintase 2 (*PTGS2*)*,* pentraxina 3 (*PTX3*) *e* proteína indutora do fator de necrose tumoral 6 (*TNFAIP6*) na presença de FSH e AREG. A adição de oócitos desnudos reverte o efeito da oocectomia sobre a expressão de RNAm de AREG às 4 horas de cultivo, HAS2 e PTGS2 às 22 horas de cultivo apenas quando os complexos foram cultivados na presença de AREG. Adicionalmente foi investigado a ação isolada do FGF10 sobre a diferenciação das células do cumulus. O FGF10 reverte o efeito da ausência do oócito sobre a expansão das células e na expressão de *HAS2* e *GFPT1*apenas às 22 horas no cumulus mas não do consumo de glicose nem da produção de lactato, o que mostra um efeito tardio do FGF10 necessitando da presença de outros OSF para que sua ação seja logo ao inicio de cultivo como visto anteriormente na literatura. Complementar aos resultados é apresentado que as células do cumulus possuem um controle intracumulus de ativação de SMAD2 pela presença de BMP15 e GDF9 nas células do cumulus. Em síntese os fatores secretados pelo oócito regulam as funções das células do cumulus bem como a expressão de RNAm de genes importantes durante a maturação do COC e esses fatores podem regular diferencialmente as células do cumulus na presença de FSH ou AREG.

Palavras Chave: Expansão do cumulus; Fatores EGF-*like*; AREG, FSH

ABSTRACT

DE LIMA, P.F. The effects of oocyte secreted factors (OSF) on the differentiation of cumulus cells during in vitro maturation (IVM) in cattle. Botucatu, 2016. Thesis (PhD – Pharmacology and Biotechnology) – Instituto de Biociências, IB, Universidade Estadual Paulista –UNESP.

The oocyte is responsible for regulating mechanisms of cumulus oocyte complex (COC) maturation mainly through secretion of paracrine factors. Including oocyte secreted factors (OSF) are the fibroblast growth factor 10 (FGF10), and two TGF beta factors most studied, bone morphogenetic protein 15 (BMP15) differentiation and growth factor 9 (GDF9), and they are associated with the oocyte development of competence. This study investigated the regulation of oocyte cumulus cells on two current scenarios in vitro maturation in the presence of follicle stimulating hormone (FSH) or ampirregulina (AREG). The absence of oocyte affects the expansion of the cumulus cells and also the glucose consumption and lactate production and addition of denuded oocytes reverses the effect of the expansion oocectomia but not glucose consumption or lactate production. The oocectomia also affects gene expression by reducing the abundance of mRNA hialurona synthetase 2 HAS2 and raising synthase prostaglandin expression 2 (PTGS2), pentraxin 3 (PTX3) and inducing protein of the tumor necrosis factor 6 (TNFAIP6) in the presence of FSH and AREG. The addition of denuded oocytes reverses the effect of AREG on oocectomia mRNA expression at 4 hours of culture, HAS2 and PTGS2 to 22 hours of cultivation only when complexes were grown in the presence of AREG. Additionally it investigated the isolated FGF10 action on the differentiation of cumulus cells. FGF10 reverses the effect of on the expansion of the cells and the expression of HAS2 and GFPT1apenas to 22 hours in cumulus but not glucose consumption or lactate production, which shows a delayed effect of FGF10 necessitating the presence of other OSF to soon effect as seen previously in the literature. Complementary to the results, it appears that the cumulus cells have an intracumulus control Smad2-activation by the presence of GDF9 and BMP15 in cumulus cells. In summary the oocyte secreted factors regulate the functions of the cumulus cells and the mRNA expression of important genes during maturation of the COC and these factors may differentially regulate cumulus cells in the presence of FSH or AREG

Keywords: cumulus expansion; EGF-like factors; AREG; FSH.

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Lista de Abreviaturas

ADAM = família desintegrina e metaloproteinases
AKT - Proteína quinase B
ALK = receptor ativina semelhante a kinase
AMPc = adenosina monofosfato cíclico
AREG = ampiregulina
BMP15 = proteína morfogênica óssea 15
BMPRII = receptor de BMP do tipo II
BSA = albumina sérica bovina
BTC = betacelulina
COC = complexo cumulus-oócito
DNA = ácido deoxiribonucleico
ECM = matriz extracelular
EGF = fator de crescimento epidermal
EGF-*like* = fatores de crescimento epidermal (EGF)-*like*
EGFR = receptores de EGF
EREG = epiregulina
ERK = quinases reguladas por sinais extracelulares
FGF = fator de crescimento dos fibroblastos
FGF = fatores de crescimento fibroblástico
FSH = hormônio folículo estimulante
GDF9 = fator de crescimento e diferenciação 9
GFPT = L-glicosamina:D-frutose-6-fosfato acetil transferase
GMPc = monofosfato cíclico de guanosina
HAS2 = hialurona sintetase 2
LH = hormônio luteinizante
MIV = maturação *in vitro*
OSF = fatores secretados pelo oócito
PDE3 = fosfodiesterase do tipo 3
PGE2 = prostaglandina E2
PI3K - Fosfatidilinositol-4,5-bisfosfato 3-quinase
PTGS2 = prostaglandina sintetase 2
PTX3 = pentraxina 3
RNA = ácido ribonucléico
RNAm = ácido ribonucléico mensageiro
S.E.M. = error of the mean
SMAD2 = Mothers against decapentaplegic homolog 2
TGFβ = fatores de crescimento transformante β
TNFAIP6 = proteína indutora do fator de necrose tumoral 6
TZP = processos citoplasmáticos trans-zonais

SUMÁRIO

Capítulo 1

“Cumulus cell-oocyte communication is an essential feature of mammalian reproduction.”
(RUSSELL et al., 2016)

1.INTRODUÇÃO

Nos anos 90 foi confirmada a importância da comunicação entre o oócito e as células somáticas adjacentes para formação folicular, regulação das funções das células do cumulus e desenvolvimento da competência oocitária (BUCCIONE et al., 1990; DONG et al., 1996; EPPIG, 2001), o que despertou o interesse da comunidade científica, pois mudou a premissa de que o oócito tinha uma ação passiva durante o desenvolvimento folicular (EPPIG, 2001; SCARAMUZZI et al., 2011)

Desde então inúmeros estudos foram realizados com foco no entendimento da interação entre o oócito e as células da granulosa, levando a um progresso no conhecimento sobre os mecanismos moleculares que regulam essa interação durante a maturação oocitária (LI et al., 2000; CAIXETA et al., 2013; FRANCIOSI et al., 2014; LUCIANO et al., 2014; DE LIMA et al., 2016).

A maturação oocitária é superior em condições fisiológicas em relação a maturação oocitária *in vitro* (RIZOS et al., 2002) e esses estudos são necessários para que ocorra uma melhoria na eficiência da maturação *in vitro* (MIV) que é uma etapa crucial na biotecnologia de reprodução assistida. Compreendendo-se melhor a fisiologia e os mecanismos moleculares da maturação do complexo cumulus oócito (COC), permite que os sistemas de cultivo se assemelhem mais as condições fisiológicas e sejam mais eficientes no aproveitamento do oócito nas biotecnologias de reprodução assistida. Recentemente a utilização de um meio mais fisiológico como o estágio de pré-maturação e a substituição do indutor de maturação hormônio folículo estimulante (FSH) pela proteína ampirregulina (AREG) mostrou-se eficiente em melhorar a qualidade embrionária (ALBUZ et al., 2010; RICHANI et al., 2013).

A interação entre o oócito e as células do cumulus ocorre de forma bidirecional via secreção de fatores parácrinos, junções do tipo *gap* e também via projeções transzonais permitindo ao oócito o desenvolvimento da competência para suportar o desenvolvimento do embrião (THOMAS et al., 2004; HUSSEIN et al., 2006; MACAULAY et al., 2014; RUSSELL et al., 2016).

Os fatores secretados pelo oócito (OSF) são cruciais para direcionar o desenvolvimento do fenótipo das células do cumulus bem como suas funções (DIAZ et al., 2006). Contudo, há diferenças importantes quanto à influência do oócito entre as espécies. O oócito é necessário para a expansão e metabolismo energético do

cumulus em camundongos (VANDERHYDEN, 1993; SUGIURA; EPPIG, 2005), mas não em bovinos (RALPH et al., 1995; SUTTON et al., 2003).

Sendo assim, este trabalho teve como objetivo avaliar a participação e os mecanismos de ação do oócito no controle das funções das células do cumulus bovinas submetidas ao processo de MIV na presença de FSH, método convencional ou na presença de AREG, que simula a condição fisiológica. Foi testada a hipótese de que a ausência do oócito influencia as funções das células do cumulus bovinas independente do estímulo para maturação utilizado. Em adição, foram avaliados os efeitos do fator de crescimento dos fibroblastos 10 (FGF10) isoladamente sobre a expressão dos genes envolvidos na expansão e no metabolismo do cumulus durante a MIV em bovinos.

2.REVISÃO DE LITERATURA

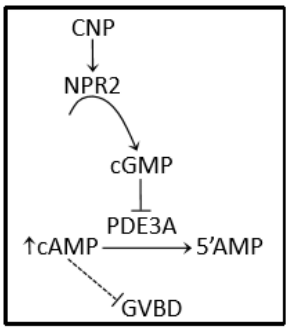
2.1.Maturação do complexo cumulus oócito

A maturação do complexo cumulus oócito compreende inúmeros eventos tanto nas células do cumulus que levam a expansão quanto no oócito que levam a retomada da meiose. Para que esses eventos ocorram de forma coordenada, a comunicação celular entre o oócito, células do cumulus e células da granulosa mural deve ser precisa, pois qualquer distúrbio pode comprometer a aquisição da competência ao desenvolvimento.

2.1.1.Controle da retomada da meiose em oócitos

Em mamíferos sabe-se que os nucleotídeis cíclicos, monofosfato cíclico de quanosina (GMPc) e adenosina monofosfato cíclica (AMPc), controlam a maturação meiótica, primeiramente descrito em camundongos (CHO et al., 1974). A produção de GMPc é dependente da sinalização entre o pepitideo natriurético do tipo C (NPPC) e o seu receptor receptor do pepitideo natriurético 2 (NPR2), o monofosfato cíclico de guanosina (GMPc) impede a ação da fosfodiesterase 3 (PDE3), que degrada o AMPc, dessa forma o AMPc se mantém elevado e não ocorre a retomada da meiose (Figura 1) mas uma vez que essa sinalização é interrompida ocorre a redução do AMPc no oócito e consequente retomada da meiose (GILCHRIST et al., 2016).

Figura 1 - Modelo de coordenação de GMPc e AMPc no controle da parada meiótica. CNP – peptídeo natriurético; NPR2 – receptor peptídeo natriurético 2; GVBD – quebra da vesícula germinativa



Fonte: Adaptado de Gilchrist et al. (2016)

O pico de hormônio luteinizante (LH) leva a uma queda nos níveis de RNAm de *NPPC*, na ausência do NPPC não ocorre sinalização via seu receptor NPR2, resultando na diminuição do GMPc dentro das células do cumulus e não sendo transferido para o oócito em camundongos (LIU, X. et al., 2014). Além disso a sinalização fator de crescimento epidermal (EGF) desencadeada pelo LH gera uma elevação nos níveis de cálcio que desestabiliza o acoplamento do NPPC com o seu receptor reduzindo a sinalização NPR2 consequentemente a produção de GMPc (HAO et al., 2016). No oócito a falta de GMPc eleva os níveis de fosfodiesterase 3 A (PDE3A) que degrada o AMPc, a redução nos níveis de AMPc no oócito permite a retomada da meiose (ZHANG, M et al., 2005, 2010; ZHANG, W et al., 2014).

A retomada da meiose estimulada pelo FSH via MAPK3/1 ainda é controversa em suínos, pois Zhang H. et al., (2005) propõe que o hormônio folículo estimulante (FSH) reduz a ação do NPPC, mas segundo Blaha et al., (2015) o FSH estimula a retomada da meiose, por fosforilar as conexinas e fechar as junções do tipo gap impedindo o transporte de GMPc do cumulus para o oócito uma vez que a adição de GMPc não impede a expansão do cumulus nem a expressão de genes responsáveis pela expansão como ácido hialurônico sintase 2 (HAS2), fator de necrose tumoral induzida proteína 6 (TNFAIP6) e prostaglandina sintase 2 (PTGS2) na presença de FSH em COCs de suínos (RICHANI et al., 2013; BLAHA et al., 2015).

Embora tanto o FSH quanto fatores de crescimento semelhantes ao EGF (EGF-*like*) tenham resultados similares sobre a maturação do COC *in vitro* existe uma diferença na sinalização, pois para FSH estimular a expansão das células do cumulus e maturação do COC é necessária a ativação da proteína 14 ativada por mitógenos (MAPK14) ao contrário da AREG que ativa quinase ativada por sinal extracelular (ERK1/2 ou MAPK3/1) e estimula a expansão sem precisar ativar a via MAPK14 em camundongos (LIU, Z. et al., 2010) em suínos a ativação da MAPK14 via AREG é importante para a expansão mas parcialmente para a progressão da meiose em oócitos (PROCHÁZKA et al., 2012). A AREG não mimifica a ação do FSH e gera uma maior competência oocitária (PROCHÁZKA et al., 2011).

Considerando isso e as ações do LH, receptor de EGF (EGFR) e FSH sobre a regulação no NPPC e da expansão das células do cumulus, nos faz pensar que a utilização da indução *in vitro* da maturação do COC via ativação EGFR se assemelhe mais ao que ocorre *in vivo* onde a indução é realizada via LH, além disso a utilização

de AREG ou EREG na MIV aumenta o número de blastocistos em comparação com FSH (RICHANI et al., 2013).

2.1.2.Controle da expansão das células do cumulus

A ovulação e maturação do COC em mamíferos é desencadeada pelo pico de LH que se liga ao seu receptor (LHR) expresso predominantemente nas células da granulosa e nulo ou raro nas células do cumulus. (PENG et al., 1991; VAN TOL et al., 1996; RiCHARDS et al., 2002; PROCHÁZKA et al., 2011; SUGIMURA et al., 2015;).

O modelo *in vitro* de maturação do COC não permite que o estímulo seja realizado via LH pela ausência de células da granulosa mural, dessa forma o estímulo para a expansão das células do cumulus e maturação do oócito ocorre via FSH ou via EGF-*like* diretamente. Uma vez que o estímulo das gonadotrofinas sobre as células da granulosa e do cumulus ativa as proteínas intracelulares proteína quinase dependente de AMPc (PKA), MAPK14 e ERK1/2 que resulta na expressão de potentes fatores de crescimento conhecidos como fatores EGF-*like* que atuam de forma autócrina e parácrina. Os fatores EGF-*like* são a AREG; a epirregulina (EREG) e a betacelulina (BTC) sendo as duas primeiras mais importantes no bovino (PARK et al., 2004; YAMASHITA et al., 2009; PROCHÁZKA et al., 2012; CAIXETA et al., 2013a).

O FSH além de estimular a expressão dos EGF-*like* também estimula nas células do cumulus a aquisição da competência em responder ao EGF/EGFR, pois COCs de folículos <4 mm expandem apenas na presença de FSH e não de EGF (PROCHÁZKA et al., 2003, 2012; RITTER et al., 2015). Embora a expressão de RNAm para EGFR em células do cumulus de folículos pequenos (<4 mm) não seja diferente das de um folículo antral médio (> 4 mm) a quantidade de proteína é maior no antral medio que é responsivo ao EGF (RITTER et al., 2015).

A ação dos EGF-*like* é dependente das desintegrinas e metaloproteinases (ADAMs), dentre elas se destacam a ADAM metalopeptidase domínio 10 (ADAM10) e a ADAM metalopeptidase domínio 17 (ADAM17) que clivam da membrana celular os EGF-*like* na sua unidade catalítica permitindo a sua liberação da membrana celular para se ligar ao seu receptor EGFR, fosforilar e então desencadear a sinalização EGFR (SAHIN et al., 2004; YAMASHITA; SHIMADA, 2012).

A sinalização EGFR é de extrema importância para a maturação do COC e desenvolvimento da competência oocitária (CONTI et al., 2016). O receptor EGFR é

do tipo tirosina quinase e ativa as proteínas intracelulares, MAPK3/1 (também referido como ERK1/2) e fosfatidilinositol-4,5-bisfosfato 3-quinase/proteína quinase B (PI3K/AKT) e em suínos também ativa MAPK14 (PARK et al., 2004; PROCHÁZKA et al., 2012; CHEN et al., 2013). A sinalização EGFR permite a expressão dos genes relacionados com a expansão do cumulus prostaglandina sintase 2 (PTGS2), hialurona sintase 2 (HAS2), fator de necrose tumoral 6 (TNFAIP6), pentraxina 3 (PTX3) (RUSSELL; ROBKER, 2007; PROCHÁZKA et al., 2012). As proteínas traduzidas por esses genes interagem no meio extracelular para que ocorra formação da matriz extracelular das células do cumulus e a consequente expansão do cumulus (Figura 2).

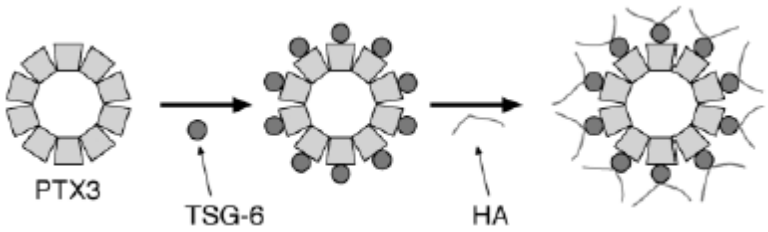
O principal componente da matriz extracelular é o ácido hialurônico, produto do gene HAS2 que é estimulado pela ação do FSH e pela presença do oócito e é de extrema importância para o desenvolvimento embrionário em bovinos (KIMURA et al., 2002; TUNJUNG et al. 2009; MAREI et al., 2013).

