UNIVERSIDADE ESTADUAL PAULISTA – UNESP
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA - BOTUCATU

INFLUÊNCIA DO FATOR DE CRESCIMENTO DOS FIBROBLASTOS 2 (FGF2) SOBRE A MATURAÇÃO *IN VITRO* DO COMPLEXO CUMULUS-OÓCITO BOVINO

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BOTUCATU – SP
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RODRIGO GARCIA BARROS

Dissertação apresentada à Faculdade de Medicina Veterinária e Zootecnia da Universidade Estadual Paulista, Campus de Botucatu para obtenção do título de Mestre na área de Biotecnologia Animal.

Orientador: Prof. Dr. José Buratini Júnior.

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Palavras-chave: Apoptose; Células do cumulus; FGF; Meiose; Oócito.

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Lista de abreviaturas

AMPc – Adenosina monofosfato cíclico
AREG – ampirregulina
ATP – Adenosina trifosfato
BAX – BCL2-associated X protein
BCL2 – B-cell CLL/Lymphoma 2
BMP15 – Proteína morfogênica óssea 15
BSA – Albumina bovina sérica
BTC – Betacelulina
CASPASE – do inglês, *cistenyl aspartate-specific protease*
CC – Células do cumulus
CIV – Cultivo *in vitro*
CMK – Clorometil cetona
COC – Complexo cumulus-oócito
DNA – Ácido desoxirribonucleico
ECM – Matriz extra celular
EGF – Fator de crescimento epidermal
EGFR – Receptor do fator de crescimento epidermal
EPM – Erro padrão da media
EREG – Epirregulina
FAS – TNF recptor superfamily member 6
FGF2 – Fator de crescimento dos fibroblastos 2
FGFb – Fator de crescimento dos fibroblastos básico
FGFR – Receptor do fator de crescimento dos fibroblastos
FIV – Fertilização *in vitro*
FLICA – do inglês, *fluorochrome-labeled inhibitors of caspase*
FMK - Fluorometil cetona
FSH – Hormônio folículo estimulante
FSO – Fatores secretados pelo oócito
GDF9 – Fator de crescimento e diferenciação 9
GMPc – Guanosina monofosfato cíclico
GVBD – Vesícula germinativa em quebra (do inglês, germinal vesicle breaking down)

HA – Ácido hialurônico

HAS2 – Hialurona sintase 2

HS – Heparan sulfato

KITL – Kit Ligant

LAV – Meio de lavagem

LH – Hormônio luteinizante

MAPK – do inglês, mitogen activated protein kinase

MIV – Maturação *in vitro*

MPF – Fator promotor da maturação

mRNA – RNA mensageiro

NPPC – do inglês, natriuretic peptide precursor C

nPR – Receptor de progesterone

NPR2 – do ingês, natriuretic peptide receptor 2

OPU – do inglês, *ovarium pick up*

PDE – Fosfodiesterase

PGE2 – Prostaglandina E2

PI – Iodeto de propídeo

PIV – Produção *in vitro*

PKCδ – Proteína qunase c delta

PS - Fosfatidilserina

PTGS2 – do inglês, *prostaglandin-endoperoxide synthase 2*

PTX3 - *pentraxin 3*

TSG6 (ou TNFAIP6) - *Tumor necrosis factor-induced protein 6*

TZP – Projeções trans-zonais

SUMÁRIO

RESUMO

BARROS, RG. **Influência do fator de crescimento dos fibroblastos 2 (FGF2) durante a maturação *in vitro* do complexo cumulus-oócito bovino.** Botucatu, 2016. Qualificação (Mestrado) - Faculdade de Medicina Veterinária e Zootecnia - Universidade Estadual Paulista - UNESP

O conhecimento sobre a interações entre células somáticas e células germinativas é importante para a melhoria na eficácia da maturação oocitária *in vitro.* O oócito é um regulador da diferenciação das células foliculares e através dos fatores secretados pelo oócito (FSO) regula eventos importantes que ocorrem dentro foliculo. O fator de crescimento dos fibroblastos 2 (FGF2) é expresso pelas células do cumulus (CC) e teca e estimula a expansão das CC em bovinos e em roedores, além de ter ação antiapoptótica em células da granulosa, mas suas funções no complexo cumulus-oócito bovino não são muito conhecidas. Uma vez que há receptores de FGF em CC e granulosa sugere que há regulação parácrina e autócrina pelo FGF2. O objetivo desse trabalho foi avaliar a adição de doses crescentes de FGF2 ao meio de maturação *in vitro* e avaliar a taxa de quebra da vesícula germinativa (GVBD), estágio da meiose, grau de expansão das CC, grau de coesividade da matriz extracelular, além de genes relacionados com a maturação. Também avaliar a apoptose das CC, através de marcadores para caspase 3 e análise de genes relacionados a apoptose. Foi observado que a taxa de GVBD é aumentada às 6 horas de maturação, no entanto não alterou os níveis de mRNA de *NPPC* ou *NPR2* às 6 horas; da mesma forma não alterou a porcentagem de oócitos em MII às 22 horas. Não houve diferenças significativas no grau de expansão das CC às 22 horas de cultivo, mas há um aumento na coesão da matriz extracelular dessas células. FGF2 diminuiu *COX2* mRNA às 6 horas de cultivo, além de diminuir a *HAS2* mRNA e elevou o *CD44* mRNA, sem interferir na expressão de *VERS* mRNA às 22 horas de cultivo. Ainda, houve uma diminuição nas CC marcadas para caspase 3 às 22 horas, mas sem alteração na marcação de anexina V. FGF2 não alterou a expressão de *FAS* mRNA, mas aumentou a quantidade de transcritos de *nPR* mRNA e a relação anti-apoptótica *BCL2/BAX*. O presente estudo sugere que FGF2 está envolvido na maturação do COC bovino e a

expressão do RNA nas CC é influenciada pelo oócito, o qual induz a retomada da meiose, alterações na matriz extra celular e atividade da caspase.

Palavras Chave: meiose, células do cumulus*,* apoptose, oócito.