O ácido hialurônico se liga ao seu receptor CD44 e desencadeia a sinalização MAPK3/1 levando a fosforilação das conexinas e consequentemente fechamento das *gap* junctions e reduz a apoptose em células da granulosa (TUNJUNG et al., 2009; YOKOO et al., 2010). Em bovinos a inibição da sinalização CD44 perturba o desenvolvimento do embrião (MAREI et al., 2013) em suínos a interação insuficiente entre ácido hialurônico e CD44 durante a MIV gera oócitos com baixa competência ao desenvolvimento (YOKOO et al., 2007). Em fibroblastos foi demonstrado que o ácido hialurônico ao se ligar ao CD44 permite que este se associe ao EGFR facilitando a sinalização MAPK3/1 a inibição do CD44 reduz a fosforilação de MAPK3/1 mas não impede a produção de ácido hialurônico, ou seja a resposta é diferente da sinalização EGF/EGFR (Fig. 3; SIMPSON et al., 2010).

A proteína TSG6 codificada pelo gene TNFAIP6 também participa na formação e estruturação da matriz extracelular. Basicamente a TSG6 se une ao ácido hialurônico e também a proteína PTX3 para estabilizar a matriz extracelular sem essa interação não há formação correta da matriz extracelular causando alterações na expansão das células do cumulus (SALUSTRI et al., 2004). Além disso foi demonstrado em linhagens de células AKR1 (linfócitos) que a TSG6 pode regular a interação do ácido hialurônico com o seu receptor CD44, a TSG6 se liga ao ácido hialurônico competindo pela ligação a seu receptor e a relação de concentração entre

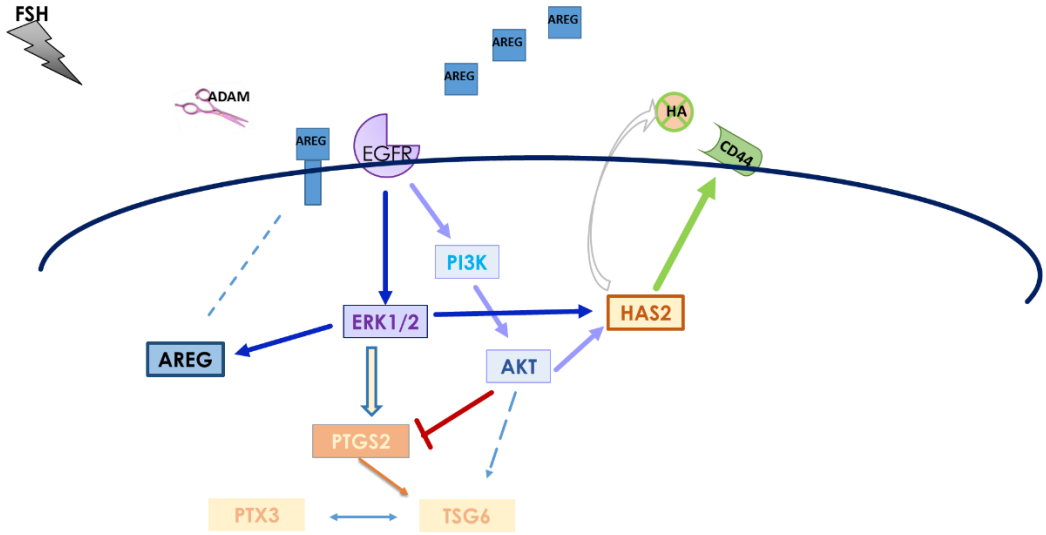
TSG6 e ácido hialurônico pode estimular ou inibir a interação ácido hialurônico/CD44 (LESLEY et al., 2004).

Figura 2 - Modelo esquemático da formação da matriz extracelular formada pela ligação entre PTX3 e TSG6 que se estabilizam e formam um ponto de ancoragem para as cadeias de HA.



Fonte: Adaptado de Salustri et al. (2004)

Figura 3 – Modelo esquemático da sinalização EGFR sobre as células do cumulus na indução da maturação do complexo cumulus oócito com FSH. Após estímulo pelo FSH a proteína ADAM cliva a AREG da membrana celular permitindo que ligue ao seu receptor, desencadeando a cascata de sinalização intracelular da ERK1/2 e PI3K/AKT estimulando a produção de PTGS2, TSG6, PTX3, HAS2 e ácido hialurônico.



2.2.Interação cumulus oócito

A comunicação bidirecional entre o oócito e as células somáticas e gera um microambiente favorável ao desenvolvimento da competência oocitária, que é definido

pela habilidade em completar a meiose, ser fertilizado e progredir na embriogênese (COTICCHIO et al., 2014; DUMESIC et al., 2015).

O efeito do oócito sobre as células do cumulus inclui prevenir contra apoptose, regular a expressão de kit ligante, um dos fatores envolvidos na regulação da maturação nuclear, além de regular a expansão (GILCHRIST et al., 2008; ASSIDI et al., 2010; DUMESIC et al., 2015 De LIMA et al., 2016).

A comunicação entre o oócito e as células adjacentes pode ser via secreção de fatores parácrinos, via junções do tipo gap e também por projeções citoplasmáticas trans-zonais (TZP), que são extensões das células adjacentes que penetram na zona pelúcida e atingem o oócito, permitindo o intercâmbio de moléculas através das junções do tipo gap (ALBERTINI et al., 2001). A presença de mRNA nas TZP e sua transferência das células do cumulus para o oócito, o que indica uma regulação no estoque de mRNA e a síntese proteica do oócito após a queda da sua atividade transcricional (MACAULAY et al., 2004).

As junções do tipo gap são compostas por seis unidades idênticas (hexamêros de proteínas pertencentes à família das conexinas), que se arranjam formando um túnel comunicante com um poro central (SANTIQUET et al., 2012). Isso permite a passagem de moléculas pequenas e hidrofílicas como o AMPc, o GMPc e o piruvato, que são fundamentais para o crescimento, metabolismo e controle da maturação do oócito. O oócito não consegue utilizar a glicose diretamente como fonte energética, necessitando das células do cumulus que realizam a glicólise e fornecem piruvato ao oócito (LODDE et al., 2007; GILCHRIST et al., 2008). Portanto, a comunicação entre o oócito e as CC é bidirecional e essencial para ambos os tipos celulares (EPPIG 2001; LODDE et al., 2007).

O oócito é dependente da fosforilação oxidativa da adenosina tri-fosfato (ATP) e tem baixa capacidade de oxidar a glicose. Já as CC tem uma significante habilidade em captar e utilizar a glicose (CETICA et al., 2002; SUTTON-MCDOWALL et al., 2004; GILCHRIST et al., 2008). O metabolismo da glicose dentro das CC fornece substrato para fosforilação oxidativa dentro do oócito (SUTTON et al., 2003; GILCHRIST et al., 2008).

2.2.1.Fatores secretados pelo oócito

Os fatores secretados pelo oócito (OSF) são membros da família dos fatores de crescimento transformante β (TGFβ) e os membros da família dos fatores de crescimento dos fibroblastos (FGF) que se ligam em seus receptores presentes nas células do cumulus desencadeando uma cascata de sinalização (SUGIURA et al., 2007; GILCHRIST et al., 2008; CAIXETA et al., 2013b; MACHADO et al., 2015), que direciona tanto o desenvolvimento do fenótipo das células do cumulus competentes à expansão bem como a expansão em si (DIAZ et al., 2006).

Os FGFs podem interferir nas funções das células do cumulus, o FGF8 juntamente com a proteína morfogenética óssea 15 (BMP15) estimulam a glicólise em camundongos, já o FGF10 estimula a expansão das células do cumulus (ZHANG et al., 2010; CAIXETA et al., 2013).

Os membros da família dos TGFβ são os mais estudados, incluindo a BMP15 e o fator de crescimento e diferenciação 9 (GDF9; SUGIURA et al., 2007; GILCHRIST et al., 2008; CAIXETA et al., 2013b; MACHADO et al., 2015;).

Em fêmeas de suínos, bovinos, ovinos, ratos, camundongos e veados GDF9 e BMP15 são fatores de crescimento específicos do oócito (CRAWFORD; MCNATTY, 2012) além disso existem vários relatos na literatura onde em camundongos e ratos o GDF9 e a BMP15 são exclusivos de oócito, uma vez que na ausência do oócito a fosforilação da SMAD2 (do inglês, *Mothers against decapentaplegic homolog 2*), proteína intracelular que participa da ativação da sinalização TGFβ, nas células do cumulus é inibida SU et al., 2010 (HAYASHI et al., 1999; ERICKSON; SHIMASAKI, 2003;) embora segundo Hosoe et al. (2011) as células do cumulus de bovinos apresentam expressão tanto de RNAm quanto proteica para GDF9 e BMP15, que também foram descritos em humanos, ovelhas e suínos (PROCHAZKA et al., 2004; SILVA et al., 2005; ASSOU et al., 2006)

A inibição da via SMAD2/3 por seu inibidor específico SB-431542 durante a MIV em camundongos (YEO et al., 2009) e suínos (GILCHRIST; RITTER, 2011; NAGYOVA et al., 2011) resulta na redução da expansão das células do cumulus, uma vez que a sinalização SMAD2/3 está envolvida na produção de ácido hialurônico (ZHANG, H. et al., 2016). Em adição a via de sinalização SMAD2/3 está envolvida na sinalização EGFR nas células do cumulus de camundongos, indicando que a resposta

ovariana ao LH e a maturação oocitária podem ser reguladas pela via SMAD2/3 (SU et al., 2010).

A sinalização TGFβ ativa SMAD2/3 e estimula tanto a expressão de EGF como a produção de ácido hialurônico, bem como a interação ácido hialurônico/CD44/EGFR (SIMPSON et al., 2010; MERAN et al., 2011). A sinalização tardia de MAPK3/1 é importante para que permita a proliferação celular induzida pelos TGFB, uma vez que MAPK pode alterar a fosforilação da SMAD, regulando a capacidade da SMAD se translocar para o núcleo (ENGEL et al., 1999; FUNABA; MURAKAMI, 2008).

Em camundongos a ativação SMAD2/3 pelos OSF juntamente com a ativação EGFR é essencial para induzir a expansão das células do cumulus. Em suínos as células do cumulus expandem sem a presença do oócito mas a inibição da SMAD2/3 impede a síntese das proteínas que formam a matriz estra celular e a participação da MAPK sobre SMAD em suínos ainda não é totalmente compreendida (GILCHIRST; RITTER, 2011; NAGYOVA et al., 2011).

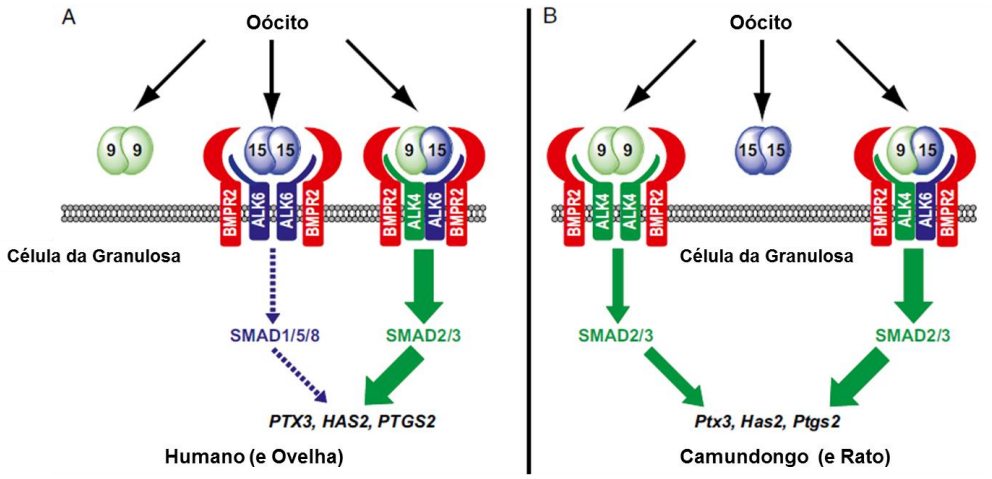
Além disso, ainda reforçando a ação do oócito sobre a sinalização EGF, na presença de oócitos desnudos de folículos maiores 4 mm a sinalização EGFR é estimulada e permite a expansão das células do cumulus de COCs de folículos menores de 4 mm (RITTER et al., 2015), adicionalmente a combinação de AMPc, proteína morfogenética 15 (BMP15) e fator de crescimento e diferenciação 9 (GDF9) estimula a sinalização AREG-EGFR e melhora a competência oocitária em COCs com baixa competência e pouco responsivos ao EGF e EGF-*like,* derivados de folículos antrais pequenos (SUGIMURA et al., 2015)*,* ou seja as células do cumulus de folículos menores respondem aos OSF mas não ao estímulo EGF/EGF-*like* (RITTER et al., 2015).

A sinalização TGFβ é complexa, pois existe a possibilidade da formação de homo e heterodímeros entre GDF9 e BMP15 e dessa forma ativar diferentemente os seus receptores principais que são as proteína quinase do tipo aurora 4 e 6 (ALK4), (ALK6) e também o receptor da proteína morfogenética tipo 2 (BMPRII) (PENG et al., 2013; MOTTERSHEAD et al., 2015).

O homodímero de BMP15 ativa o heterodímero de receptores BMPRII e ALK6 em humanos e ovinos, o homodímero de GDF9 ativa o heterodímero de ALK4 e BMPRII em roedores. O heterodímero de BMP15 e GDF9 ativa heterodímero de ALK4, ALK6 e BMPRII em mamíferos. Excetuando-se o homodímero de BMP15, todas as outras combinações ativam a via SMAD2 que estimula a expressão de genes

que são importantes para formação da matriz extracelular das células do cumulus durante a expansão (PENG et al., 2013).

Figura 4 - Modelo para homo e heterodímeros de BMP15 e GDF9 na regulação das funções das células da granulosa em humanos e ovelhas (A) e em camundongos e ratos (B).



Fonte: Adaptado de Peng et al., (2013)

Ainda, mais recentemente o heterodímero de GDF9 e BMP15 foi nomeado de cumulina, sendo a união das partes maturas de ambas proteínas, que ativa receptor ALK4, ALK6 e BMPRII sinalizando preferencialmente via SMAD2 e estimulando a expressão dos genes envolvidos na expansão do cumulus (MOTTERSHEAD et al., 2015).

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Capítulo 2

"The current IVM techniques yield oocytes with low developmental competence as compared to their counterparts matured in vivo. The molecular mechanisms underlying this insufficiency are largely unknown."
(BLAHA et al., 2015)

Artigo preparado para ser submetido para publicação na revista Biology of Reproduction

O artigo está apresentado de acordo com as normas da revista exetuando-se a numeração das linhas

Título: Addition of oocyte can differentially regulate cumulus cells functions in the presence of FSH or AREG

TITLE: Oocytes can differentially regulate cumulus cell function in the presence of FSH or AREG

Short Title: Cumulus cells function: regulation by oocyte

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ABSTRACT

Cumulus-oocyte complex (COC) *in vitro* maturation is an assissted reproductive technology that is not eficiente in cattle, thus there is great interest in the development of new approaches to improve maturation and fertilization rates and this involves knowlege of COC physiology to develop oocyte competence. In *in vitro* maturation FSH or AREG can be used as inductor of COC maturation but they activate different pathways in cumulus cells. Thus to investigate the influence of oocyte on cumulus cells under different maturation inductors the oocytectomy model were used. We demosntrated that cumulus expansion and glucose metabolism decreases only under FSH stimulus but not under AREG stimulus. The oocytectomy also influence the mRNA levels of genes related to cumulus expansion, we demonstrate that OOX reduced abundance of *HAS2* mRNA and increased *PTGS2, PTX3* and *TNFAIP6* mRNA levels in cumulus cells and the addition of denuded oocytes restored expansion but did not alter levels of *HAS2* and *ADAM10* mRNA at 4 hours. Although, addition of denuded oocytes restored *CD44, PTX6 and TNFAIP6* mRNA levels to those of intact COC controls at 22 h IVM, and of *HAS2* and *PTGS2* mRNA under AREG but not FSH stimulation. Thus the results shown that oocyte influence differentely cumulus expansion, glucose metabolism and gene expression

1. INTRODUCTION

The development of oocyte competence is dependent on the microenvironment created by the bidirectional communication between cumulus cells and the oocyte. The oocyte is the lead actor and cumulus cells are the supporting actor of the cumulus-oocyte maturation process. Cumulus cells provide nutritional support and contribute to resumption of meiosis of the oocyte, and the oocyte controls aspects of cumulus function including LH surge-induced expansion and gene expression. The impact of the oocyte on cumulus cell function differs between species, as the oocyte is necessary for cumulus expansion and metabolism in mice [1, 2] but not in cattle [3, 4].

In mice, the oocyte acts at least in part through the secretion of oocyte-specific factors (OSF) including bone morphogenetic protein 15 (BMP15) and growth differentiation factor 9 (GDF9) that bind to receptors on cumulus cells and activate SMAD2/3 and SMAD1/5/8 intracellular pathways. This in turn that leads to increased expression of genes involved in cumulus cells expansion, including CD44, pentraxin 3 (PTX3), hyaluronan synthase 2 (HAS2), tumor necrosis factor alpha induced protein 6 (TNFAIP6) and prostaglandin-endoperoxide synthase 2 (PTGS2) [5-8] [9, 10]. In addition, SMAD signaling can influence EGF expression in fibroblasts and the production of hyaluronic acid (HA), as well as the HA/CD44/EGFR interaction [10, 11]. The inhibition of SMAD signaling reduced cumulus expansion in mice as well as in pigs [12-14].

Two major ligands are used in in-vitro maturation (IVM) to induce expansion and resumption of meiosis: FSH and amphiregulin (AREG) [15, 16]. FSH activates mitogen-activated protein kinase pathways, particularly MAPK3/1 and MAPK14, stimulating the expression of AREG, epiregulin (EREG) and beta-cellulin (BTC) in

cumulus cells [15, 17, 18]. These growth factors are synthesized as transmembrane proteins that require cleavage of the bioactive extracellular domain by proteases, particularly members of the 'a disintegrin and metalloproteinase domain' (ADAM) family; the extracellular domain then binds to their receptor, EGFR, and activates MAPK signaling [19].

EGFR signaling is crucial to cumulus oocyte complex (COC) maturation and the development of oocyte competence [20]. EGFR on cumulus cells activates MAPK3/1, MAPK14 and PI3K/AKT pathways [21-23] leading to phosphorylation of connexins, and the expression of genes involved in the cumulus cell expansion (HAS2, TNFAIP6, PTGS2 and PTX3 [15, 23-25]. Although FSH and AREG can be used to induce COC maturation *in vitro,* AREG has been shown to result in improved oocyte quality and better embryo development compared with FSH [24, 26].

The role of the oocyte cumulus function in cattle is unclear. Numerous studies have shown that the oocyte is not necessary for cumulus glycolysis or expansion [3, 4], although the oocyte may enhance expansion (refs). We have recently shown that the oocyte is as important for the expression of cumulus cell Kit-ligand mRNA levels in cattle [27]. It is possible that the received notion that the bovine oocyte does not regulate cumulus function is a function of the IVM conditions used. The present study was designed to assess the effect of FSH and of AREG on cumulus-oocyte interactions during IVM in cattle.

2. MATERIALS AND METHODS

Unless specified, all chemicals and reagents were purchased from Sigma (St. Louis, MO, USA).

2.1. *In vitro* maturation

Ovaries of adult cows (predominantly Nellore, *Bos indicus*) were obtained at an abattoir local to the Sao Paulo State University campus in Botucatu and transported to the laboratory in saline solution (0.9% NaCl) at 37°C. COCs were aspirated from 3 to 8 mm diameter follicles with an 18 gauge needle and pooled in a 15 ml conical tube. After sedimentation, COCs were recovered and selected using a stereomicroscope.

Only COCs with homogeneous cytoplasm and compact multilayer of cumulus cells were used (Grade 1 and 2). COCs were washed and transferred in groups of 20 into 24 well plates in TCM199 containing Earle's salts supplemented with 1 µg/ml porcine FSH (equivalent to 1.6 mU/ml; Folltropin-V® Bioniche Animal Health, Belleville ON, Canada) or recombinant human AREG at 10 and 100 ng/ml (R&D Systems), 22 µg/ml sodium pyruvate, 75 µg/ml amicacin, 4 mg/ml BSA. COCs and and incubated at 38.5 °C in 5% CO_2 in humidified air.

2.2. Oocytectomy

Cumulus-oocyte complexes were placed in 200 µl drops of TCM199 partially covered with mineral oil and the cytoplasm of the oocytes was removed with a micromanipulator as described [28] with modifications [29]. The resulting oocyte-free cumulus complexes (OOX) were cultured as for COCs. To produce denuded oocytes, 100 imature COCs of grade 1 and 2 were denuded mechanically by successive pipetting.

2.3 Experimental design

To assess the effects of the oocyte on FSH- or AREG-stimulated cumulus cells, COCs and OOXs were cultured with FSH or AREG (10, 100 ng/ml) for 22 h in

400 µl of medium. At the end of culture, cumulus expansion was assessed visually, and total RNA was extracted from cumulus cells. Glucose uptake and lactate production were measured in the spent medium.

The role of oocyte-specific secreted factors in cumulus expansion and glycolysis was then assessed by incubating COC and OOX with FSH (1µg/ml) or AREG (100 ng/ml) in 100 µl medium, with the addition of an OOX group cocultured with denuded oocytes (1DO/µl; OOX+DO).

The effect of oocyte-specific secreted factors on cumulus cell gene expression was tested by culturing COC, OOX and OOX+DO groups with FSH or AREG for 4 h (for AREG, EGFR, ADAM10, PTGS2) or 22 h (for HAS2, PTX3, TNFAIP6, CD44); these times were selected based on our previous studies with bovine IVM [30].

2.4 Assessment of cumulus expansion

Cumulus expansion was visually assessed at 22 hours of culture according to a subjective scoring system. Grades 1 to 3 were attributed to increasing degrees of expansion (1, poor expansion, characterized by few morphological changes compared with before maturation; 2, partial expansion, characterized by fair expansion but notable clusters lacking expansion; 3, complete or nearly complete expansion [8]).

2.5. Glucose uptake and lactate production

Glucose and lactate concentrations were determined in spent media (including media that were not used for IVM) after 22 h culture with FSH or AREG (10 and 100 ng/ml). At the end of culture, spent media were collected, frozen and stored at – 80°C. Glucose and lactate levels were measured using a Hitachi 912 chemical analyzer (F. Hoffmann-La Roche Ltd.). To determine glucose uptake, the measured glucose

concentration was subtracted from the concentration of glucose in media blanks (drops of media, cultured without cells). The base medium (TCM199) did not contain lactate. Glucose uptake and lactate production were expressed as pmol/COC per h [31, 32].

2.6. Gene expression analysis

After culture, COCs were mechanically separated by repeated pipetting in PBS without calcium and magnesium. Cumulus cells from COC, OOX and OOX+DO groups were transferred to 1.5 ml tubes, collected by centrifugation for 5 min at 700g and frozen at -80 °C in 350 µl of RNA extraction lysis buffer from the RNeasy® kit (Qiagen, Mississauga, ON, Canada). Total RNA was extracted using the RNeasy® kit as recommended by the manufacturer. After purification, RNA samples were eluted in 30 µl of RNAse free water. Total RNA concentrations were measured by spectrophotometry using a NanoDrop ND® 1000 (Thermo Scientific, Wilmington, DE, USA). Total RNA (100 ng/reaction for cumulus cells and 9µl of the RNA sample for oocytes) was incubated with DNAse I (1 U/µg; Invitrogen, São Paulo, Brazil) and then reverse transcribed using Oligo-dT primers and Omniscript for cumulus cells.

The reagents were incubated at 37 ºC for 60 min and then at 93 ºC for 3 min for enzyme inactivation. Relative real time RT-PCR analysis was performed with an ABI 7500 thermocycler using Power Sybr Green PCR Master Mix (Applied Biosystems, São Paulo, Brazil). The final volume of the PCR mix was 25 µl and thermocycling conditions were: 95 °C for 10 min (1 cycle), denaturing at 95 °C for 10 sec followed by annealing for 1 min (40 cycles). Primers for the housekeeping gene *CYCA* and for target genes *PTGS2, HAS2, PTX3, CD44, ADAM10, EGFR* and *AREG* were as previously used and validated [7, 30].

The relative expression values for each gene were calculated using the ΔΔCt method with efficiency correction and using one control sample as calibrator [33]. All information of primers is presented in table 1.

2.7. Statistical Analysis

Maturation and cumulus expansion data were arcsine transformed before analysis, and gene expression data were transformed to logarithms when not normally distributed. The effects of treatments with growth factors on cumulus cell expansion, maturation and gene expression were tested by analysis of variance (ANOVA), and means were compared with the Tukey-Kramer HSD test using JMP software (SAS Institute, Cary, NC, USA). Differences were considered significant when $P \leq 0.05$.

3. RESULTS

3.1. Effect of the oocyte on expansion, glucose uptake and lactate production of cumulus cells in the presence of FSH or AREG

The rate of cumulus expansion in COCs was similar under FSH and AREG (100 ng/ml) stimulation, although the lower dose of AREG (10 ng/ml) resulted in lower rates of expansion (Fig. 1). Both doses of AREG tested were as effective as FSH in stimulating glycolysis.

Removal of the oocyte significantly reduced cumulus expansion, glucose uptake and lactate production when cultured with FSH, however none of these parameters was affected in the presence of AREG (Fig. 1).

3.2. Effect of the oocyte on *TNFAIP6, PTGS2, PTX3* and *HAS2* mRNA expression in cumulus cells in the presence of FSH or AREG

Abundance of mRNA encoding *PTGS2* did not differ under FSH or AREG but *HAS2 and TNFAIP6* were higher under FSH compared with AREG stimulation, whereas conversely, *PTX3* mRNA levels were higher under AREG compared with FSH stimulation (Fig. 2).

Removal of the oocyte significantly reduced cumulus cell *HAS2* mRNA levels, and increased abundance of *TNFAIP6* and *PTGS2* mRNA in FSH- and AREG-stimulated groups (Fig 2). Oocytectomy increased *PTX3* mRNA levels under FSH stimulation but not under AREG stimulation. (Fig. 2).

3.3. Effects of oocyte secreted factors on expansion, glucose metabolism and gene expression of cumulus cells

As removal of the oocyte affected cumulus expansion and glycolysis under FSH stimulation, we assessed whether this was a result of the loss of oocyte secreted factors by adding back denuded oocytes to OOX cumulus cells. As above, oocytectomy reduced expansion, glucose uptake and lactate production, and addition of denuded oocytes reversed the effect of expansion (Fig. 3). Addition of denuded oocytes did not reverse the effects of OOX on glycolysis. Under AREG stimulation, neither OOX nor the addition of denuded oocytes to OOX cumulus cells affected cumulus expansion or glycolysis.

3.4. Effects of oocyte secreted factors on abundance of mRNA of genes involved in the preovulatory cascade.

As putative oocyte secreted factors impacted expansion of the cumulus under FSH stimulation, we sought to determine where in the preovulatory cascade of gene expression the oocyte might act. After 4 h of IVM, oocytectomy significantly reduced the relative mRNA abundance of *EGFR*, *ADAM10* and *HAS2*. The addition of denuded oocytes did not restore these mRNA levels to those seen in intact COC (Fig. 4).

The effects of removal of the oocyte and replacement with denuded oocytes under AREG stimulation were very similar to those observed under FSH stimulation, although the response of AREG was different; OOX increased AREG-stimulated AREG mRNA levels, and addition of denuded oocytes reduced AREG mRNA levels to those seen in controls (Fig. 4).

By 22 h of IVM, oocytectomy decreased *HAS2* and *CD44* mRNA levels in FSH-induced IVM, and increased *PTX3*, *PTSG2* and *TNFAIP6* mRNA levels. Addition of denuded oocytes restored *CD44, TNFAIP6* and *PTX3* mRNA abundance to levels not different from intact COC, but did not restore *HAS2 and PTGS2* mRNA levels (Fig. 5).

The effects of OOX and OOX+DO under AREG-stimulation were very similar to those observed under FSH-stimulation, except that addition of denuded oocytes restored levels of all mRNA measured to those not different from COCs.

4. DISCUSSION

Although it is widely ackowledged that the oocyte secretes factors essential for cumulus function and expansion in mice, the current dogma is that in cattle, the oocyte has little influence on cumulus cells. The data reported in this paper refute this tenet, and demonstrate that the bovine oocyte regulates cumulus cell levels of mRNA encoding proteins critical to the preovulatory cascade, including HAS2 and TNFAIP6.

In the mouse, the oocyte is essential for cumulus expansion and expression of *Has2, Ptx3* and *Tnfaip6* mRNA [9, 34], whereas it is widely considered that in the cow expansion proceeds in the absence of the oocyte [28, 35]. However, recent evidence has suggested that removal of the oocyte can impact gene expression in cattle: OOX increased cumulus *KITL* mRNA levels and decreased *NPPC* mRNA levels [27]. We explored this further in the present study and demonstrate that OOX reduced abundance of *HAS2* mRNA and increased *PTGS2, PTX3* and *TNFAIP6* mRNA levels in cumulus cells. This response is different from that in the mouse, in which *Ptgs2, Ptx3* and *Tnfaip6* as well as *Has2* mRNA levels are suppressed after OOX. We also measured the expression of CD44, which is a hyaluronan receptor and regulates gap junction communication between cumulus cells [36, 37] and which increases during IVM in cattle [38]. In the present study, OOX reduced the abundance of CD44 mRNA.

The increase in *PTGS2, PTX3* and *TNFAIP6* mRNA levels after OOX are consistent with the increase in *AREG* mRNA, and suggests that the EGFR-activated signaling pathway was upregulated by the removal of the oocyte. The decrease in *HAS2* mRNA is inconsistent with this, as the *HAS2* gene is under the same control by MAPK as is *PTGS2*, at least in mice [39]. However, the potential action of CD44 may explain this discrepancy. It is known that hyaluronan increases *CD44* mRNA and protein in granulosa cells [40, 41] and in porcine granulosa cells [42], and that CD44 inhibits *PTGS2* expression in fibroblasts and macrophages [43, 44] but does not alter *Has2* mRNA levels [11]. We therefore propose that removal of the oocyte decreases *HAS2* mRNA levels and HA production, leading to a reduction in CD44 levels. This in turn would allow increased *PTGS2* expression and PG secretion, explaining the increase in *AREG* mRNA levels and the increased *TNFAIP6* mRNA level, as these

genes are PG-responsive [45]. High PTX3 mRNA levels may be maintained by MAPK3/1 activity [23] as a result of increased AREG activation of the EGFR.

Addition of denuded oocytes restores cumulus function in mice [9], and partly restored cumulus gene expression in the present study. Addition of denuded oocytes to OOX cumulus cells did not alter levels of *HAS2* and *ADAM10* mRNA, key genes that are early responders to FSH/AREG [30], although denuded oocytes could restore *AREG* mRNA levels after AREG but not FSH stimulation. This discrepancy is possibly linked to stronger or earlier signaling through MAPK by AREG relative to FSH [23]. For those genes that change at later time points during IVM [30], addition of denuded oocytes restored *CD44, PTX6 and TNFAIP6* mRNA levels to those of intact COC controls at 22 h IVM, and of *HAS2* and *PTGS2* mRNA under AREG but not FSH stimulation. Thus these data suggest that OSFs play a role in modulating some functions of cumulus cells in cattle.

Although the identity of the OSF responsible for altering bovine cumulus function is(are) unknown, we can speculate that it (they) act at least in part by controling hyaluronan and/or CD44 expression or activity. To expand our model developed above, removal of the oocyte reduces HA-CD44 interaction that in turn relieves a break on PTGS2 activity and PG secretion. By replacing OSFs in the medium, HA-CD44 interactions are restored, and this inhibits PTGS2 mRNA and/or activity leading to reduced PG secretion and lowered *PTX6 and TNFAIP6* mRNA levels additionaly there is a coregulation of PTX3 and TSG6 [46]. It is less likely that OSFs act upon *HAS2* gene expression, as mRNA levels were not consistently altered by denuded oocytes, and the fact that CD44-HA interactions did not alter Has2 mRNA in fibroblasts [11] suggests that OSF actions are downstream of HAS2 (Fig. 6).

Recent studies have shown that the physiologically relevant AREG may be beneficial to IVF outcomes such as blastocyst development rates compared with the use of supraphysiological levels of FSH [24, 47]. In agreement with previous studies in mice and cattle, glucose uptake and lactate production were higher [48] and abundance of mRNA encoding *HAS2* in cumulus cells was lower under AREG compared with under FSH stimulation, whereas *PTGS2* mRNA levels were not different [47]. Previous studies with cattle and pigs have shown that cumulus expansion rates are higher under FSH compared to AREG stimulation [18, 23, 47], whereas there was no difference in the present study. These differences are likely to be due in part to the dose (100 vs 1.6mU/ml) and type (recombinant vs purified) of FSH used. In contrast, the mouse appears to be different: under FSH stimulation, cumulus rates and abundance of mRNA encoding *Has2* and *Ptgs2* are lower than under AREG stimulation [48].

Removal of the oocyte diminished FSH-stimulated cumulus expansion and glycolysis but had no impact on AREG stimulated cumulus expansion and glycolysis. One possible explanation is that the induction of AREG signaling by FSH results in a weaker or later activation of EGFR than does the direct addition of recombinant AREG [23]. It is also of note that removal of the oocyte in AREG-stimulated cells altered *HAS2, ADAM10, CD44, PTX3* and *TNFAIP6* mRNA levels without impacting cumulus expansion. Most changes in gene expression were noted at 22 h, after expansion had occurred, it is likely therefore that these changes in mRNA were too late to impact the process of expansion. This is in contrast to the mouse, for which expansion is critically dependent on the oocyte, and raises the possibility that two distinct mechanisms are involved: in both species, the oocyte signals to cumulus cells and influences the expression of genes involved in the preovulatory cascade, and in mice but not in cattle,

the oocyte may influence the protein-protein interactions within the cumulus extracellular matrix that are necessary for expansion independently of subsequent changes in gene expression.

In conclusion this work presents new evidence to suggest that bovine oocytes, like mouse oocytes, secrete OSFs that control gene expression in cumulus cells. However, unlike in the mouse, the process of cumulus expansion is largely unaffected by the absence of the oocyte in cattle, suggesting species-specific regulation of the remodeling of the cumulus extracellular matrix.