ABSTRACT

BARROS, RG. **Influence of fibroblast growth factor 2 (FGF2) during *in vitro* maturation of bovine cumulus oocyte complexes** Botucatu, 2016. Qualificação (Mestrado) - Faculdade de Medicina Veterinária e Zootecnia - Universidade Estadual Paulista - UNESP

The knowledge about interactions between somatic cells and germ cells is important to improve the oocyte *in vitro* maturation. The oocyte is a regulator of the differentiation of follicle cells and through oocyte secreted factors (OSF) regulates important events that occurs inside follicle. The Fibroblast growth factor 2 (FGF2) is expressed by cumulus cells (CC) and theca cells and stimulates the expansion of CC in bovine and rodents, also have anti-apoptotic action on granulosa cells, but it functions on cumulus-oocyte complexes remains unclear. Once there are receptors for FGF on CC and granulosa cells it suggest that there is a paracrine and autocrine regulation by FGF2. The aim of this study was evaluate the addition of grade doses of FGF2 on maturation medium and assess the germinal vesicle break down (GVBD) rate, meiosis stage, expansion grade of CC, grade of cohesiveness of extra cellular matrix, besides genes related with maturation. Also assess apoptosis on CC, through markers for caspase 3 and analysis of genes related to apoptosis. It was observed an increase in GVBD rate at 6 hours of maturation, but did not alter the rates of mRNA of *NPPC* or *NPR2* ate 6 hours, in the same way, did not alter the percentage of oocytes in metaphase II (MII) ate 22 hours. Did not have significance in expansion grade of CC at 22 hours of maturation, but there is an increase in cohesiveness of extra cellular matrix of these cells. FGF2 decreased *PTGS2* mRNA at 6 hours of culture, and *HAS2* mRNA and increased *CD44* mRNA, without alter expression of *VERSICAN* mRNA at 22 hours of culture. There is a decrease in CC marked for caspase 3 at 22 hours, but no alteration on anexin V was observed. FGF2 did not alter expression of *FAS* mRNA expression, but increased *nPR* mRNA and *BCL2/BAX* ratio. The present data suggest that FGF2 is involved in bovine COC maturation and it is mRNA expression in CC is influenced by oocyte which induces meiosis resumption, alteration of ECM and decrease of caspase activity.

Palavras Chave: meiose, células do cumulus, apoptose, oócito.

CAPÍTULO 1

1. Introdução

O Brasil é líder mundial na produção *in vitro* de embriões (PIV) bovinos e o segundo colocado na produção *in vivo* convencional (Pontes *et al.*, 2011). Esse sucesso se deve à adequação da PIV às raças zebuínas, as principais componentes do rebanho nacional (Pontes *et al.*, 2009). Fêmeas zebuínas usualmente apresentam grande número de folículos antrais nos ovários e, consequentemente, grande disponibilidade de oócitos passíveis de recuperação por OPU (do inglês, *ovum pick up*) para PIV.

O aperfeiçoamento das biotecnologias na reprodução tem como objetivo melhorar e explorar o potencial reprodutivo das fêmeas, além do aumento na produtividade dos rebanhos de corte e leite. Com isso, o uso da PIV de embriões tende a ser intensamente pesquisado, assim aperfeiçoando protocolos e melhorando cada vez mais as taxas de produção.

A PIV compreende a maturação *in vitro* (MIV), fecundação *in vitro* (FIV) e cultivo *in vitro* (CIV). Apesar dessas biotecnologias serem bem estabelecidas, os índices de desenvolvimento ainda são insatisfatórios quando comparados com a produção *in vivo* (RIZOS et al., 2002). Esse desempenho inferior da PIV é associado a diversos fatores como a perda da sincronização entre maturação citoplasmática e nuclear durante a MIV e alterações epigenéticas nos embriões provocadas pelo sistema de cultivo (Blanco et al., 2011). Dessa forma, acredita-se que as biotecnologias envolvidas na PIV podem ser otimizadas aproximando o sistema de cultivo às condições fisiológicas.

Os oócitos estão circundados por células somáticas especializadas, conhecida como células do cumulus (CC). Há uma comunicação birecional entre as CC e o oócito que é crítica para o desenvolvimento do oócito, uma vez que as CC provêm nutrientes, aminoácidos, segundos mensageiros, fatores necessários à maturação, promovendo um ambiente estável e favorável para a maturação do complexo cumulus oócito (COC) (Gilchrist *et al.*, 2008; Assidi *et al.*, 2010; Sutton-Mcdowall *et al.*, 2015).

A maturação nuclear é caracterizada por diversos eventos como remodelagem da cromatina e quebra da vesícula germinativa (VGBD), extrusão dos corpúsculos polares e, consequentemente, o fim da meiose (Meinecke et al.,

2001). Já na citoplasmática ocorre a reorganização das organelas e acúmulo de RNA, proteínas e fatores trascricionais (Watson, 2007).

É de extrema importância que as CC estejam viáveis e não iniciem a apoptose, que é um dos fatores que mais influenciam a competência oocitária (Zaraza *et al.*, 2010). A apoptose é um evento que ocorre em células individuais (Kerr *et al.*, 1972) e pode ser iniciada por fatores intrínsecos (estímulos internos) ou extrínsecos (estímulos externos) (Chang *et al.*, 2002). Esses estímulos desencadeiam respostas que levam a ativação da caspase-3, provocando os eventos finais da morte celular programada (Jin e El-Deiry, 2005).

Parte das CC iniciam o processo de apoptose durante a MIV, sendo que os níveis de apoptose são inversamente associados a competência do oócito (Hussein *et al.*, 2005). A apoptose das CC pode ser reduzida com a adição de fatores secretados pelo oócito (FSO) ao meio de cultivo.

O fator de crescimento dos fibroblastos 2 (FGF2) ou fator de crescimento dos fibroblastos básico (FGFb), mostrou-se efetivo em reduzir a apoptose das células da granulosa em cultivo (Peluso *et al.*, 2001). Há evidências de que uma das ações é a inibição da apoptose pela manutenção da homeostase de íons cálcio livres dentro da célula, através da proteína quinase c δ (PKCδ) (Peluso *et al.*, 2001; Chaves *et al.*, 2012).

Em folículos antrais a maior expressão FGF2 é apresentada nas células da teca (Berisha *et al.*, 2004) e após o pico de hormônio luteinizante (LH) há estímulo para expressão de FGF2 nas CC bovinas (Assidi *et al.*, 2010). Ainda, segundo (Caixeta *et al.*), a expressão dos principais receptores do FGF2 foi detectada nas CC, sendo fortemente estimulada pelo hormônio folículo estimulante (FSH) durante a MIV.

A sinalização FGF é feita através de quatro receptores de FGF (FGFR1 – 4) do tipo tirosina quinase e ocorre arranjos transcricionais alternativosformando as isoformas “b” e “c” apresentando diferentes graus deafinidades por distontos FGFs (Ornitz *et al.*, 1996; Zhang *et al.*, 2006). E o FGF2 ativa o FGFR1 e as isoformas ”c” dos FGFR2 e 3 (Ornitz *et al.*, 1996).

O presente estudo teve como objetivo verificar o padrão de expressão de mRNA de *FGF2* e sua reguação pelo FSH e FSO nas CC bovinas submetidas a MIV. Também verificar os efeitos da adição de FGF2 sobre maturação nuclear de oócitos, expansão e apoptose das CC de COC submetidos a MIV.

2. Revisão de Literatura

2.1. Interação entre o oócito e as células do cumulus

As CC estão próximas do oócito e essa relação de proximidade provê nutrientes e fatores necessários à maturação, proporcionando um micro ambiente que assegura o sucesso da maturação e o desenvolvimento posterior. Em contrapartida, o oócito previne ativamente a morte das CC, regula a expressão de kit ligante, responsável pela maturação nuclear, nas células da granulosa, regula a diferenciação das células da granulosa e CC, expansão das CC. (Gilchrist *et al.*, 2008; Assidi *et al.*, 2010).