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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

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FIGURE 1

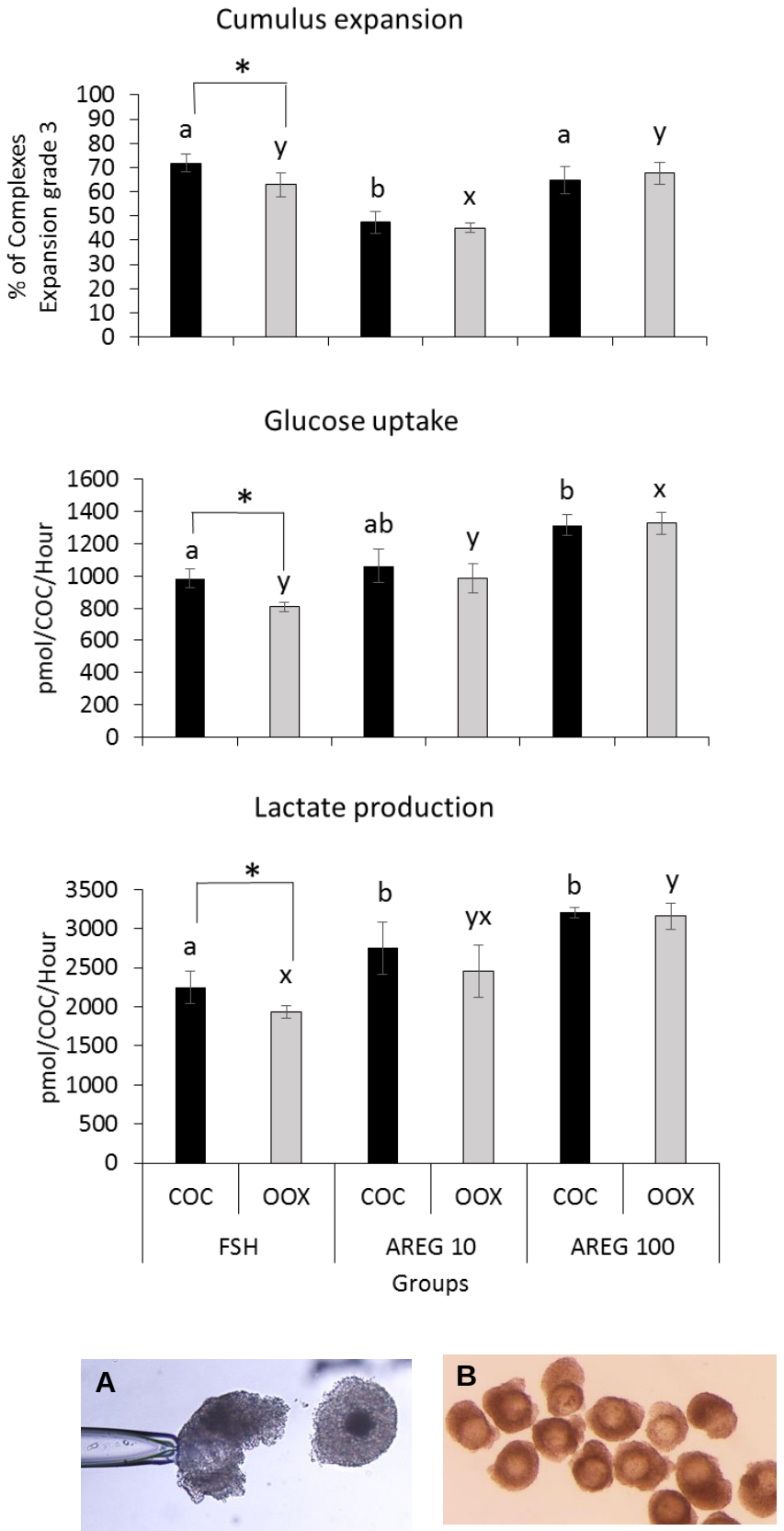
Cumulus expansion
% of Complexes Expansion grade 3
100
90
80
70
60
50
40
30
20
10
0
*
a
y
b
x
a
y
Glucose uptake
pmol/COC/Hour
1600
1400
1200
1000
800
600
400
200
0
*
a
y
ab
y
b
x
Lactate production
pmol/COC/Hour
3500
3000
2500
2000
1500
1000
500
0
*
a
x
b
yx
b
y
COC
OOX
COC
OOX
COC
OOX
FSH
AREG 10
AREG 100
Groups
A
B

FIGURE 2

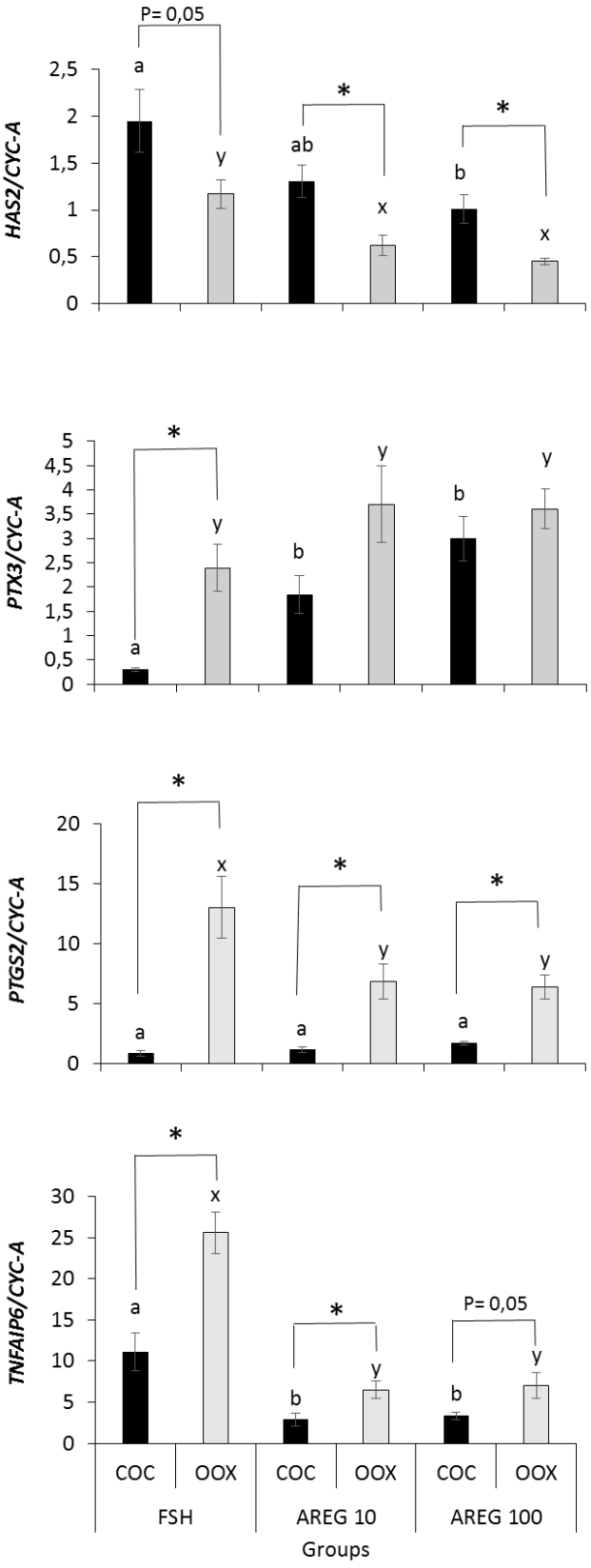
P= 0,05
HAS2/CYC-A
PTX3/CYC-A
PTGS2/CYC-A
TNFAIP6/CYC-A
P= 0,05
COC
OOX
FSH
AREG 10
AREG 100
Groups

FIGURE 3

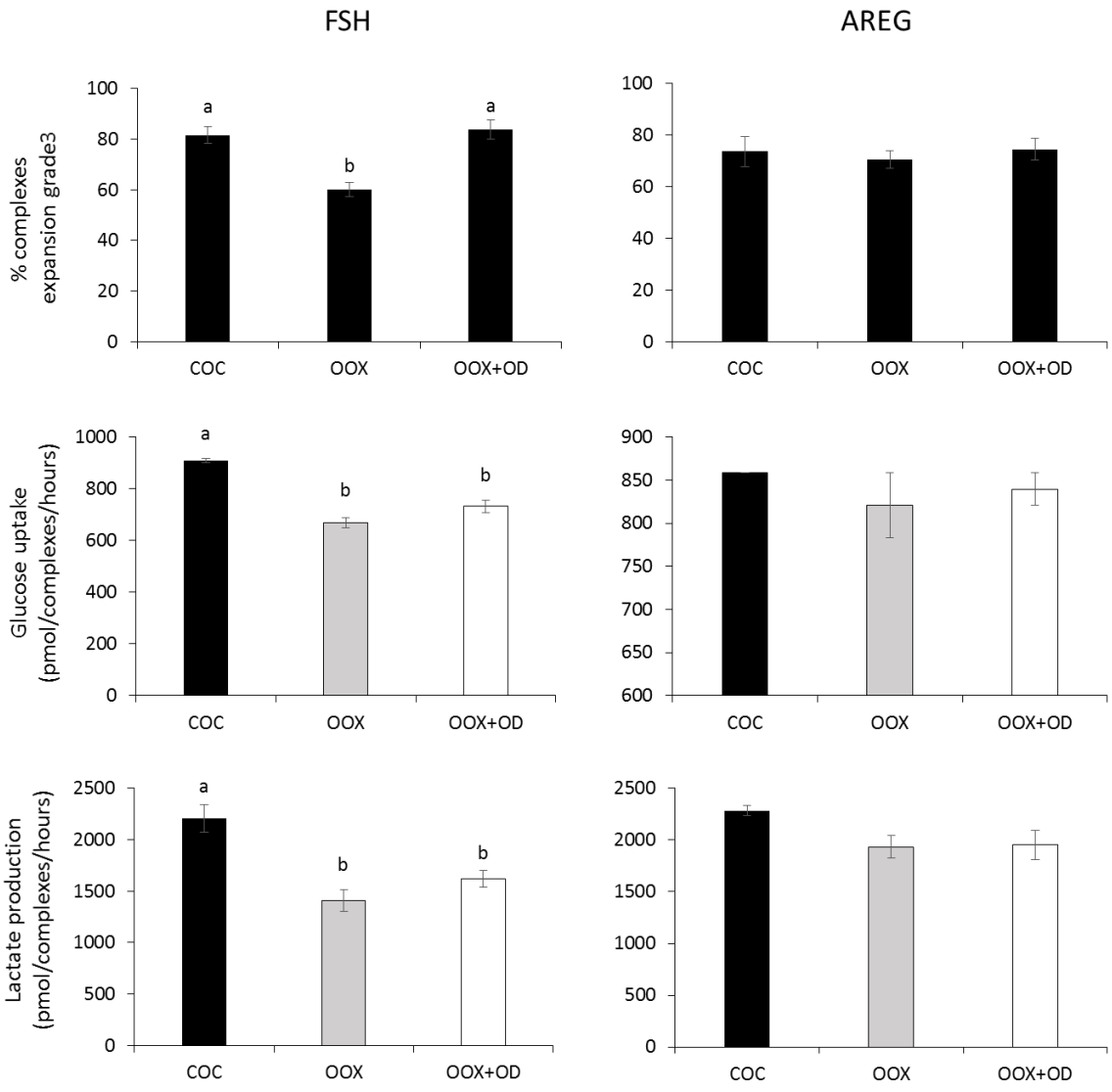
FSH
AREG
% complexes expansion grade3
Glucose uptake (pmol/complexes/hours)
Lactate production (pmol/complexes/hours)
COC
OOX
OOX+OD
a
b

FIGURE 4

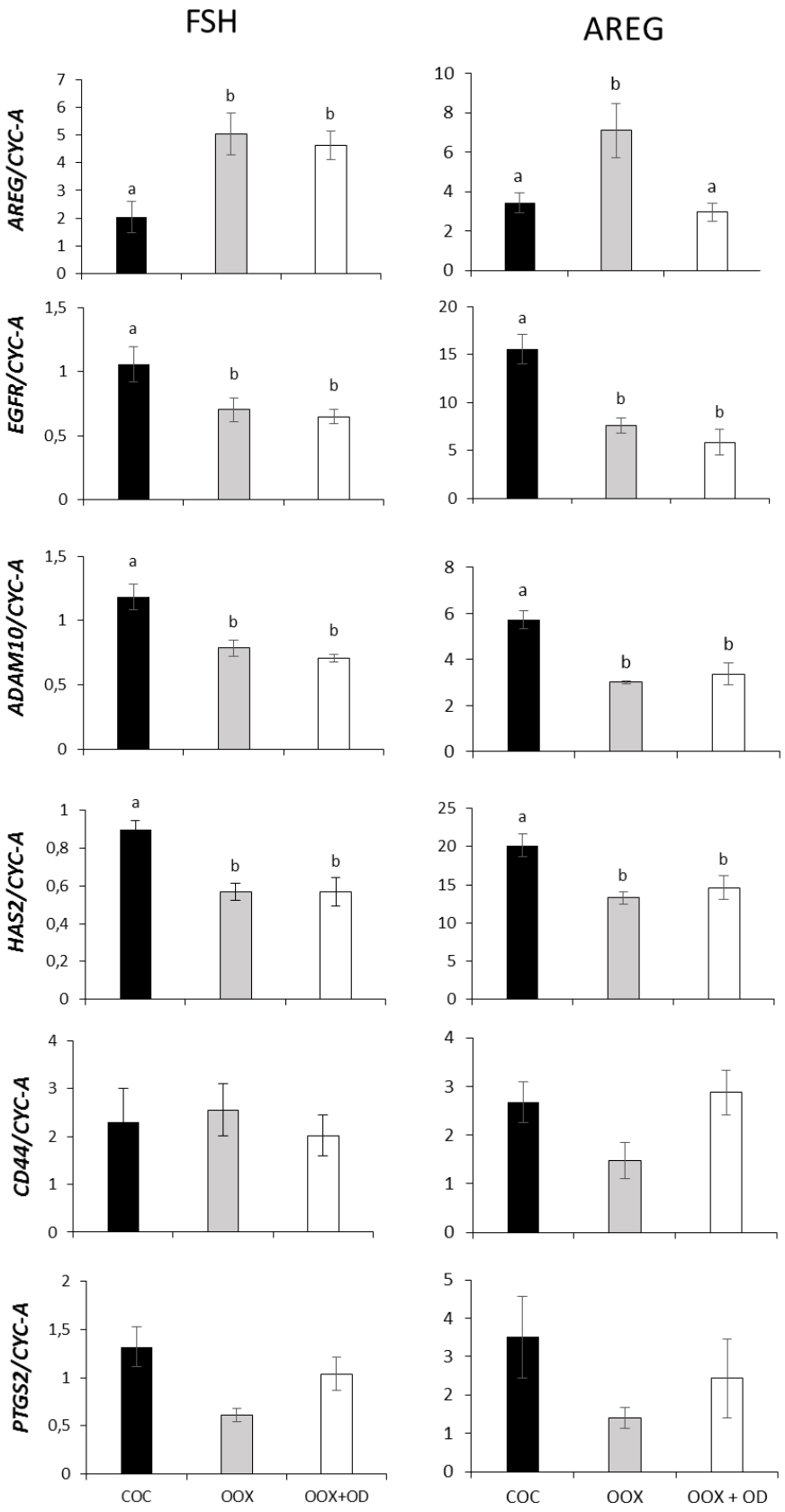
4 HOURS
FSH
AREG
AREG/CYC-A
EGFR/CYC-A
ADAM10/CYC-A
HAS2/CYC-A
CD44/CYC-A
PTGS2/CYC-A
COC
OOX
OOX+OD
COC
OOX
OOX + OD

FIGURE 5

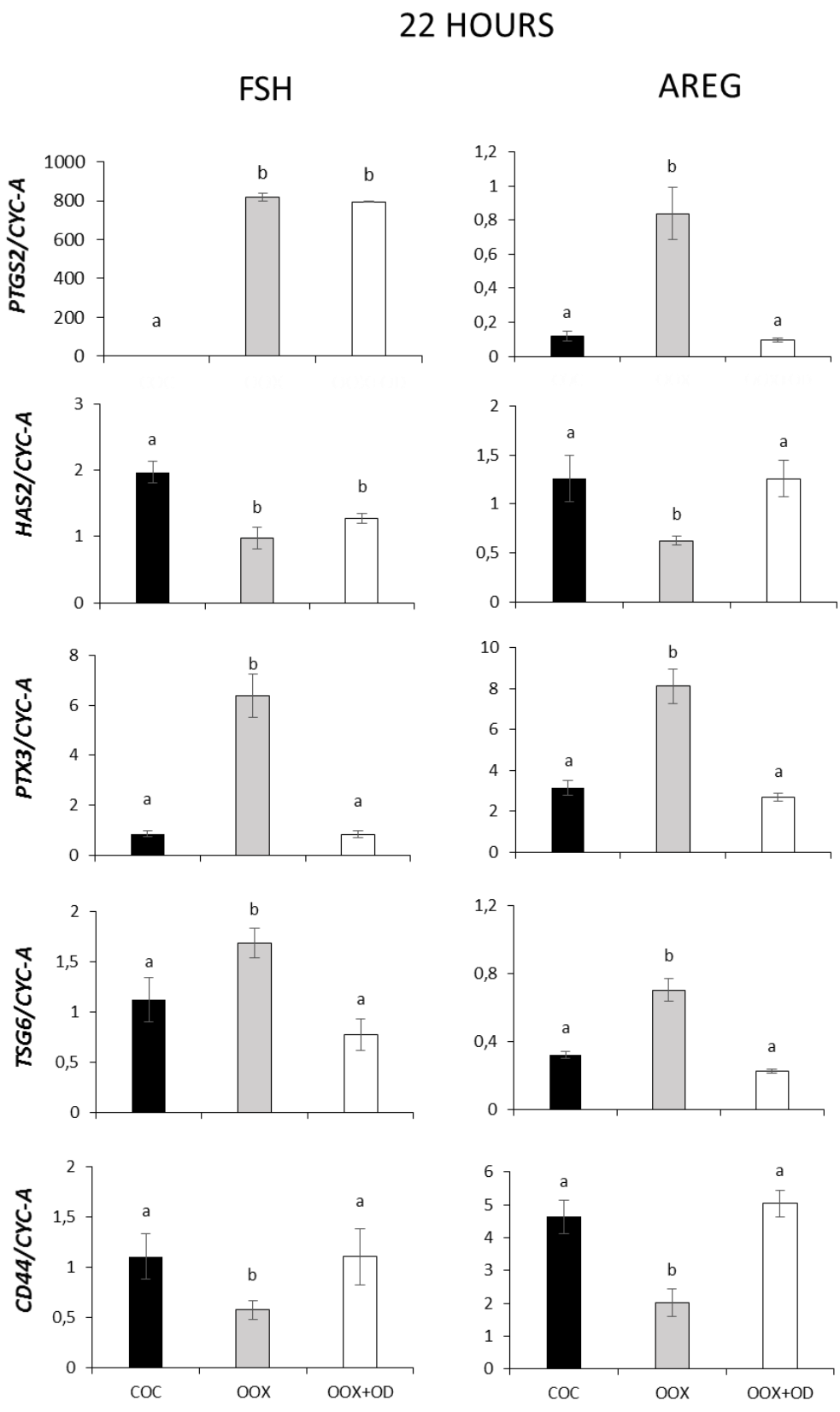
22 HOURS
FSH
AREG
PTGS2/CYC-A
HAS2/CYC-A
PTX3/CYC-A
TSG6/CYC-A
CD44/CYC-A
COC
OOX
OOX+OD

FIGURE 6

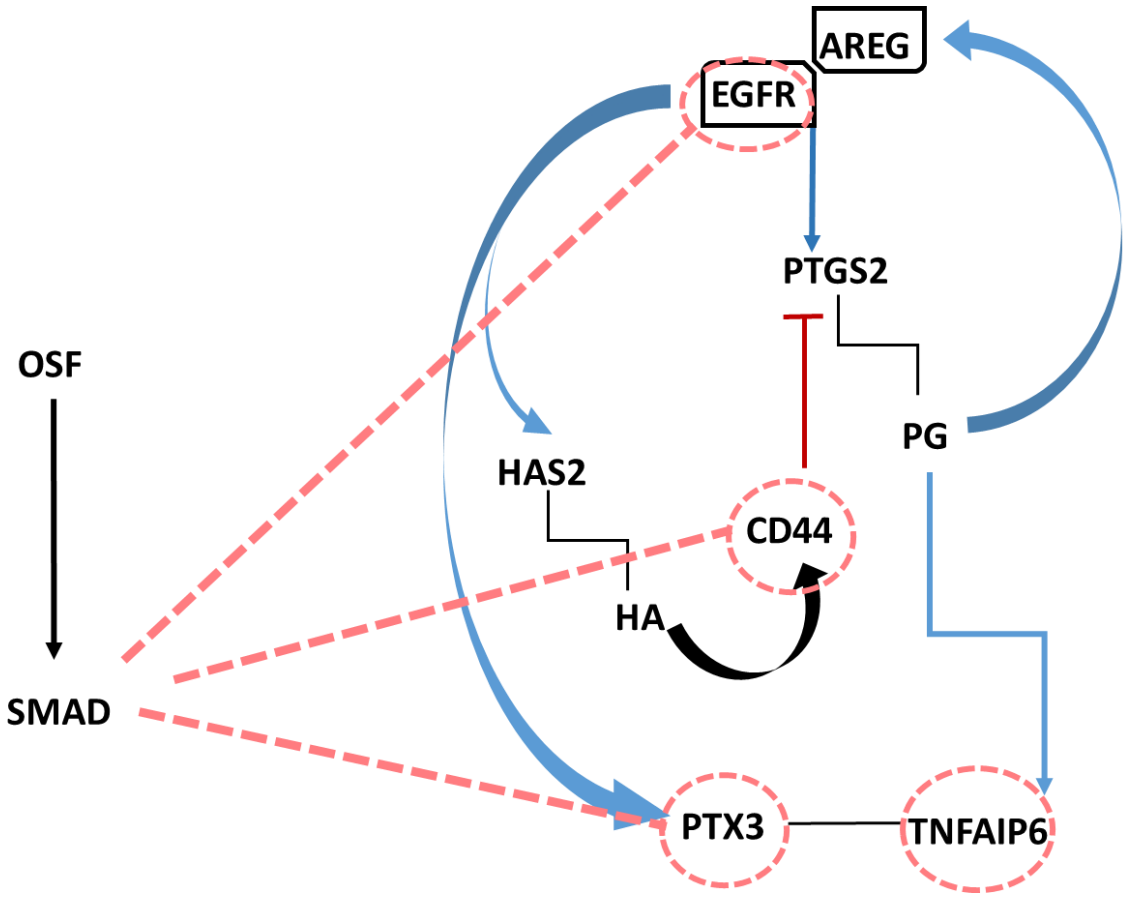

FIGURE LEGENDS

Figure 1: Effect of oocyte removal on cumulus expansion, glucose uptake and lactate production from intact COC (black bars) and OOX (grey bars) submitted to 22 hours of IVM in the presence of FSH or AREG at concentration of 10ng/ml or AREG at concentration of 100ng/ml. Siginificative diference between COC and OOX are represented with "*" and significative diference between treatments are presented with different letters ($p<0.05$). Data were derived from five independente replicates. Representation of the Oocytectomy procedure (A) and OOX group (B).

Figure 2: Effects of oocyte removal in the presence of FSH, AREG at 10 and 100ng/ml on *HAS2*, *PTX3*, *PTGS2* and *TNFAIP6* mRNA levels in cumulus cells. COCs were cultured intact (COC, black bars) or oocytectomized (OOX, grey bars) for 22 hours. Messenger RNA abundance was measured by real-time PCR. Data are presented as mean values (±S.E.M.) relative to a calibrator sample by the ΔΔCt method with efficiency correction. Siginificative diference between COC and OOX are represented with "*" and significative diference between treatments are presented with different letters ($P<0.05$). Data were derived from five independente replicates.

Figure 3: Effect of oocyte removal and oocyte replacement in the presence of FSH or AREG on cumulus expansion, glucose uptake and lactate production. COCs were cultured intact (COC), oocytectomized (OOX) or oocytectomized with denuded oocytes (OOX + DO) for 22 hours. Siginificative diference between COC and OOX are represented with "*" and significative diference between treatments are presented with different letters ($p<0.05$). Data were derived from four independente replicates

Figure 4: Effects of oocyte removal and oocyte replacement in the presence of FSH or AREG on *AREG*, *EGFR*, *ADAM10*, *HAS2*, *CD44* and *PTGS2* mRNA levels in cumulus cells. COCs were cultured intact (COC), oocytectomized (OOX) or oocytectomized with denuded oocytes (OOX + DO) for 4 hours. Messenger RNA abundance was measured by real-time PCR. Data are presented as mean values (±S.E.M.) relative to a calibrator sample by the ΔΔCt method with efficiency correction. Bars with different letters are significantly different ($P < 0.05$). Data were derived from four independent replicates.

Figure 5: Effects of oocyte removal and oocyte replacement in the presence of FSH or AREG on *PTGS2*, *HAS2*, *PTX3*, *TNFAIP6* and *CD44* mRNA levels in cumulus cells. COCs were cultured intact (COC), oocytectomized (OOX) or oocytectomized with denuded oocytes (OOX + DO) for 22 hours. Messenger RNA abundance was measured by real-time PCR. Data are presented as mean values (±S.E.M.) relative to a calibrator sample by the ΔΔCt method with efficiency correction. Bars with different letters are significantly different ($P < 0.05$). Data were derived from four independent replicates.

Figure 6: Illustrative model of the AREG signaling in cumulus cells and the influence of OSFs.

TABLE

Table 1: Information of specific primers used for amplification in real-time RT-PC

Gene	Primer Sequence	Fragment size (bp)	Annealing temperature (°C)	Reference
EGFR	F: 5' AAAGTTTGCCAAGGGACAAG3' R: 5' AAAGCACATTTCCTCGGATG3'	253	53	[7]
ADAM10	F: 5'ACCCCCCAAAGTCTCTCACA3' R: 5'AATCATGCGGAGATCCAAAGTT3'	210	60	[7]
AREG	F: 5'CTTTCGTCTCTGCCATGACCTT3' R: 5'CGTTCTTCAGCGACACCTTCA3'	100	60	[7]
EREG	F: 5'ACTGCACAGCATTAGTTCAAACTGA3' R: 5'TGTCCATGCAAACAGTAGCCATT3'	100	60	[7]
PTX3	F: 5'CCTCAGCTATCGGTCCATAA3' R: 5'ATTGAAGCCTGTGAGGTCTGC3'	294	54	[7]
TNFAIP61	F: 5'GCAAAGGAGTGTGGTGGTGTGTTT3' R: 5'ACTGAGGTGAATGCGCTGACCATA3'	135	60	[7]
HAS2	F: 5'ACACAGACAGGCTGAGGACAACTT3' R: 5'AAGCAGCTGTGATTCCAAGGAGGA3'	133	60	[7]
CD44	F: 5' GACCCTCAATCTTCCTCTTCAC 3' R: 5' TTTCCTACCAGGTGCCAATC 3'	94	60	NM_174013.3
PTGS2	F: 5'AAGCCTAGCACTTTCGGTGGAGAA3' R: 5'TCCAGAGTGGGAAGAGCTTGCATT3'	168	60	[7]
CYC-A	F: 5'GCCATGGAGCGCTTTGG3' R: 5'CCACAGTCAGCAATGGTGATCT3'	65	60	[49]

F, forward primer; R, reverse primer

Capítulo 3

Artigo preparado para ser submetido para publicação na revista Theriogenology

O artigo está apresentado de acordo com as normas da revista exetuando-se a numeração das linhas

Título: Effects of FGF10 on expansion and glucose metabolism of oocytectomized bovine cumulus-oocyte complexes

TITLE: Effects of FGF10 on expansion and glucose metabolism of oocytectomized bovine cumulus-oocyte complexes

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Abstract

The *in vitro* maturation (IVM) of COC in catlle is not efficient, thus intraovarian signaling molecules are being explored as potential additives to IVM media. Once fibroblast growth factor 10 (FGF10) is an oocyte secreted factor the aim of the present study was to investigate the effects of FGF-10 on cumulus cells functions without the presence of the oocyte during *in vitro* maturation. The results shown that the oocyte regulates glucose metabolism and expansion in bovine cumulus cells. Removal of the oocyte by oocytectomy did not abolish cumulus expansion but decreased the percentage of oocytes reaching full expansion and that addition of FGF10 to the IVM medium completely restores expansion but not glucose metabolism in oocytectomized bovine COCs. Furthermore, removal of the oocyte decreases *HAS2* and *GFPT1* mRNA abundance we demonstrated an increase in *ADAM10, HAS2* and *GFPT1* mRNA abundance and FGF10 restored *HAS2* and *GFPT1* mRNA at the same levels of intact COC this is associated with the positive effect on cumulus expansion.Taken together, the present data shown that FGF10 is involved in the oocyte control of cumulus expansion in bovine COCs undergoing IVM in cattle. The data specifically suggest that FGF10 is involved in the regulation of cumulus expansion via mechanisms involving enhanced expression of *HAS2*.

1. Introduction

Fibroblast growth factors (FGF) are involved in numerous biological processes and are present in different cell types [1, 2]. They are grouped into seven subfamilies, which members activate common receptors encoded by four genes (*FGFR1, 2, 3* and *4*) with 55-72% homology at the protein level [3-5]. There is evidence that FGFs play important roles in follicular development and mediating the communication between the oocyte and cumulus cells [6-10]. For example, oocyte derived FGF8 cooperates with BMP15 to increase cumulus glycolytic activity in mice [11, 12].

The communication between cumulus cells and the oocyte is essential for developmental competence [13]. Oocyte secreted factors such as BMP15, GDF9 that have impact on cumulus apoptosis and embryo production [14, 15]and FGFs control cumulus expansion and metabolism via mechanisms including increased expression of major regulatory genes such as prostaglandin sintase 2(*PTSG2*), hyaluronan sintase 2 (*HAS2*), tumor necrosis factor alpha induced protein 6 (*TNFAIP6*) and pentraxin 3 (*PTX3*) for expansion, and phosphofructokinase, platelet (*PFKP*), lactate dehydrogenase A (*LDHA*) and glutamine--fructose-6-phosphate transaminase 1 (*GFPT*) for glucose metabolism [16]. Oocyte secreted factors also regulate genes involved in the control of oocyte nuclear maturation such as *KITL* and *NPPC*; FGFs appear to be involved in the regulation of both genes [17].

Fibroblast growth factor 10 is a member of the FGF7 subfamily expressed in preantral follicles and in oocytes and theca cells of antral follicles in cattle [10, 18]. Receptors for FGF10 (FGFR2B and FGFR1B) are predominantly expressed by mural and cumulus granulosa cells in the bovine ovary[10, 19]. Fibroblast growth factor 10 inhibits aromatase expression and estradiol production in bovine granulosa cells, which has been interpreted as a consequence of suppressed FSH and IGF signaling as expression of FSH and IGF receptors was also reduced

by FGF10 [20]. Interestingly, this inhibitory action is enhanced in future subordinate follicles suggesting that FGF10 is involved in the mechanisms of dominant follicle selection [18]. On the other hand, in the bovine cumulus-oocyte complex (COC) undergoing *in vitro* maturation, FGF10 has stimulatory effects on cumulus expansion and glucose uptake; both processes are directly related to oocyte developmental competence [7]. In fact, supplementation of the IVM medium with FGF10 was reported to improve embryo production in cattle [21]. The influence of FGF10 on cumulus expansion has been interpreted as a consequence of increased *PTGS2* expression and greater use of glucose for hyaluronan production due to increased expression of *GFPT*, a rate-limiting enzyme in the glycolytic pathway leading to hyaluronan production in cumulus cells [7].

Interestingly, the influence of the oocyte on cumulus function appears to vary between different species. While the oocyte is absolutely required for cumulus expansion in mice, the bovine cumulus can expand in its absence [17, 22]. However, we have recently demonstrated that, although cumulus expansion does occur in oocytectomized cumulus-oocyte complexes, the oocyte does enhance expansion and glycolysis in cattle [16]. To date, the mechanisms underlying the influence of the oocyte on cumulus expansion and metabolism are mostly unknown. To shed light onto these mechanisms, in the present study we aimed to assess the effects of FGF10, an oocyte derived factor, on cumulus expansion, glucose metabolism and related mRNA expression in bovine oocytectomized COCs. We tested the hypothesis that FGF10 would restore cumulus expansion and glucose metabolism.

2. Materials and Methods

Unless specified, all chemicals and reagents were purchased from Sigma (St. Louis, MO, USA).

2.1. *In vitro* maturation

Ovaries of adult cows (predominantly Nellore, *Bos indicus*) were obtained at an abattoir local to the Sao Paulo State University campus in Botucatu and transported to the laboratory in saline solution (0.9% NaCl) at 37°C. COCs were aspirated from 3 to 8 mm diameter follicles with an 18 gauge needle and pooled in a 15 ml conical tube. After sedimentation, COCs were recovered and selected using a stereomicroscope. Only COCs with homogeneous cytoplasm and compact multilayer of cumulus cells were used (Grade 1 and 2). COCs were washed, alternatively oocytecomized (as described below in experimental design) and transferred in groups of 20 to 100µl of maturation medium [TCM199 containing Earle's salts supplemented with 1 µg/ml porcine FSH (equivalent to 0.002 IU; Folltropin-V® Bioniche Animal Health, Belleville ON, Canada), 22 µg/ml sodium pyruvate, 75 µg/ml amicacin and 4 mg/ml BSA] in 96 well plates. FGF10 was supplemented as described below. Groups of intact or oocytectomized COCs were incubated at 38.5 °C in 5% CO_2 in humidified air.

2.2. Oocytectomy

Cumulus-oocyte complexes were placed in 200µl drops of TCM199 partially covered with mineral oil and the cytoplasm of the oocytes was removed with a micromanipulator as previously described [22] with modifications [23]. The resulting oocyte-free cumulus complexes (OOX) were cultured as for COCs.

2.3. Experimental design

To assess the effects of FGF10 on cumulus expansion, metabolism and gene expression, pools of 20 OOXs were cultured with 0, 1 or 10 ng/ml of FGF10 for 22 hours. Pools of 20 intact

COCs were also cultured simultaneously in base media without FGF10 as a control group. All groups were done in 4 (4 hours experiment) or 5 (22 hours experiment) replicates. Since FGF10 only affected expansion at 10ng/ml, we then repeated this experiment with only the effective dose of FGF10 (10ng/ml) during 4 hours of IVM to assess the effects of FGF10 on mRNA abundance of genes that regulate expansion, particularly those acting early in the ovulatory cascade (*AREG, EREG, ADAM10,* n=4 or 5 replicates). The effect of FGF10 on glucose uptake and lactate production was also assessed at 4 hours of IVM.

At the end of culture, cumulus expansion was assessed visually and glucose uptake and lactate production were measured in the spent medium as decribed below, Total RNA was extracted from cumulus cells for gene expression analysis. The effect of FGF10 on cumulus cell gene expression was tested at 4 hours of IVM for *GFPT1, GFPT2 AREG, EREG, ADAM10, PTGS2*, *TNFAIP6, HAS2, PTX3* and *CD44,* and at 22 hours of IVM for *GFPT1*, *GFPT2, PTGS2*, *HAS2, PTX3, TNFAIP6* and *CD44*.

2.4. Assessment of cumulus expansion

Cumulus expansion was visually assessed at 22 hours of culture according to a subjective scoring system. Grades 1, 2 and 3 were attributed to increasing degrees of expansion (1-pool expansion, characterized by few morphological changes compared with before maturation; 2-partial expansion, characterized by fair expansion but notable clusters lacking expansion; 3-complete or nearly complete expansion [8].

2.5. Assessment of glucose uptake and lactate production

Glucose and lactate concentrations were determined in spent media after 4 and 22 hours of culture. At the end of culture, spent media were collected, frozen and stored at – 80°C. Glucose and lactate levels were measured using a Hitachi 912 chemical analyzer (F. Hoffmann-La Roche Ltd.). To determine glucose uptake, the value measured at the end of culture was subtracted from the concentration of glucose in media blanks (media incubated without cells). The base medium (TCM199) did not contain lactate. Glucose uptake and lactate production were expressed as pmol/COC per hour [24, 25].