A comunicação entre o oócito e as células adjacentes é realizada 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). Recentemente foi descrito a presenca de mRNA nas TZP e sua transferência das CC para o oócito. A presenca de mRNA nas TZP e seu fluxo na direção do oócito em foliculos antrais, reforçam a importância das TZP na comunicação intercelular no COC e indicam que as CC regulam o estoque de mRNA e a síntese proteica do oócito após a queda da sua atividade transcricional (Macaulay et al., 2004).

A comunicação também ocorre via secreção de fatores parácrinos (FSO) (Lodde *et al.*, 2007). Dentre os FSO se destacam o fator de crescimento e diferenciação 9 (GDF9) e a proteína morfogênica óssea 15 (BMP15), que são os dois fatores mais extensamente investigados nesse contexto. Agindo de forma parácrina, eles regulam a diferenciação das células das granulosa (Gilchrist, 2011). E também podem ocorrer através de junções do tipo gap (Lodde *et al.*, 2007).

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 a adenosina monofosfato cíclico (AMPc), o monofosfato cíclico de guanosina (GMPc) e o piruvato, que são fundamentais para o crescimento, metabolismo e

controle da maturação do 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).

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).

A aquisição de competência do oócito para conclusão da meiose, fecundação e desenvolvimento de um embrião viável depende da diferenciação adequada do COC, regulada através desses mecanismos de comunicação intercelular (Coticchio *et al.*, 2004).

2.2. Maturação do complexo cumulus-oócito

O processo de maturação oocitária compreende a maturação nuclear e citoplasmática, sendo que estas devem acontecer em sincronia para que não haja prejuízo celular. Se uma das etapas for incompleta ou assincrônica o desenvolvimento posterior – fecundação e desenvolvimento embrionário – ficam prejudicados (Xu e Greve, 1988).

A maturação nuclear é caracterizada por diversos eventos, como remodelagem da cromatina, VGBD, desaparecimento do nucléolo, extrusão do primeiro corpúsculo polar e formação do segundo fuso meiótico e extrusão do segundo corpúsculo polar, finalizando a meiose (Meinecke *et al.*, 2001). Na maturação citoplasmática ocorre reorganização de organelas, acúmulo de RNA, proteínas e fatores transcricionais (Watson, 2007).

Os oócitos de mamíferos encontram-se bloqueados em prófase I, também conhecido como estado de vesícula germinativa (GV). Após o pico de LH *in vivo,* há retomada da meiose e a maturação progride até a metáfase II, quando é novamente bloqueada até que ocorra a fecundação (Pincus e Enzmann, 1935). Ainda, o pico de LH leva a uma queda nos níveis de mRNA de *NPPC*, o NPPC quando não se liga ao seu receptor, NPR2, resulta em uma diminuição no cGMP dentro das CC, não sendo transferido para o oócito. No oócito a falta de cGMP

eleva os níveis de PDE3A, diminuindo o nível de cAMP, levando a retomada da meiose (Zhang, M. *et al.*, 2010; Zhang *et al.*, 2011).

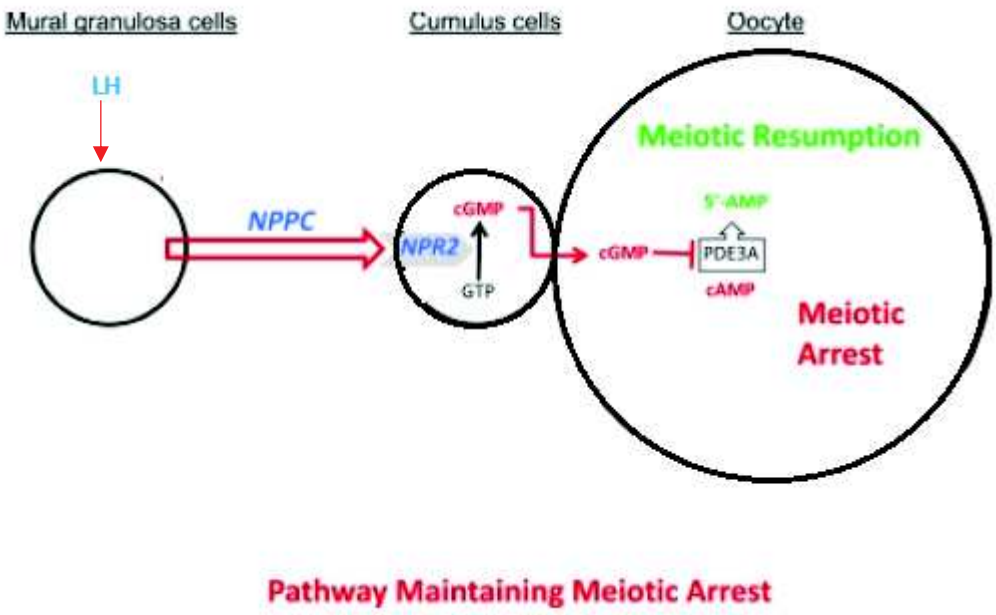

Figura 1. Participação do LH, NPPC e NPR2 nos mecanismos da retomada da meiose. Adaptado de Zhang *et al.* (2011).

Também, as gonadotrofinas induzem a expressão dos fatores EGF-like, nas células da granulosa, como ampirregulina (AREG), epiregulina (EREG) e betacelulina (BTC) (Freimann *et al.*, 2004). Assim que liberados, esses fatores se ligam aos receptores de EGF (EGFR), induzindo a fosforilação das ERK1/2 (Sakaguchi *et al.*, 2000; Su *et al.*, 2003; Conti *et al.*, 2006) e ativação de vias que induzem a fosforilação das conexinas, causando o fechamento das junções gap, levando à perda dos mecanismos inibitórios do AMPc (Gilchrist, 2011). A diminuição dos níveis de AMPc no oócito ativa o fator promotor da maturação (MPF) que promove a fosforilação das proteínas que formam o envelope nuclear, levando a VGBD e consequente retomada da meiose (Millar e Russell, 1992).

Foi demonstrado que a expansão volumétrica do COC está relacionada com a progressão da na maturação nuclear (Yokoo *et al.*, 2010), dessa forma é de extrema importância que ocorra a expansão das CC. Durante a expansão das CC há formação da matriz extracelular (ECM), cujo principal componente dessa ECM é o ácido hialurônico (HA), proveniente do gene hialurona sintase 2 (*HAS2;*

(Tunjung *et al.*, 2009). Quando há produção de HA, este interage com seu receptor na superfície celular, CD44, e desencadeia interações envolvendo fosforilação de MAPK nos oócitos e interrupção de conexina 43, gap junctions presente entre as CC (Tunjung *et al.*, 2009). Assim, fortalecendo a premissa da participação da ECM na maturação celular e processos subsequentes.