2.6. Gene expression analysis

After culture, COCs were mechanically separated by repeated pipetting in PBS without calcium and magnesium. Cumulus cells from COC and were transferred to 1.5 ml tubes, collected by centrifugation for 5 min at 700g and frozen at -80 °C in 350µl of RNA extraction lysis buffer from the RNeasy® kit (Qiagen, Mississauga, ON, Canada). Total RNA was extracted using the RNeasy® kit as recommended by the manufacturer. After purification, RNA samples were eluted in 30µl of RNAse free water. Total RNA concentrations were measured by spectrophotometry using a NanoDrop ND® 1000 (Thermo Scientific, Wilmington, DE, USA). Total RNA (100ng/reaction) was incubated with DNAse I (1 U/µg; Invitrogen, São Paulo, Brazil) and then reverse transcribed using Oligo-dT primers and Omniscript (Qiagen, Mississauga, ON, CA). The reagents were incubated at 37 ºC for 60 min and then at 93 ºC for 3 min for enzyme inactivation. Relative real time RT-PCR analysis was performed with an ABI 7500 thermocycler using Power Sybr Green PCR Master Mix (Applied Biosystems, São Paulo, Brazil). The final volume of the PCR mix was 25 µl and thermocycling conditions were: 95 °C for 10 min (1 cycle), denaturing at 95 °C for 10 sec followed by annealing for 1 min (40 cycles). Primers for the housekeeping gene *CYCA* and for target genes *GFPT1*, *GFPT2*, *PTGS2*, *TNFAIP6, HAS2, PTX3, CD44, ADAM10, EREG* and *AREG* were as previously used and

validated [7, 19]. The relative expression values for each gene were calculated using the ΔΔCt method with efficiency correction and using one control sample as calibrator [26].

2.7. Statistical Analysis

Maturation and cumulus expansion data were arcsine transformed before analysis, and gene expression data were transformed to logarithms when not normally distributed. The effects of FGF10 on cumulus cell expansion, glucose uptake, lactate production and gene expression were tested by analysis of variance (ANOVA), and means were compared with the Tukey-Kramer HSD test using JMP software (SAS Institute, Cary, NC, USA). Differences were considered significant when $P \leq 0.05$.

3. Results

Removal of the oocyte by oocytectomy significantly reduced the percentage of complexes with full expansion (grade 3). Addition of FGF10 at 10ng/ml to the IVM media, but not at 1ng/ml, restored the percentage of fully expanded complexes, determining values not different than those observed in intact COCs (Fig. 1).

Treatment with FGF10 for 4 hours of IVM reduced the abundance of mRNA encoding *CD44* and *PTX3,* leading to values not different than those observed in intact COCs (Fig. 2). This reduction was not observed after 22 hours of IVM, when FGF10 at 10ng/ml increased abundance of *HAS2* mRNA, although it did not fully restore gene expression in relation to intact COCs (Fig. 3). In addition, also after 22 hours of IVM, FGF10 increased *GFPT1* mRNA abundance in a dose dependent manner, leading to expression values higher than those provided by intact COCs (Fig. 5). Ocytectomy significantly reduced glucose uptake at 4 hours of IVM

and lactate production after 4 and 22 hours of IVM. Addition of FGF10 did not alter these parameters in oocytectomized COCs (Fig. 4).

4. Discussion

The data presented herein demonstrate that the oocyte regulates glucose metabolism and expansion in bovine cumulus cells, that confirms previously data in cattle [16], and that addition of FGF10 to the IVM medium restores expansion but not glucose metabolism in oocytectomized bovine COCs. Moreover, we report increases in *HAS2* and *GFPT1* mRNA abundance associated with the positive effect of FGF10 on cumulus expansion. Taken together, the present data suggest that FGF10 is involved in the oocyte control of cumulus expansion, but not of energy metabolism in bovine COCs undergoing IVM.

Removal of the oocyte by oocytectomy did not abolish cumulus expansion but decreased the percentage of oocytes reaching full expansion in the present work. This confirms that differently than in mice, expansion occurs in the absence of the oocyte in cattle, although the oocyte can enhance it (data not published). Ocytectomy decreased mRNA levels of *ADAM10*, *HAS2* and *CD44* suggesting that reduced expression of these genes may be involved in the mechanisms by which the oocyte affects cumulus expansion. ADAM10 is necessary to cleave and release AREG and EREG from the cell membrane and thus regulates EGFR signaling. Therefore, decreased *ADAM10* may impair ERK1-2 signaling, a major pathway leading to expansion [27]. HAS2 is a rate-limiting enzyme for the production of hyaluronan and thus reduced expression of *HAS2* would reduce the production of the main component of the extracellular matrix that is accumulated during cumulus expansion [28]. CD44 is a hyaluronan receptor that regulates PTGS2 activity and production, which is required for cumulus expansion [29]. Surprisingly, oocytectomy increased mRNA abundance of *AREG* at 4 hours of IVM and of *PTGS2*, *TNFAIP6* and *PTX3* at 22 hours of IVM. The reasons for the

increase in *AREG* expression in oocytectomized COCs are unknown and intriguing but we suggests that the EGFR-activated signaling pathway was upregulated by the removal of the oocyte. On the other hand, the increase in *PTGS2*, *TNFAIP6* and *PTX3* expression may be a consequence of decreased HAS2/CD44 signaling in oocytectomized COCs, as CD44 was reported to inhibit PTGS2 activity. Therefore, attenuated HAS2/CD44 signaling may possibly restrain the break on the expression of *PTGS2* and down-stream genes.

Supplementation of the IVM medium with FGF10 at 10ng/ml restored cumulus expansion, which was accompanied by increased mRNA abundance of *HAS2* and *GFPT1*. These observations suggest that FGF10 enhances cumulus expansion at least in part by increasing the expression of these genes and, consequently the availability of the encoded enzymes, which are crucial in the hexosamine pathway of glucose metabolism that leads to hyaluronan production [7, 24, 30]. However, *HAS2* mRNA abundance was not fully restored by FGF10 at 10ng/ml, whereas cumulus expansion was. This observation suggests that FGF10 activates other mechanisms that cooperate with increased *HAS2* mRNA abundance to fully restore expansion. Enhanced expression of *GFPT1* may be a potential cooperative mechanism. Oocytectomy decreased *GFPT1* expression at 4 hours of IVM and FGF10 increased it at 22 hours of IVM. Although the removal of the oocyte did not alter *GFPT1* expression at 22 hours, treatment with FGF10 could compensate weaker expression at the beginning of IVM in oocytectomized oocytes. Effects of FGF10 on cumulus expansion and abundance of *GFPT1* mRNA are in agreement with our previous study using only intact oocytes [7]. However, in our previous study FGF10 did not significantly increase *HAS2* mRNA levels in bovine intact COCs undergoing IVM. Conversely, FGF10 stimulated *PTGS2* mRNA expression in intact COCs [7], but not in oocytectomized COCs (present study). These discrepancies might be due to the interaction of FGF10 with other OSFs active in intact COCs.

Cumulus glucose metabolism was also affected by the oocyte in the present study; both glucose uptake and lactate production were decreased by oocytectomy. Addition of FGF10 to the IVM could not reverse these effects. This is somehow in contrast with our previous study with intact COCs where FGF10 increased glucose uptake without altering lactate production [7]. Again, we interpret this discrepancy as a possible consequence of the interaction of FGF10 with other OSFs in intact COCs.

In conclusion, the present study reinforce the concept that the oocyte does influence cumulus function in cattle and suggest that FGF10 is one of the oocyte secreted factors mediating this influence. The data specifically suggest that FGF10 is involved in the regulation of cumulus expansion via mechanisms involving enhanced expression of *HAS2*.

Acknowledgements

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FIGURE 1

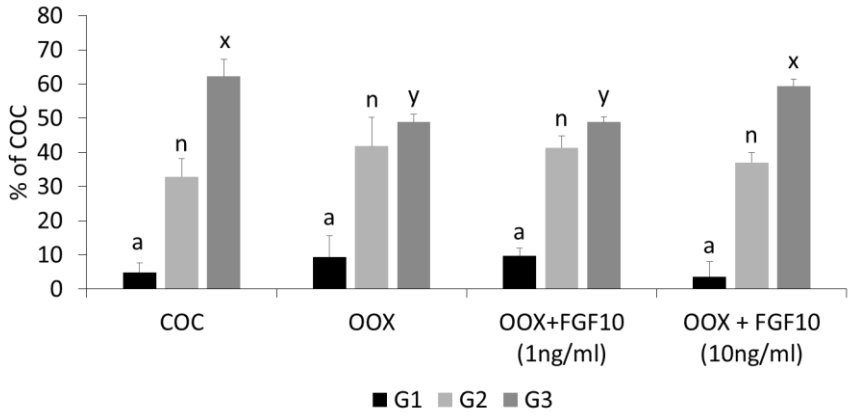

80
70
60
50
40
30
20
10
0
% of COC
a
n
x
a
n
y
a
n
y
a
n
x
COC
OOX
OOX+FGF10 (1ng/ml)
OOX + FGF10 (10ng/ml)
G1
G2
G3

FIGURE 2

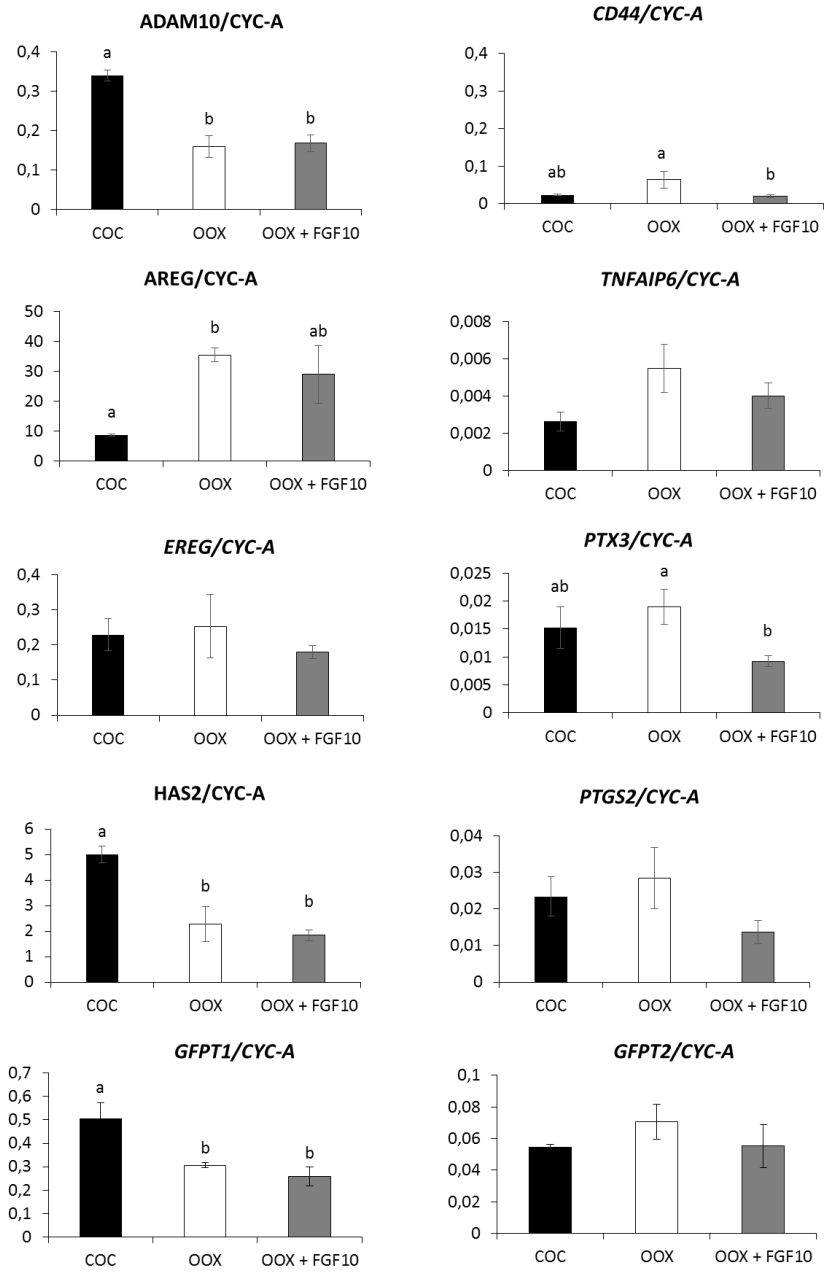

ADAM10/CYC-A
0,4
0,3
0,2
0,1
0
a
b
b
COC
OOX
OOX + FGF10
CD44/CYC-A
0,4
0,3
0,2
0,1
0
ab
a
b
COC
OOX
OOX + FGF10
AREG/CYC-A
50
40
30
20
10
0
a
b
ab
COC
OOX
OOX + FGF10
TNFAIP6/CYC-A
0,008
0,006
0,004
0,002
0
COC
OOX
OOX + FGF10
EREG/CYC-A
0,4
0,3
0,2
0,1
0
COC
OOX
OOX + FGF10
PTX3/CYC-A
0,025
0,02
0,015
0,01
0,005
0
ab
a
b
COC
OOX
OOX + FGF10
HAS2/CYC-A
6
5
4
3
2
1
0
a
b
b
COC
OOX
OOX + FGF10
PTGS2/CYC-A
0,04
0,03
0,02
0,01
0
COC
OOX
OOX + FGF10
GFPT1/CYC-A
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
a
b
b
COC
OOX
OOX + FGF10
GFPT2/CYC-A
0,1
0,08
0,06
0,04
0,02
0
COC
OOX
OOX + FGF10

FIGURE 3

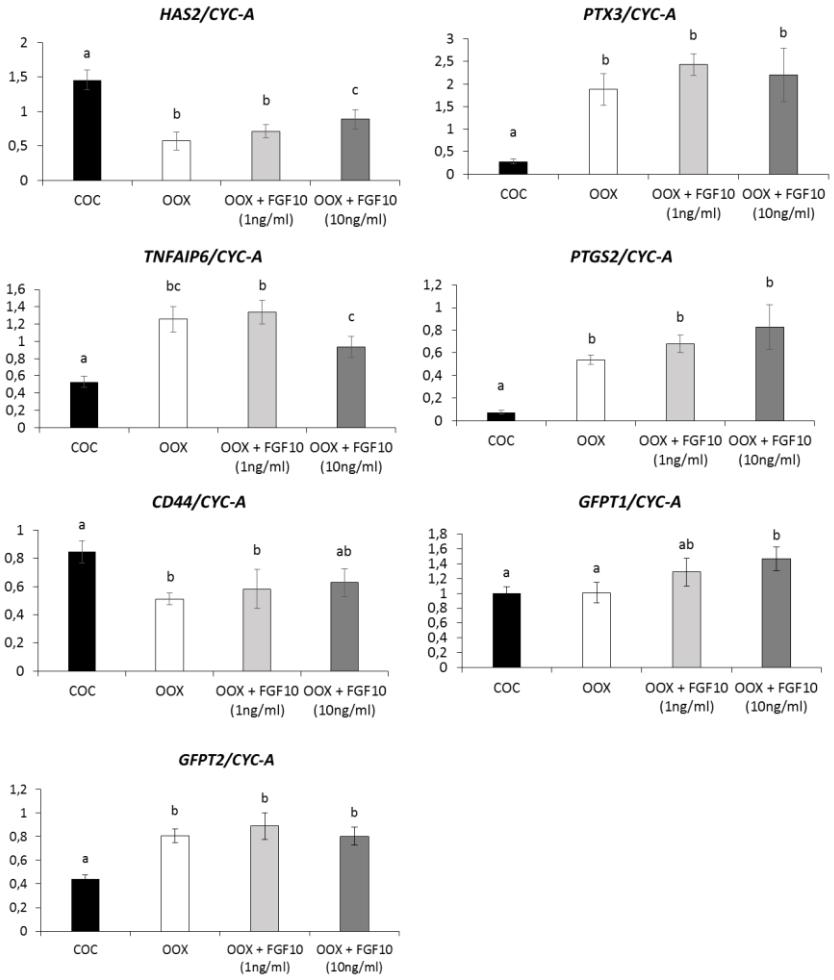

HAS2/CYC-A
2
1,5
1
0,5
0
a
b
b
c
COC
OOX
OOX + FGF10 (1ng/ml)
OOX + FGF10 (10ng/ml)
PTX3/CYC-A
3
2,5
2
1,5
1
0,5
0
a
b
b
b
COC
OOX
OOX + FGF10 (1ng/ml)
OOX + FGF10 (10ng/ml)
TNFAIP6/CYC-A
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
a
bc
b
c
COC
OOX
OOX + FGF10 (1ng/ml)
OOX + FGF10 (10ng/ml)
PTGS2/CYC-A
1,2
1
0,8
0,6
0,4
0,2
0
a
b
b
b
COC
OOX
OOX + FGF10 (1ng/ml)
OOX + FGF10 (10ng/ml)
CD44/CYC-A
1
0,8
0,6
0,4
0,2
0
a
b
b
ab
COC
OOX
OOX + FGF10 (1ng/ml)
OOX + FGF10 (10ng/ml)
GFPT1/CYC-A
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
a
a
ab
b
COC
OOX
OOX + FGF10 (1ng/ml)
OOX + FGF10 (10ng/ml)
GFPT2/CYC-A
1,2
1
0,8
0,6
0,4
0,2
0
a
b
b
b
COC
OOX
OOX + FGF10 (1ng/ml)
OOX + FGF10 (10ng/ml)