A formação da matriz extracelular depende de vários fatores que trabalham em conjunto para a sua correta formação. Então, ocorre um complexo entre PTX3/TSG6 (*pentraxin 3/ Tumor necrosis factor-induced protein 6*) que são responsáveis por se ligar ao HA e estabilizar essa rede (Salustri *et al.*, 2004). Essa interação entre o HA e as proteínas é necessária para a formação e estabilização da ECM (Salustri *et al.*, 2004).

Ainda, a interação HA-CD44 tem a capacidade de diminuir a *prostaglandin-endoperoxide synthase 2* (*PTGS2*), e consequentemente leva a uma diminuição da prostaglandina E2 (PGE2; (Salustri *et al.*, 2004; Liu, C. *et al.*, 2014), promovendo um ambiente adequado aos processos subsequentes. Assim, a formação correta da ECM das CC é dependente de eventos intracelulares e extracelulares complexos que são regulados por fatores parácrinos e autócrinos.

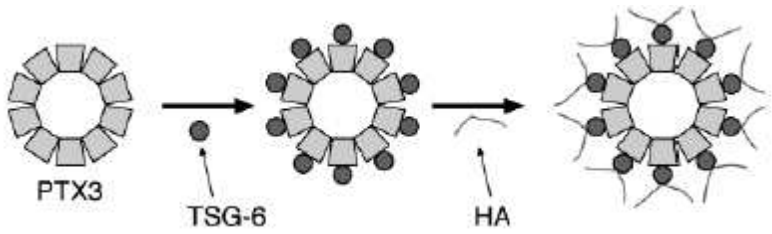

Figura 2. Modelo esquemático da formação da matriz extracelular formada pela ligação entre PTX3 e TSG6 que se estabilizam e formam um ponto de ancoragem para as cadeias de HA. Adaptado de (Salustri *et al.*, 2004).

2.3. Apoptose

Existem dois tipo de morte celular clássica: a necrose e a apoptose. Na necrose os componentes celulares são liberados de forma descontrolada no micro ambiente da célula, resultando em danos às células vizinhas e uma intensa resposta inflamatória no tecido correspondente. Já na apoptose, a célula que

sofre a injúria tem perda da integridade da membrana, inchaço e rompimento, terminando em engolfamento por macrófagos ou células vizinhas, evitando o processo inflamatório (Savill e Fadok, 2000; Leist e Jäättelä, 2001; Jin e El-Deiry, 2005).

A apoptose foi descrita pela primeira vez por (Kerr *et al.*, 1972) como um fenômeno que afeta células individuais e se manifesta, histologicamente, pela formação de pequenos fragmentos citoplasmáticos esféricos a ovais, alguns dos quais contem restos picnóticos dos núcleos. No entanto, tem que ocorrer de forma regulada e balanceada em um contexto fisiológico, uma vez que falhas nesse mecanismo resultam em processos patológicos como tumores (Thompson, 1995). Desde então, intensos esforços têm sido feitos para explorar os mecanismos moleculares e as vias de sinalização da apoptose incluindo iniciação, mediação, execução e regulação (Jin e El-Deiry, 2005).

A apoptose é a morte programada fisiologicamente e está entre os fatores que influenciam a competência oocitária (Zaraza *et al.*, 2010). É caracterizada por alteração na permeabilidade da membrana mitocondrial externa, desorganização da camada bilipídica com translocação da fosfatidilserina (PS) e redução no potencial de membrana mitocondrial regulado por proteínas da família Bcl-2 (Martin *et al.*, 1995; Goldstein *et al.*, 2000; Patterson *et al.*, 2000). Enquanto que no núcleo ocorre ativação da cascata das caspases, que desencadeiam ativação de DNAses que levam à fragmentação do DNA (Saraste e Pulkki, 2000).

Para preservar as funções fisiológicas de cada célula, a membrana plasmática deve permanecer intacta. A preservação da membrana plasmática até estágios avançados da morte celular é o que diferencia apoptose de morte acidental ou necrose (Wlodkowic *et al.*, 2011). Em condições normais, há uma distribuição assimétrica dos fosfolipídeos da membrana plasmática. Durante a apoptose, há translocação da PS, tornando-se exposta e iniciando uma sinalização para que macrófagos fagocitem essa célula apoptótica (Wlodkowic *et al.*, 2011).

Há duas vias que iniciam a cascata da apoptose, conhecidas como via extrínseca, que ocorre através da ativação dos receptores de morte, com ativação da caspase iniciadora 8 ou intrínseca, que ocorre através da via mitocondrial, com ativação da caspase iniciadora 9 e liberação do citocromo c,

presente nas mitocôndrias, para o citosol e refletindo o estresse celular (Chang *et al.*, 2002; Jin e El-Deiry, 2005; Tunjung *et al.*, 2009). No fim, as duas vias convergem para a ativação da caspase-3 e subsequentemente de outras nucleases e proteases que levam aos eventos finais da morte celular programada (Jin e El-Deiry, 2005).

A via extrínseca é ativada por receptores de morte, que pertencem à família do receptor do fator de necrose tumoral, um dos receptores mais estudados e conhecidos pertencentes a essa família é o FAS. Esses receptores têm várias funções, dentre elas iniciar a apoptose (Baud e Karin, 2001). Quando há ligantes nesses receptores, há ativação da caspase-8, formando um complexo de sinalização para indução de morte. No fim da cascata, há ativação da caspase-3, promovendo a apoptose (Green e Evan, 2002; Jin e El-Deiry, 2005). Há evidências de que o aumento nos receptores de progesterona (nPR) faz com que as células se tornem resistentes a apoptose induzida pelo Fas (Quirk *et al.*, 2004).

A via intrínseca da apoptose é iniciada dentro da célula, o ponto mais importante da via ocorre na mitocondria. O principal evento dessa via é a permeabilização da membrana externa da mitocondria, que é regulada pelos membros da família BCL-2; sendo Bax um fator pró-apoptótico e Bcl2 anti apoptótico. Para que ocorra a desestabilização da membrana mitocondrial deve ocorrer uma homodimerização entre Bax, quando o Bcl2 se liga ao Bax, este complexo impede que ocorra a desestabilização da membrana (Jin e El-Deiry, 2005). Com a desestabilização da membrana a apoptose é iniciada por liberação de moléculas da cascata ou por perda de função da mitocôndria, que é essencial à sobrevivência da célula (Green e Kroemer, 2004; Jin e El-Deiry, 2005). Essas situações promovem a translocação das proteínas pró-apoptóticas para a membrana da mitocôndria, levando à liberação do citocromo-c e, subsequentemente, à ativação da caspase iniciadora 9 e da caspase efetora 3, promovendo a apoptose (Green e Evan, 2002; Smaili *et al.*, 2003).