FIGURE 4

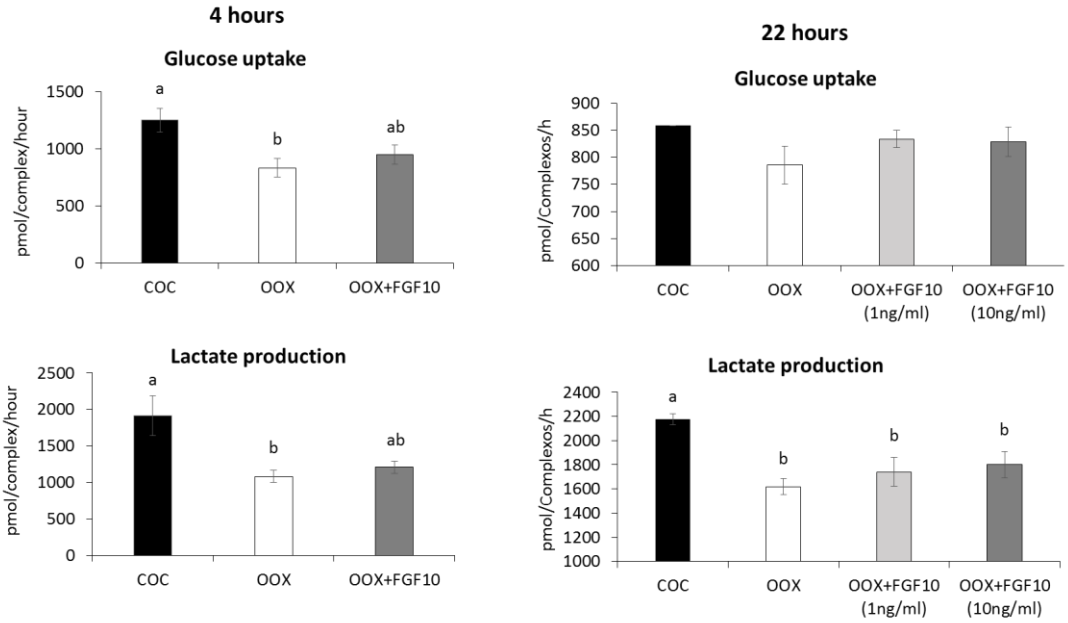

4 hours
Glucose uptake
pmol/complex/hour
1500
1000
500
0
a
b
ab
COC
OOX
OOX+FGF10
22 hours
Glucose uptake
pmol/Complexos/h
900
850
800
750
700
650
600
COC
OOX
OOX+FGF10 (1ng/ml)
OOX+FGF10 (10ng/ml)
Lactate production
pmol/complex/hour
2500
2000
1500
1000
500
0
a
b
ab
COC
OOX
OOX+FGF10
Lactate production
pmol/Complexos/h
2400
2200
2000
1800
1600
1400
1200
1000
a
b
b
b
COC
OOX
OOX+FGF10 (1ng/ml)
OOX+FGF10 (10ng/ml)

Table 1: Information of specific primers used for amplification in real-time RT-PCR

Gene	Primer Sequence	Fragment size (bp)	Annealing temperature (°C)	Reference
GFPT1	F: 5'GTTGAAACATGGCCCTCTGGCTTT3' R: 5'TGCCTAGCAACCACTTGCTGTAGA3'	117	60	[6]
GFPT2	F: 5'GCCTTGTACCCAAGTGCTTTGCTT3' R: 5'TGCACGGTATTGGAAGAGTCTGCT3'	123	60	[6]
ADAM10	F: 5'ACCCCCCAAAGTCTCTCACA3' R: 5'AATCATGCGGAGATCCAAAGTT3'	210	60	[6]
AREG	F: 5'CTTTCGTCTCTGCCATGACCTT3' R: 5'CGTTCTTCAGCGACACCTTCA3'	100	60	[6]
EREG	F: 5'ACTGCACAGCATTAGTTCAAACTGA3' R: 5'TGTCCATGCAAACAGTAGCCATT3'	100	60	[6]
PTX3	F: 5'CCTCAGCTATCGGTCCATAA3' R: 5'ATTGAAGCCTGTGAGGTCTGC3'	294	54	[6]
TNFAIP61	F: 5'GCAAAGGAGTGTGGTGGTGTGTTT3' R: 5'ACTGAGGTGAATGCGCTGACCATA3'	135	60	[6]
HAS2	F: 5'ACACAGACAGGCTGAGGACAACTT3' R: 5'AAGCAGCTGTGATTCCAAGGAGGA3'	133	60	[6]
CD44	F: 5' GACCCTCAATCTTCCTCTTCAC 3' R: 5' TTTCCTACCAGGTGCCAATC 3'	94	60	NM_174013.3
PTGS2	F: 5'AAGCCTAGCACTTTCGGTGGAGAA3' R: 5'TCCAGAGTGGGAAGAGCTTGCATT3'	168	60	[6]
CYC-A	F: 5'GCCATGGAGCGCTTTGG3' R: 5'CCACAGTCAGCAATGGTGATCT3'	65	60	[6]

F, forward primer; R, reverse primer

FIGURE LEGENDS

Figure 1: Effect of oocyte removal and FGF10 on OOX cumulus expansion. The cumulus cells expansion from intact COC; OOX; OOX+FGF10 at 1 and 10 ng/ml were avaluated after 22 hours of IVM in the presence of FSH. Significative diference between treatments are presented with different letters ($p<0.05$). Data were derived from five independent replicates.

Figure 2: Effect of oocyte removal and grading doses of FGF10 (1 and 10 ng/ml) on OOX cumulus cells mRNA abundance of *ADAM10*, *AREG*, *EREG*, *CD44*, *TSG6*, *PTX3*, *PTGS2*, *HAS2*, *GFPT1* and *GFPT2* after 4 hours of IVM. Messenger RNA abundance was measured by real-time PCR. Data are presented as mean values (±S.E.M.) relative to a calibrator sample by the ΔΔCt method with efficiency correction. Significative difference between treatments are presented with different letters ($P<0.05$). Data were derived from four independent replicates.

Figure 3: Effect of oocyte removal and grading doses of FGF10 (1 and 10 ng/ml) on OOX cumulus cells mRNA abundance of *CD44*, *TNFAIP6*, *PTX3*, *PTGS2*, *HAS2*, *GFPT1* and *GFPT2* after 22 hours of IVM. Messenger RNA abundance was measured by real-time PCR. Data are presented as mean values (±S.E.M.) relative to a calibrator sample by the ΔΔCt method with efficiency correction. Significative difference between treatments are presented with different letters ($P<0.05$). Data were derived from five independent replicates.

Figure 4: Effect of oocyte removal and FGF10 on OOX glucose uptake and lactate production. The culture media from intact COC; OOX; OOX+FGF10 at 1 and 10 ng/ml were avaluated after 4 and 22 hours of IVM in the presence of FSH. Significative diference between treatments are presented with different letters ($p<0.05$). Data is expressed in pmol/complex/hour.

Capítulo 4

"...it is striking that the large numbers of studies from the 1970s-1990s, principally in mice, were largely limited to investigating oocyte meiosis and did not follow the oocyte's subsequent capacity to support embryo development, or oocyte developmental competence. Curiously, even today, this central function of oocytes is not typically studied by mouse oocyte developmental biologists, but rather is the subject of major research efforts conducted by domestic animal oocyte biologists,"
(GILCHRIST et al., 2016)

Resultados complementares

A regulação da expansão das células do cumulus em bovinos é dependente da sinalização intra-cumulus?

1.INTRODUÇÃO

O progresso do conhecimento sobre os mecanismos moleculares que regulam a maturação do complexo cumulus-oócito (COC), incluindo a interação cumulus oócito e a importância das células do cumulus para o desenvolvimento do oócito, é necessário para melhorar a eficiência da maturação in vitro (MIV) que é uma etapa crucial na biotecnologia de reprodução assistida. Compreendendo-se melhor a fisiologia e os mecanismos moleculares da maturação do COC, permite que os sistemas de cultivo sejam mais eficientes, no aproveitamento do oócito nas biotecnologias da reprodução assistida.

Embora o protagonista da fertilização seja o oócito, este é dependente das células do cumulus, que atuam como coadjuvantes, permitindo ao oócito desempenhar o seu papel de forma ideal e se tornar apto tanto à fertilização quanto o subsequente desenvolvimento. A importância das células do cumulus para oócito vem sendo relatada à mais de 50 anos, em 1964 os pesquisadores Marston e Chang, demonstraram que as células do cumulus são importantes para manter a viabilidade do oócito em camundongos (MARSTON; CHANG, 1964).

A relevância fisiológica das células do cumulus direciona o critério de seleção de complexos cumulus-oócito para o processo de produção in vitro (PIV), onde se opta por complexos que possuam mais camadas de células do cumulus e que estas não apresentem espaços entre elas. Com isso o interesse nos mecanismos fisiológicos da expansão das células do cumulus e também na comunicação bidirecional entre o cumulus e o oócito cresce no meio científico, visto artigos recentes que mostram as células do cumulus regulando a maturação oocitária via peptídeo natriurético C (NPPC; ZHANG et al., 2010; FRANCIOSI et al., 2014) e o oócito via fosforilação da SMAD2 nas células do cumulus regula estágios finais da maturação oocitária e aumenta a responsividade ao fator de crescimento epidermal (EGF) que leva à expansão do cumulus e a retomada da meiose no oócito (SALHAB et al., 2011; RITTER et al., 2015).

A SMAD2 é fosforilada em resposta aos receptores TFGβ onde se liga BMP15 e GDF9. A sinalização TGFβ é complexa, pois existe a possibilidade da formação de homo e heterodímeros entre GDF9 e BMP15 e dessa forma ativar diferentemente os seus receptores principais que são ALK4, ALK6 e BMPRII (PENG et al., 2013; MOTTERSHEAD et al., 2015). De forma simplificada, a sinalização desencadeada

fosforila as proteínas SMAD2/3 e SMAD 1/5/8 permite a expressão de genes relacionados com expansão das células do cumulus como PTX3, HAS2 e PTGS2 (PENG et al., 2013).

A inibição da via SMAD2/3 durante a MIV em camundongos (YEO et al., 2009) e suínos (GILCHRIST; RITTER, 2011; NAGYOVA et al., 2011) resulta na redução da expansão das células do cumulus. Em adição a via de sinalização SMAD2/3 está envolvida na expressão do receptor de fator de crescimento epidermal (EGFR) nas células do cumulus de camundongos, indicando que a resposta ovariana ao LH e a maturação oocitária podem ser reguladas pela via SMAD2/3 (SU et al., 2010).

Esse dado é muito importante pois o pico de LH (in vivo) e o FSH e AREG/EREG (in vitro) desencadeiam a maturação do COC via células do cumulus (PENG et al., 1991; VAN TOL et al., 1996; RICHARDS et al., 2002; PROCHÁZKA et al., 2011; SUGIMURA et al., 2015). O que induz a expressão dos fatores EGF-like, nas células do cumulus (FREIMANN et al., 2004; PARK et al., 2004) e ativam o seu receptor EGFR nas células do cumulus e estimulam pela via MAPK/ERK, AKT e p38 a retomada da meiose, a maturação do oócito e a expressão de genes promotores da expansão do cumulus (ASHKENAZI et al., 2005; CONTI et al., 2006; GILCHRIST, 2011).

Contudo, há diferenças importantes quanto à influência do oócito entre as espécies. O dogma actual é que, ao contrário do rato, nos bovinos não é necessária a presença do oócito para a expansão das células do cumulus (HUSSEIN et al., 2005; DRAGOVIC et al., 2007). Isso poderia implicar em uma regulação intra-cumulus da expansão. Assim, a hipótese de que o sistema TGFβ poderia estar envolvido no controle intra-cumulus da expansão, uma vez que na literatura a presença de BMP15 e GDF9 nas ;células do cumulus é muito controversa (HOSOE et al. 2011; CRAWFORD; MCNATTY, 2012).

O objetivo deste estudo foi investigar a presença do RNAm de BMP15 e GDF9 e a sua regulação pelo oócito nas células do cumulus, bem como a regulação das vias intracelulares, baseado na hipótese de que existe, no bovino, uma regulação intra-cumulus de expansão das células do cumulus que pode ser atribuída à presença de fatores TGFβ nas células do cumulus, o que leva a uma menor dependência do oócito para expansão, diferindo dos ratos e camundongos onde o oócito é indispensável para que ocorra a expansão das células do cumulus.

2.MATERIAL E MÉTODOS

2.1.Cultivo dos Complexos Cumulus-Oócitos (COCs)

Ovários bovinos obtidos em abatedouro foram transportados em recipiente térmico contendo solução salina estéril acrescida de antibióticos e aquecida a 35-37°C.

No laboratório, foram lavados em solução salina aquecida a 35°C, esterilizados com álcool 70%, e os folículos com diâmetro entre 3 e 8 mm aspirados. Após sedimentação, os COCs foram recuperados e avaliados sob lupa para seleção daqueles de grau 1 e 2. Foram divididos entre 3 grupos experimentais: COCs, OOX e OOX cocultivados com oócitos desnudos (OD) na concentração de 1OD/µl. A IVM foi realizada por 4 e 22 horas em placas de 96 poços com 100µl de TCM199 com sais de Earl suplementado quer com FSH (1 ug/ml) ou com AREG (100 ug/mL), 0,4 % de BSA, 22 ug/ml de piruvato de sódio, 75 µg/ml de amicacina a 38,5 °C em atmosfera húmida e 5,5 % de CO2. Cada momento de cultivo foram realizadas quarto replicatas.

2.2.Oocectomia

Após a seleção, os oócitos foram colocados em placa de Petri contendo meio Hepes coberto parcialmente com óleo e levados ao microscópio invertido acoplado ao micromanipulador (Eppendorf) para realização da oocectomia, ou seja, retirada do conteúdo citoplasmático do oócito. Para tanto, utilizou-se pipeta “holding” padrão e “tip” de 20µm com bizel. O complexo cumulus oócito foi fixado pela holding por pressão de sucção e, em seguida, perfurado com “transfer tip”, que automaticamente aspira todo o conteúdo oocitário, como demonstrado abaixo.

2.3..Investigação da fosforilação da proteína intracelular SMAD2, MAPK14 e MAPK3/1

A expressão das proteínas foi avaliada por Western Blotting com anticorpos comerciais adequados a essa técnica. As células do cumulus foram cultivadas em meio de cultivo como descrito, na presença de AREG, mas foram cultivas por 30

minutos, 1 hora e 3 horas e então recuperadas, foram colocadas em solução RIPA buffer (SIGMA®) contendo cocktail de inibidor de protease e fosfatase (SIGMA®) e armazenadas em -80°C para posterior utilização. O volume de 2µl de amostra (cada amostra continha células do cumulus provenientes de grupos de 40 COCs) foi adicionada a solução tampão (50 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 12.5 mM EDTA, com 0.02% bromophenol blue) e colocadas à 95°C por 5 minutos. As proteínas foram então separadas em 10 %SDS-Poliacrilamida gels e transferidas por eletroforese para a membrana Hybond-P PVDF (GE Amersham, Piscataway, NJ) em 39mM de glicina, 48Mm Tris-Base, 1%SDS, 20% metanol com pH. 8.3. Após a transferência as membranas passaram pelo processo de bloqueio (de acordo com a padronização de cada anti-corpo).

As membranas foram subsequentemente incubadas com os anticorpos primários diluídos em Tris-HCl contendo 0,2% de Tween 20 (TTBS) *overnight* à 4°C. Após lavagem, as membranas foram então incubadas com o anticorpo secundário em TTBS por 1h em temperatura ambiente. As bandas foram reveladas utilizando horseradish peroxidase (HRP) e fotografadas, analizadas e quantificadas pelo software Image Lab. 5.2.1 BIO-RAD. Os resultados de expressão da SMAD2 foram analisados seguindo a relação de expressão da porteína fosforilada com a proteína total. (*)Para esse experimento os COCs foram divididos nos seguintes grupos: COC-, grupo controle sem estímulo de AREG; COC+ grupo com estímulo de AREG e OOX+ grupo sem oócito com estímulo de AREG, com n=4 réplicas/grupo/tempo.

2.4.Investigação da expressão gênica

As células do cumulus provenientes de 20 COCs foram separadas mecanicamente por pipetagem e recuperadas para extração de RNA total ou proteínas, as células do cumulus dos OOX não passaram pelo processo de pipetagem. O RNA total foi extraído com utilização dos kit RNEasy (Qiagen).

Após a extração, o RNA total foi avaliado (pureza e concentração) por espectrofotômetro (NanoDrop ND 1000), tratado com DNase (Invitrogen) e submetido à transcrição reversa (RT-PCR) com a enzima Omniscript (Qiagen) e oligonucleotídeos poli T. A expressão dos RNAm alvos (*BMP15* e *GDF9*) foi investigada por ensaio de PCR em tempo real com o sistema Power SybrGreen PCR Master Mix no ABI Prism 7500 Sequence Detection System (Applied Biosystem). Os

oligonucleotídeos iniciadores foram obtidos na literatura ou delineados a partir de sequências disponíveis no banco de dados GenBank e estão descritos na tabela 1. Quando delineados a identidade dos amplicons foi confirmada pela análise de seu tamanho por meio de eletroforese em gel de agarose 2,5% e, se necessário, por sequenciamento do DNA amplificado.

A expressão relativa de cada gene alvo foi calculada utilizando o método ΔΔCt e corrigida pela eficiência de amplificação das reações utilizando-se a equação descrita por Pfaffl (PFAFFL, 2001). Os valores médios de eficiência para cada gene foram calculados pelo perfil de amplificação de amostras individualmente pelo programa LinRegPCR (RAMAKERS et al., 2003). Para cada primer, a linha de threshold foi fixada automaticamente no ponto médio da janela de linearidade, determinada pelos valores mínimos e máximos de fluorescência. A expressão da ciclofilina (CYC-A) foi utilizada como controle endógeno, uma vez ela foi a mais estável nas células do *cumulus* em estudos anteriores realizados em nosso laboratório

Tabela 1: Informações de primers específicos utilizados para amplificação em real-time RT-PCR.

Gene	**Sequência do Primer**	**Tamanho do fragmento (bp)**	**Temperatura de anelamento (°C)**	**Referência**
BMP15	F: 5'GTCAGCAGCCAAGAGGTAGTG3' R: 5'CCCGAGGACATACTCCCTTAC3'	360	59	CAIXETA et al., 2013
GDF9	F: 5'TGGTCCTTGCTGAAGCATCTAGA3' R: 5'ACAGTGTTGTAGAGGTGGCTTCT3'	202	59	CAIXETA et al., 2013
CYC-A	R: 5'TCCAGAGTGGGAAGAGCTTGCATT3' F: 5'GCCATGGAGCGCTTTGG3' R: 5'CCACAGTCAGCAATGGTGATCT3'	65	60	MACHADO et al., 2009

F, forward primer; R, reverse primer

2.5.Análise Estatística

Os efeitos dos tratamentos sobre os parâmetros elencados acima seguiram a seguinte sequência:

1-Teste de normalidade dos dados e de homogeneidade de variâncias com transformação em LOG de dados se necessário para realização de análise paramétrica.

2-Aplicação de ANOVA ou análise não paramétrica (caso dados não estejam em distribuição normal) ou as variâncias não sejam homogêneas para determinação dos efeitos principais.