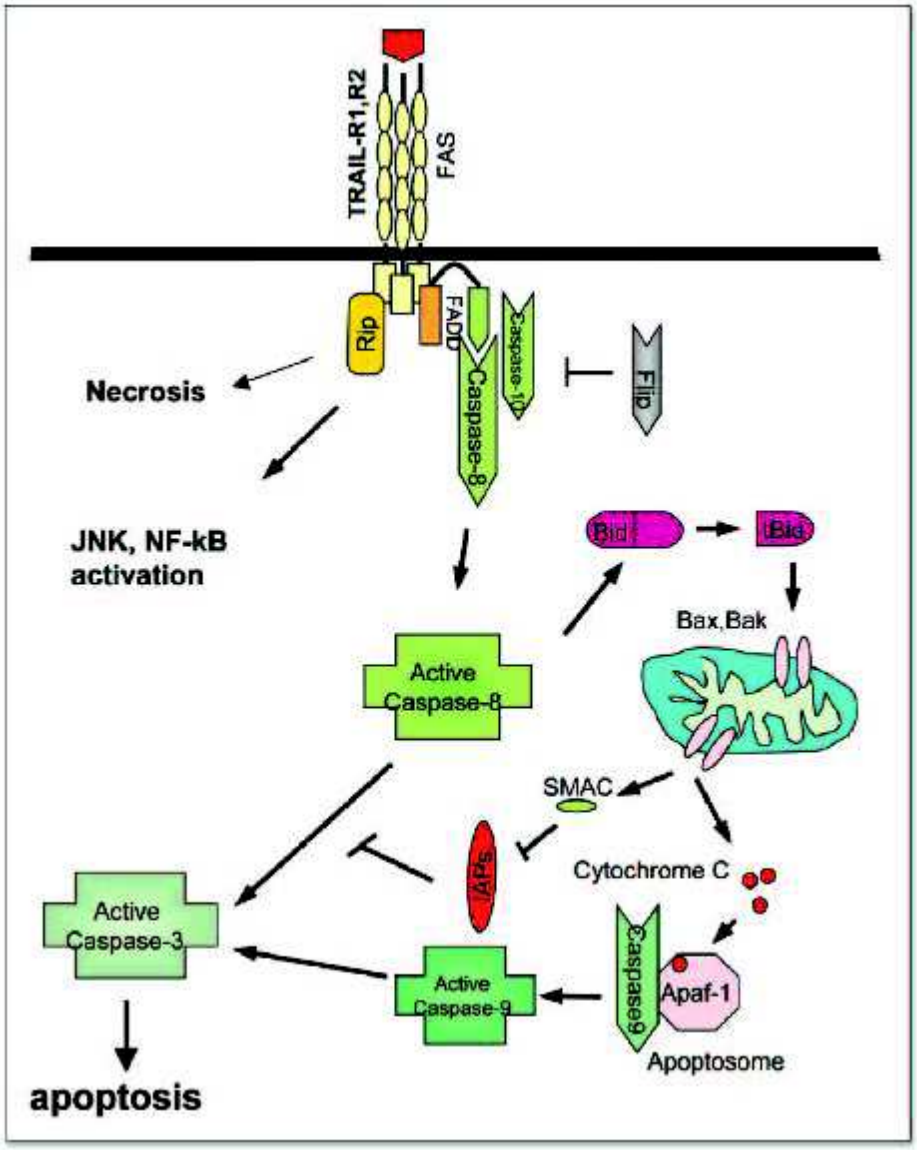

Figura 3. Esquema da apoptose pela via intínseca iniciada pela receptor FAS levando a ativação da pró-caspase 8 e por fim a ativação da caspase efetora 3. Também o esquema de apoptose pela via intrínseca onde há um dano a mitocondria por diversos fatores, dessa forma ocorre uma heterodimerização entre fatores pró-apoptóticos (Bax-Bax) que formam um poro na mitocôndria, levando a sua desestabilização e consequente exposição do citocromo c, que tem capacidade de ativar a caspase efetora 9, que por fim ativa a caspase efetora 3, levando aos evento finais da apoptose. Adaptado de (Jin e El-Deiry, 2005).

Parte das CC entram em apoptose durante a MIV, sendo que os níveis de apoptose são inversamente associados com a competência do oócito e são diminuídos com a adição de fatores secretados pelo oócito ao meio de cultivo (Hussein *et al.*, 2005). A BMP15 parece ser um dos fatores responsáveis por essa ação oocitária anti-apoptótica, uma vez que ela inibiu a expressão de BAX e aumentou a expressão de Bcl2 nas células do cumulus (Hussein *et al.*, 2005).

Também, há evidências de que o FGF2 adicionado ao meio MIV melhora as taxas de sobrevivência das CC, além de limitar a porcentagem de CC TUNEL-positivas (Ikeda *et al.*, 2003; Zhang e Ealy, 2012). Ainda, o tratamento com FGF2 durante a MIV aumentou a maturação do oócito e a formação de blastocistos 7 dias pós FIV (Zhang e Ealy, 2012).

2.4. Marcadores para apoptose em citometria de fluxo

2.4.1. Ativação das caspases

Um dos marcadores da apoptose clássica é a ativação da caspase (do inglês, *cistenyl aspartate-specific protease*). Nos mamíferos, há 14 caspases, das quais apenas 8 participam da execução da apoptose (capase 1, 2, 6, 7, 8, 9, 10 e 12). Em condições normais, as caspases estão expressas no citoplasma com baixa atividade (Boyce *et al.*, 2004; Wlodkowic *et al.*, 2011). Elas são ativadas por uma clivagem trans-catalítica seguida de uma dimerização e, após sua ativação, elas iniciam uma cascata de auto-amplificação, que leva à quebra de substratos vitais das células (Earnshaw *et al.*, 1999).

Um dos métodos para marcação de caspase é a marcação com fluorocromo do inibidor de caspase (FLICA, do inglês, *fluorochrome-labeled inhibitors of caspase*), que permite que a apoptose seja estimada tanto no citômetro de fluxo como em microscópio de fluorescência (Smolewski *et al.*, 2001; Wlodkowic *et al.*, 2011). Cada molécula de FLICA possui três domínios: o fluorocromo (FITC; FAM; SR), o elemento reconhecedor da caspase, específico para cada caspase, composto por quatro peptídeos de aminoácidos e o cloro- ou fluorometil cetona (CMK ou FMK). A porção FMK reage com a cisteína ativa e forma uma ligação covalente irreversível, de maneira que a porção fluorescente

(FITC) permite a sua detecção (Bedner *et al.*, 2000; Pozarowski *et al.*, 2003; Wlodkowic *et al.*, 2011).

O FLICA é extremamente permeável na membrana plasmátiva e relativamente não tóxico, permitindo detectar apoptose em células vivas. (Wlodkowic *et al.*, 2011). Quando o FLICA é usado em conjunto com iodeto de propídeo (PI), diferentes estágios de apoptose podem ser detectados (Pozarowski *et al.*, 2003).

Outra forma, é a detecção da quebra de PARP, que são substratos específicos das caspases. A PARP é uma enzima nuclear envolvida na reparação do DNA e é ativada em resposta a danos ao DNA (De Murcia e Ménissier De Murcia, 1994). A PARP é quebrada pelas caspases 3 e 7, que são marcadores clássicos de apoptose. No entanto, para que esse método seja possível é necessária a fixação da célula (com formaldeído) e permeabilização (normalmente com etanol) (Wlodkowic *et al.*, 2011).

2.4.2. Alterações na membrana plasmática

Para preservar as funções fisiológicas, as células têm que manter a membrana plasmática intacta. A principal característica da membrana plasmática é a assimetria entre os fosfolipídeos da camada interna e externa. Essa assimetria é embaralhada durante a apoptose, quando a PS, que fica na camada interna da membrana plasmática, torna-se exposta (Fadok *et al.*, 1992; Wlodkowic *et al.*, 2011). A exposição da PS inicia a sinalização para que macrófagos fagocitem as células apoptóticas e e corpos apoptóticos (Van Engeland *et al.*, 1998).

Assim, uma das formas de detecção dessas alterações na membrana plasmática é através da proteína anexina V, ligada a um fluorocromo. Essa probe se liga a resíduos de PS na presença de íons cálcio. A anexina V pode ser conjugada com diferentes fluorocromos de diferentes graus de absorção e comprimentos de onda, tornando esse método possível em citometria de fluxo (Van Engeland *et al.*, 1998; Wlodkowic *et al.*, 2011).

As células se tornam reativas à anexina V antes de perderem a habilidade da membana plasmática de excluir corantes como o PI. Sendo assim, ao usar a marcação com anexina V é possível distinguir entre células vivas, apoptóticas e

em apoptose tardia/necrose. Células vivas não apresentam marcação para anexina V ou PI; células em estágio inicial de apoptose, apresentam marcação para anexina V, mas não para PI; e por fim, células em estágios avançados de apoptose/necrose apresentam marcação para ambas as probes (Wlodkowic *et al.*, 2011).

2.5. Fator de crescimento dos fibroblastos (FGFs)

Nos mamíferos, a família dos fatores de crescimento dos fibroblastos contém 22 membros, dos quais 18 são proteínas secretadas que agem através de receptores dos fatores de crescimento dos fibroblastos (FGFR) 1. Os FGFs podem ser classificados em subfamílias de acordo com a função bioquímica, similaridade das sequências e relações evolutivas: FGF1, FGF4, FGF7, FGF8, FGF9, iFGF e hFGF (Ornitz e Itoh, 2015).

Os FGFR são do tipo tirosina quinase e contém um domínio de ligação extracelular, um domínio transmembrana e um domínio tirosina-quinase intracelular. A região extracelular contém um domínio de ligação de heparina, o heparan sulfato (HS), que é uma longa cadeia de carboidratos linear capaz de interagir com ambos FGF ou FGFR, aumentando a afinidade e estabilidade do dímero FGF-FGFR no complexo FGF-HS-FGFR, cuja fosforilação em resíduo de tirosina específico inicia a via de sinalização (Itoh e Ornitz, 2004; Ornitz e Itoh, 2015).

Esses receptores são derivados de 4 genes principais: FGFR1, FGFR2, FGFR3 e FGFR4. Transcritos alternativos do FGFR1, FGFR2 e FGFR3 resultam em duas isoformas distintas “b” e “c”, que apresentam diferentes graus d e afinidade pelos diversos FGFs (Ornitz *et al.*, 1996; Zhang *et al.*, 2006; Price, 2016).

Os FGFs estão envolvidos na oôgenese, foliculogênese e maturação do complexo cumulus oócito, além de regularem processos celulares fundamentais que incluem regulação da proliferação, sobrevivência, migração, diferenciação e metabolismo. O bloqueio dos FGFRs prejudica as taxas de fertilização e desenvolvimento embrionário em vacas (Zhang, K. *et al.*, 2010; Ornitz e Itoh, 2015).

2.5.1. Fator de crescimento dos fibroblastos 2 (FGF2)

O FGF2, também conhecido como FGFb, pertence a subfamília do FGF1 e ativa preferencialmente as isoformas “c” dos FGFRs, mas também é capaz de ativar as isoformas FGFR1b e FGFR4 (Itoh e Ornitz, 2004). O FGF2 é produzido em vários tipos celulares de diferentes tecidos e está envolvido na embriogênese, morfogênese e, principalmente, na angiogênese (Arnaud *et al.*, 1999).

Em folículos antrais de bovinos, as células da teca e da granulosa apresentam expressão do FGF2 (Berisha *et al.*, 2004; Hammadeh *et al.*, 2004). No entanto, foi observado que o pico pré-ovulatório de LH estimula a expressão de FGF2 nas CC bovinas (Assidi *et al.*, 2010). Ainda, a expressão dos mRNA que codificam os principais receptores do FGF2 foi detectada nas CC, sendo fortemente estimulada pelo FSH durante a MIV (Caixeta *et al.*, 2013).

As principais funções do FGF2 na esfera reprodutiva são: estimular a proliferação das células da granulosa em folículos antrais, estimular a angiogênese e diminuir a apoptose e a esteroidogênese em folículos (Gospodarowicz e Bialecki, 1979; Lavranos *et al.*, 1994; Vernon e Spicer, 1994; Berisha *et al.*, 2004; Cao *et al.*, 2006; Woad *et al.*, 2009). Além disso, o FGF2 é capaz de potencializar a ativação de folículos primordiais em ratos, ruminantes e humanos (Matos *et al.*, 2007).

O mRNA do FGF2 apresenta uma extensa região 3’ não traduzida, cerca de 90%. Sendo assim, grande parte do mRNA não é processada e há geração de transcritos alternativos de 18, 21, 22, 22,5, 25 e 34 kDa, todos com terminal C comum. Somente a isoforma de baixo peso molecular (18 kDa) é secretada e tem função parácrina e autócrina, enquanto as demais isoformas permanecem no interior da célula e participam de funções intrácrinas (Arnaud *et al.*, 1999).

A maioria dos FGFs são secretados através da via convencional retículo endoplasmático-complexo de Golgi. No entanto, o FGF2 é secretado através de uma via não usual. Ele parece ter a capacidade de se translocar através da membrana plasmática para atingir o ambiente extracelular (Schäfer *et al.*, 2004; Steringer *et al.*, 2015).

Há evidências de que o FGF2 inibe a apoptose através da manutenção dos íons cálcio (Ca^{2+}) livres dentro da célula através da proteína quinase C, desta

forma, mantendo a homeostase necessária a sobrevivência das células (Peluso *et al.*, 2001).