3-Testes de Tukey ou Kruskall-Wallis foram utilizados para a comparação das médias quando diferenças indicadas pela ANOVA ou análise não paramétrica, respectivamente.

3. RESULTADOS

3.1.*Expressão de RNAm para BMP15 e GDF9 nas células do cumulus de bovinos*

A oocectomia reduziu significativamente a expressão RNAm *BMP15* e *GDF9* no tratamento com AREG às 4 e 22 horas, e co-cultivo de OOX com DO restaurou a abundância de mRNA de *BMP15* e *GDF9*, não diferindo do grupo controle (Figuras 1 e 2).

Por outro lado, no tratamento com FSH o oócito afetou significativamente a expressão do RNAm de *BMP15* e de *GDF9* às 22 horas e a presença do oócito desnudo não reverteu esse efeito. Embora a expressão de RNAm *GDF9* e *BMP15* às 4 horas não seja diferente significativamente existe uma redução numérica expressiva nos grupos OOX e OOX+OD em relação ao controle (Figuras 1 e 2).

Figura 1 - Expressão relativa de RNAm de BMP15 e GDF9 em células do cumulus cultivadas por 4 horas tratadas com FSH ou AREG. COC – complex cumulus oócito intacto; OOX – oocectomizados; OOX+OD oocectomizados cocultivados com oócitos desnudos;(n=5, FSH e n=4, AREG).

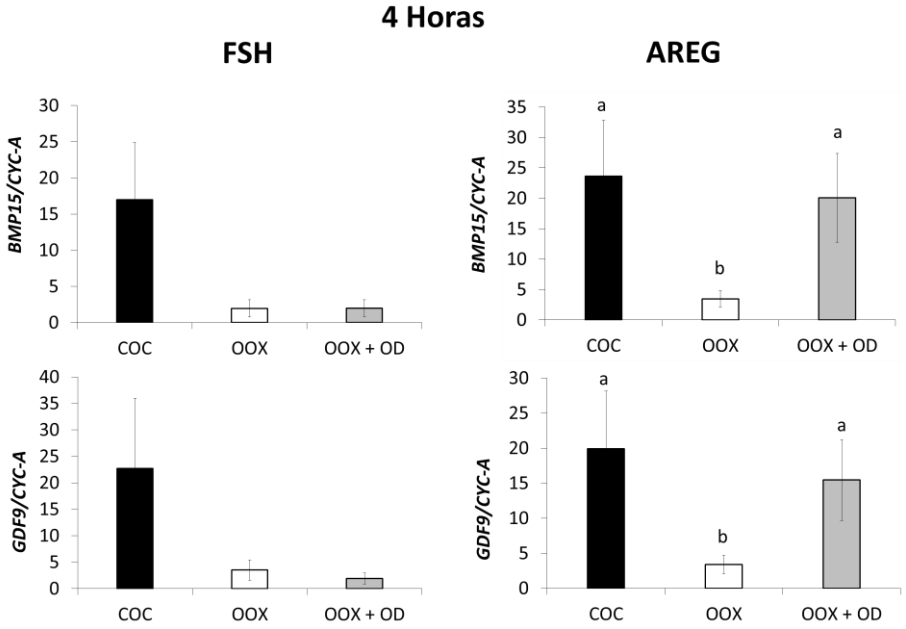
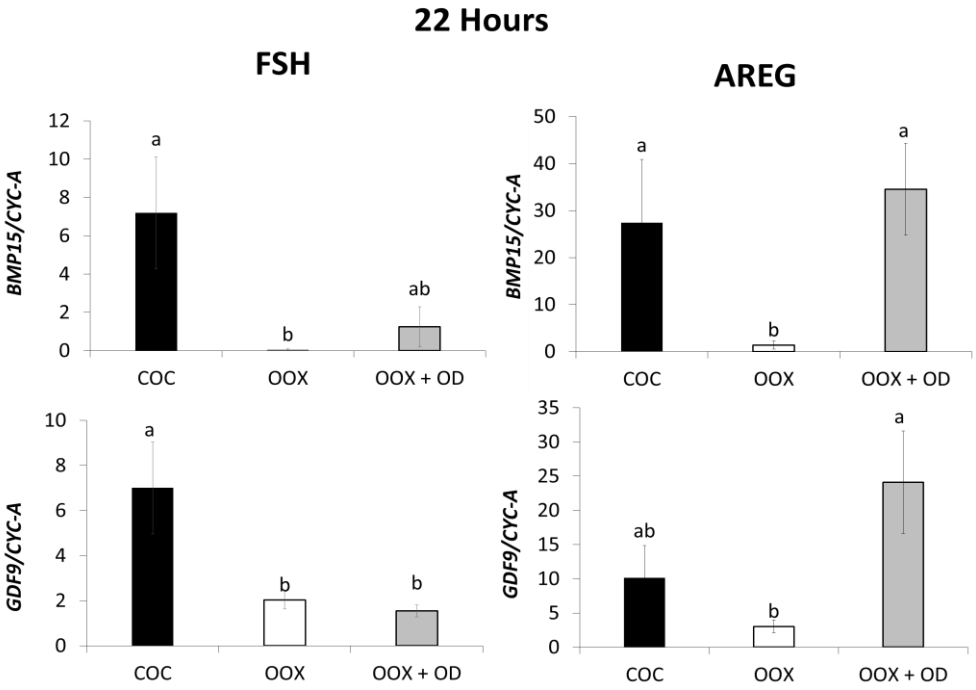


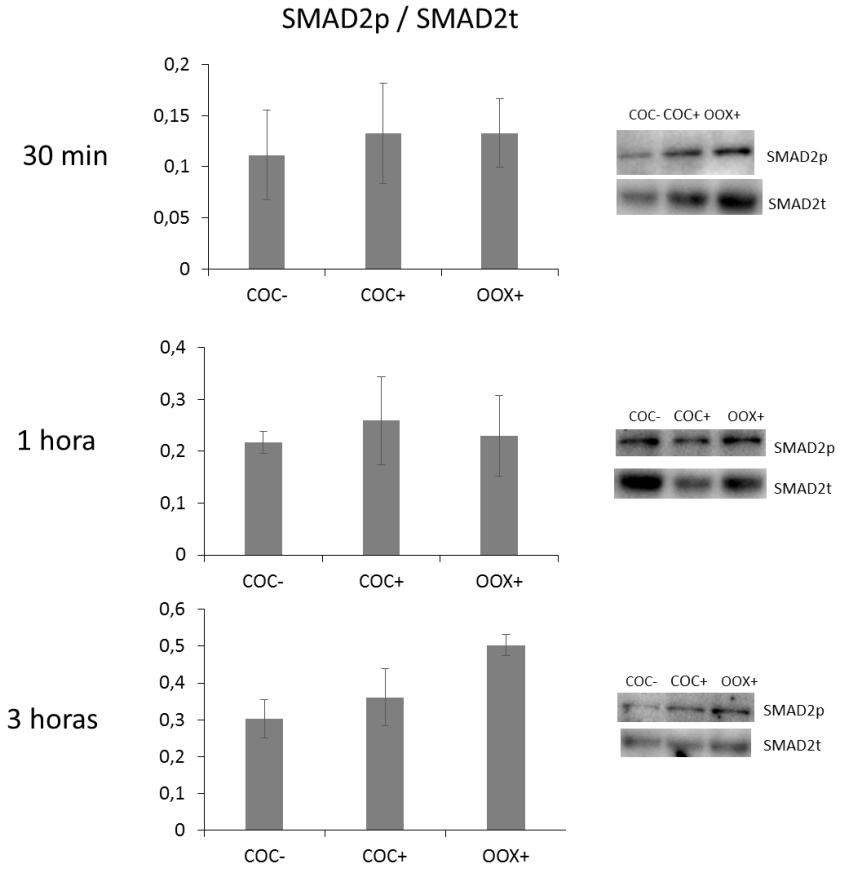
Figura 2 - Expressão relativa de RNAm de BMP15 e GDF9 em células do cumulus cultivadas por 22 horas tratadas com FSH ou AREG. COC – complex cumulus oócito intacto; OOX – oocectomizados; OOX+OD oocectomizados cocultivados com oócitos desnudos; n=3, FSH; n=4, AREG.



3.2.Fosforilação de SMAD2 nas células do cumulus

A fosforilação de SMAD2 nas células do cumulus não foi alterada pela ausência do oócito (grupo OOX). Além disso a adição de AREG ao meio de cultivo durante a maturação não influenciou a fosforilação de SMAD2, não houve diferença entre os grupos COC- e COC+.

Figura 3 - Expressão relative de SMAD2 nas células do cumulus cultivadas por 30 minutos, 1 hora e 3 horas. COC-: complexo cumulus oócito intacto sem presença de AREG; COC+ complex cumulus oócito intacto na presença de AREG; OOX+ oocectomizado na presença de AREG, n=4/momento.

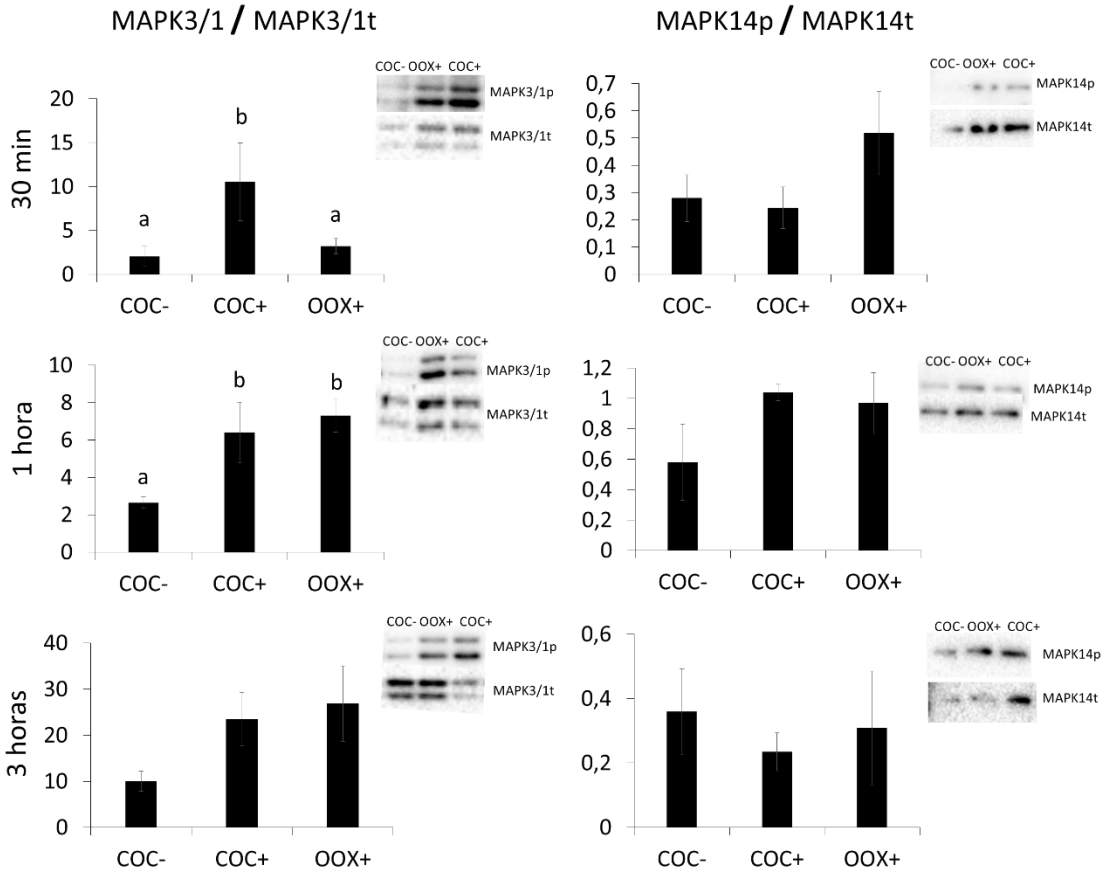


3.3.Fosforilação da MAPK14 e MAPK3/1

A expressão relativa de MAPK14 não foi alterada nem pela presença de AREG nem pela ausência do oócito em nenhum dos momentos de cultivo avaliados (Figura 3).

A expressão relative de MAPK3/1 com 30 minutos de cultivo sofreu influência da presença de AREG e também da ausência do oócito. A presença de AREG estimula a fosforilação de MAPK3/1 no grupo COC+ em relação ao grupo COC- mas não no grupo OOX+ onde a fosforilação de MAPK3/1 se iguala aos níveis do grupo COC- que não sofreu influencia da AREG mas essa redução da fosforilação de MAPK3/1 no grupo OOX+ foi revertida com 1 hora de cultivo. Nenhuma diferença entre os grupos foi encontrada às 3 horas de cultivo (Figura 3).

Figura 4 - Expressão relativa de MAPK3/1 e MAPK14 nas células do cumulus cultivadas por 30 minutos, 1 hora e 3 horas. COC-: complexo cumulus oócito intacto sem presença de AREG; COC+ complex cumulus oócito intacto na presença de AREG; OOX+ oocectomizado na presença de AREG, n=4/momento. Letras diferentes mostram diferenças estatísticas com valor de $p<0,05$.



4. DISCUSSÃO

O s resultados mostram a presença de RNAm de *BMP15* e *GDF9* nas células do cumulus de bovinos, corroborando com estudos anteriores (HOSOE et al., 2011) e descrevemos pela primeira vez que essa expressão é regulada pelo oócito diferentemente quando na presença de FSH ou de AREG.

Os fatores secretados pelo oócito conseguem estimular a expressão de *BMP15* e *GDF9* em OOX apenas quando tratados com AREG, sugerindo que a sinalização AREG é um fator limitante para a ação dos OSF em regular a expressão de RNAm de *BMP15* e *GDF9* nas células do cumulus de bovinos, mas esse controle de expressão talvez não ocorra via ativação de SMAD2 uma vez que ela é independente da presença de AREG,

A fosforilação de SMAD2 no grupo OOX é intrigante pois sugere que a sinalização TGFβ está ativa mesmo sem oócito e com a expressão de RNAm de *BMP15* e *GDF9* reduzida, além disso a fosforilação de SMAD2 mostrou-se não

dependente da presença de AREG. Esse resultado contrasta com os dados encontrados em camundongos, onde a sinalização EGFR é importante para que ocorra a fosforilação de SMAD2 (SASSEVILE et al., 2010), o que evidencia que existem diferenças na sinalização intracelular entre as espécies.

Outro ponto importante é que não só BMP15 e GDF9 podem ativar a via SMAD2, pode se que outras BMPs presentes nas células do cumulus sejam responsáveis por essa ativação como BMP4, BMP6 e BMP7 (ZHANG et al., 2016).

Um ponto a ser considerado é o momento de avaliação da fosforilação de SMAD2, pode ser que os momentos avaliados sejam muito iniciais e que os OOXs ainda estejam sofrendo influencia dos OSF secretados pelo oócito antes da micromanipulação, talvez uma avaliação mais tardia no cultivo seja interessante para confirmer os resultados obtidos.

Em camundongos os OOX tem redução da fosforilação de SMAD2 consequente redução da sinalização EGFR (SU et al., 2010), nossos resultados mostraram que a ausência do oócito não afeta a sinalização da MAPK14 mas houve uma redução na fosforilação de MAPK3/1 apenas nos primeiros 30 minutos de cultivo no grupo OOX, como não houve redução da fosforilação de SMAD2 sugerimos que no bovino, diferente do camungongo a sinalização EGFR não parece totalmente dependente da fosforilação da SMAD2. Resultados anteriores do nosso grupo mostram que na presença de AREG a expansão das células do cumulus não afetada pela ausência do oócito o que poder explicado por não ocorrer redução drástica e constant na sinalização de EGFR quando na ausência do oócito.

Em conclusão, As células de cumulus possuem RNAm de *BMP15* e de *GDF9* e sua expressão nas células do cumulus é regulada pelo oócito. Além disso o estímulo utilizado para provocar a expansão do OOX altera a capacidade do oócito para regular a função das células do cumulus. Além disso a fosforilação de SMAD2 é independente da presença do oócito e não interfere na sinalização EGFR no bovino. Assim, podemos inferior que em bovinos a regulação da sinalização intracellular nas células do cumulus pode ser influenciada de forma diferente de outras espécies.

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CONSIDERAÇÕES FINAIS

Considerações Finais

O Brasil é um grande produtor e exportador de carne bovina, de acordo com dados da EMBRAPA entre 2000 e 2015 o rebanho brasileiro cresceu cerca de 25% e a produção de carne foi de 9,2 milhões de toneladas e em julho deste ano a exportação de carne bovina somou US$423 milhões, além disso a cadeia produtiva gera em torno de 7 milhões de empregos. Sendo assim, a busca na melhoria da eficiência da maturação *in vitro* em bovinos por motivos econômicos e sociais estimula a comunidade científica no desenvolvimento de novas abordagens técnicas para o desenvolvimento de novos meios de cultivo bem como tempo de cultivo para reduzir a perda de oócitos durante o processo de maturação e aumentar a competência do oócito em suportar o desenvolvimento embrionário. Mas o sucesso da técnica depende do conhecimento dos mecanismos fisiológicos que regulam a competência do oócito. Por esse motivo existem muitos estudos envolvendo essa interação e sinalização celular, grande parte em roedores e suínos o que limita a decisão da utilização do método para a maturação *in vitro*. O entendimento dos mecanismos regulatórios da maturação oocitária no bovino se faz necessária. A comunicação entre o oócito e as células do cumulus é de extrema importância para o desencadeamento da maturação do COC e este estudo mostrou que embora ambos, FSH e AREG, estimulem a maturação do COC existem diferenças no processo de expansão das células do cumulus, do metabolismo da glicose e alteração na expressão de genes relacionados com a competência oocitária, essas observações podem ser relevantes e devem levados em consideração na decisão de qual método mais adequado para maturação *in vitro* ou ainda como manipular os mecanismos intracelulares para obter melhores resultados após a fertilização.