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CAPITULO 2

Fibroblast growth factor 2 regulates *in vitro* maturation of bovine cumulus oocyte complexes: evidences for a role mediating the influence of the oocyte and FSH

Abstract

In this study we first assessed regulation of *FGF2* mRNA abundance in bovine cumulus cells during IVM, testing the influence of time in culture, FSH and oocyte secreted factors. Then we tested the hypothesis that FGF2 regulates nuclear maturation, cumulus expansion, extracellular matrix (ECM) cohesiveness and apoptosis in cumulus-oocyte complexes (COC) undergoing IVM. During IVM *FGF2* mRNA expression is regulated, the higher expression occur at 4 hours of culture and are maintained until the end of maturation, also *FGF2* mRNA expression is increased with the presence of FSH. The removal of the oocyte decreased *FGF2* mRNA expression at 22 hours of IVM and co-culture with denuded oocytes (DO) restored mRNA expression. The addition of FGF2 to the maturation medium increased the percentage of oocytes showing germinal vesicle breakdown (GVBD) at 6 hours, but did not influence the percentage of oocytes at meiosis II (MII) at 22 hours, nor the abundance of *NPPC* or *NPR2* mRNA. Moreover, FGF2 did not influence the percentage of COC fully expanded at the end of IVM, but increased ECM cohesiveness and *CD44* mRNA abundance, while decreasing *PTGS2* and *HAS2* mRNA levels. FGF2 also decreased the percentage of cumulus cells staining for caspase 3/7, which was accompanied by increase in the ratio *BCL2/BAX* and *nPR* mRNA abundance. These results suggest that FGF2 controls nuclear maturation, cumulus expansion and apoptosis during IVM, mediating influences of the oocyte and FSH.

1. Introduction

Oocyte developmental competence is acquired during folliculogenesis and final stages of follicle growth before ovulation. Any disturbance in this process compromises oocyte developmental competence (Krisher, 2004). Therefore, *in vitro* maturation (IVM) is a crucial stage for artificial reproductive technologies. It has been long known that cumulus cells (CC) are important to maintain oocyte viability and for ovulation and fertilization (Chen *et al.*, 1993).

Cumulus oocyte bidirectional communication by paracrine factors is crucial to acquisition of oocyte competence, cumulus cells secretes KITL that regulates oocyte maturation and the oocyte secretes BMP15, FGF8 and FGF10 that regulates KITL mRNA expression in cumulus cells (Hussein *et al.*, 2006) oocyte also regulates other cumulus cells function as glucose metabolism and expansion (Sutton *et al.*, 2003)

In vitro, meiosis resumption and cumulus expansion are usually initiated by FSH, which can directly stimulate the production of EGF-like factors in cumulus cells (Gilchrist, 2011). Differently, *in vivo,* LH first activates the EGF-like system in mural granulosa cells, and EGF-like factors then signal to cumulus cells to stimulate their own production (Gilchrist, 2011). EGF-like factors decrease *NPPC* mRNA expression somatic cells of preovullatory follicle (Liu, X. *et al.*, 2014; Zhang *et al.*, 2014; Hao *et al.*, 2016), and reduced NPPC/NPR2 signalling decreases cGMP production in CC, augmenting the activity of PDE3A on cAMP. Reduced cAMP levels allow activation of MPF and thus meiosis resumption (Zhang *et al.*, 2011). In addition, via MAPK-ERK1/2, EGF-like factors lead to phosphorylation of connexins and increased expression of genes involved in CC

expansion and organization of the extra cellular matrix (ECM; (Diaz *et al.*, 2006; Procházka *et al.*, 2011; Zhang *et al.*, 2014).

Many genes/proteins are involved in ECM formation. Hyaluronan synthase 2 (HAS2) is the key enzyme required for production of hyaluronic acid (HA) by cumulus cells, the fundamental component of the cumulus matrix (Chen *et al.*, 1993). Tumor necrosis factor-induced protein 6 (TSG6) is a protein that binds both HA and PTX3, and thus is important for matrix structure/organization (Fulop et al., 2003). Pentraxin 3 (PTX3), is secreted by CC and is responsible to stabilize TSG6 maintaining the ECM structure (Salustri *et al.*, 2004). Also, HA has the ability to bind to its receptor CD44 in the CC surface, which signals via intracellular cascades such as MAPK cascade involved in oocyte maturation (Yokoo *et al.*, 2010). Prostaglandin synthase 2 (PTGS2) responsible for prostaglandin E2 production in cumulus cells, that is important to stimulate cumulus ECM disassembly, which contributes to successful fertilization, furthermore prostaglandin E2 also stimulates AREG and EREG leading to MAPK3/1 activation in CC (Yamashita *et al.*, 2011).

Apoptosis is regulated in a physiological context and any disturbance of this process can lead to pathological conditions (Jin e El-Deiry, 2005). When the apoptosis cascade is triggered, it leads to caspase 3/7 activation and terminal events of apoptosis (Jin e El-Deiry, 2005). There are two major pathways to reach caspase 3/7 activation, the first known as the mitochondrial pathway (intrinsic), which involves *BCL2* and *BAX,* genes responsible for mitochondrial dysfunction and activation of caspase 3/7. The second is known as the death receptor pathway (extrinsic), which results from activation of FAS, a cell surface receptor that initiates this cascade (Jin e El-Deiry, 2005).

Fibroblast growth factor 2 (FGF2), also known as basic FGF (bFGF), is expressed predominantly by theca cells during follicle growth in humans (Hammadeh *et al.*, 2004) and cattle (Berisha *et al.*, 2004). The ovulatory LH peak increases *FGF2* mRNA expression in CC (Assidi *et al.*, 2010) and FSH increases the expression of fibroblast growth factor receptors (FGFR) during IVM in cattle (Caixeta *et al.*, 2013). Activation of FGF receptors enable phosphorylation of mitogen activated protein kinase (MAPK) and Akt (Dailey *et al.*, 2005; Jiang *et al.*, 2011). FGF2 stimulates granulosa cell proliferation and angiogenesis in antral follicles, and inhibit apoptosis and steroidogenesis in granulosa cells (Gospodarowicz e Bialecki, 1979; Baird e Hsueh, 1986; Lavranos *et al.*, 1994; Vernon e Spicer, 1994; Peluso *et al.*, 2001).

The first aim of this study was to assess the expression patterns and regulation of *FGF2* mRNA by FSH and oocyte secreted factors in bovine cumulus cells during IVM. The second aim was to assess the effects of FGF2 on oocyte nuclear maturation, cumulus expansion and apoptosis in bovine cumulus-oocyte complexes undergoing IVM.

2. Material and Methods

Unless where specified, all chemicals and reagents were purchased from Sigma.

Ovaries and COC culture

Ovaries of adult cows (predominantly Nellore, *Bos indicus*) were obtained from an abattoir local to the São Paulo State University campus in Botucatu and transported to the laboratory within 2 hours in sterile saline solution (0.9% NaCl)

maintained at 26 °C. COCs were aspirated from 3–8 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 homogenous cytoplasm and multiple compact layers of cumulus cells were used. COCs were washed and transferred in groups of 20 to a drop of basic maturation medium: TCM199, containing Earle's salts supplemented with porcine FSH (Folltropin-V Bioniche Animal Health, Belleville, ON, Canada; concentrations specified below for each experiment), 22 μg/ml sodium pyruvate, 75 μg/ml amicacin, 4 mg/ml BSA. Drops were incubated at 38.5 °C in 5% CO2 in humidified air.

Expression patterns of *FGF2* mRNA during IVM: effects of time, FSH and oocyte secreted factors

To assess the effect of time in culture on FGF2 mRNA abundance, 20 COC were cultured in drops of 100 μL of maturation medium covered by mineral oil and containing 10^{-2} μg/mL of FSH (Folltropin-V Bioniche Animal Health, Belleville, ON, Canada) for 0, 4, 8, 12, 16 and 20 hours (4 replicates).

To assess the effect of FSH concentration on FGF2 mRNA abundance, 20 COC were cultured for 12 hours in 100 μL drops of maturation medium supplemented with graded concentrations of FSH (0, 10^{-4}, 10^{-3}, 10^{-2} and 10^{-1} μg/mL; 4 replicates).

Oocytectomy was performed to test the effects of the presence of the oocyte on *FGF2* mRNA expression. To assess whether the influence of the oocyte would be mediated by oocyte secreted factors, oocytectomized COCs were cultured in the presence or absence of denuded oocytes, and compared

with intact COCs. Therefore, the three experimental groups: intact COCs, oocytectomized COCs (OOX) and OOX + denuded oocytes (OD; 1 oocyte/μL). Groups of 20 COCs were cultured in 96 wells plates with 100 μL of base medium with FSH (1 μg/mL) for 22 hours (5 replicates) and for 4 hours (4 replicates). For oocytectomy, COC 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 previously described by (Lima et al. 2016)). After culture, CC were recovered to assess *FGF2* mRNA abundance.

Effects of FGF2 on nuclear maturation dynamics

To assess the effects of FGF2 on germinal vesicle breakdown (VGBD) dynamics and meiosis progression, groups of 20 COC were cultured for 6 (5 replicates) and 22 hours (8 replicates) respectively, in drops of 450 μL of basic medium supplemented with graded concentrations of FGF2 (0, 1, 10 and 100 ng/mL). After culture, oocytes were denuded mechanically by repeated pipetting in PBS, fixed in methanol 60% overnight at 4°C, stained for 15 minutes with 5μl Hoechst 33342 (Roche, Indianapolis, USA) and analyzed under UV light using epifluorescence microscopy. Oocytes cultured for 6 hours were classified as GV (germinal vesicle) or GVBD (germinal vesicle breakdown) and oocytes cultures for 22 hours was classified as metaphase I (MI) or telophase I/metaphase II (TI/MII).

Effects of FGF2 on cumulus expansion, matrix cohesiveness and related genes

To assess the effects of FGF2 on cumulus expansion, groups of 20 COCs were cultured in drops of 450 μL supplemented with graded concentrations of FGF2 (0, 1, 10 and 100 ng/mL) for 22 hours (5 replicates). Expansion degree was visually assessed according to a subjective scoring system. Grades 1–3 were attributed to increasing degrees of expansion (1 – poor expansion, characterized by a few morphological changes; 2 – partial expansion, characterized by fair expansion but notable clusters lacking expansion; 3 – complete or nearly complete expansion; (Kobayashi et al. 1994)).

Matrix cohesiveness was assessed by counting the number of pipetting movements necessary to completely denude the expanded COCs after IVM, which was performed by the same individual throughout the experiment (5 replicates).

To assess mRNA abundance of genes regulating cumulus expansion, cumulus cells were mechanically separated from COCs by repeated pipetting in PBS after six (*CD44, PTGS2, PTX3, TNFAIP6, HAS2, NPPC, NPR2* and *VERSICAN;* 5 replicates) or 22 hours of IVM (*CD44*, *PTGS2, HAS2* and *VERSICAN;* 5 replicates).

Effects of FGF2 on apoptosis/CC survival and related genes

Pools of 20 COCs were cultured in drops of 450 μL of basic medium supplemented with graded concentrations of FGF2 (0, 1, 10 and 100 ng/mL) for 22 hours. After culture, oocytes were denuded mechanically by repeated pipetting in drops of 100 μL PBS and the CC were transferred to a 5mL tube (Sarstedt, Nümbrecht, Alemanha), where 100 μL of PBS, 50 μg/mL of propidium iodide (PI - BD Pharmingen; 0,5 μg/mL), 100 μg/mL of Hoechst 33342, 0.2μL

CellEvent™ caspase 3/7 flow cytometry assay kit (Thermo Scientific, Wilmington, DE, USA; according to manufacturer's protocol), 2µL of Anexina V-APC (BD Pharmingen; according to manufacturer's protocol) were added. The resulting solution was incubated at 37.5 °C for 15 minutes, and then analyzed in a flow cytometer (BD LSR Fortessa; Becton Dickinson, Mountain View, CA, USA). The data were analyzed by the software FACSDiva™ V6.2 (6 replicates).

To assess mRNA abundance of genes regulating apoptosis (*nPR, FAS, BAX* and *BCL2)*, cumulus cells were mechanically separated from COCs by repeated pipetting in PBS after 22 hours (5 replicates).

RT-PCR

Cumulus cells were transferred to 1.5 mL tubes, collected by centrifugation for 5 min at 700 G, 350 mL of the RNA extraction lysis buffer from the RNeasy® kit (Qiagen, Mississauga, ON, Canada) was added to the cell pellets and samples were stored at -80 °C until RNA extraction. Total RNA was extracted from cumulus cells 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) was incubated with DNAse I to prevent interference of DNA contamination with the PCR analysis (1 U/mg; Invitrogen, São Paulo, Brazil) and then reverse transcribed using Oligo-dT primers and Omniscript reverse transcriptase (Qiagen, Mississauga, ON, Canada). The reagents were incubated at 37 °C for 60 min and then at 93 °C 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 seconds followed by annealing for 1 min (40 cycles). The primers for the housekeeping gene *CYC-A* and target genes were used and validated according to table 1. The primer sequences, amplicon sizes, and annealing temperatures for each target gene are given in table 1. Primers to amplify bovine *FAS and FGF2 were* designed with PrimerQuest Tool and are shown in table 1. The relative expression values for each gene were calculated using the ΔΔCt method with efficiency correction and using one control sample as calibrator (Pfaffl 2001).

Statistical analysis

Meiosis progression, maturation, cumulus expansion and apoptosis data were arcsine transformed before analysis, and gene expression and matrix cohesiveness data were transformed to logarithms when not normally distributed. The effects of treatments were tested by analysis of variance (ANOVA), and means were compared with the Fisher-Protected test, using JMP software (SAS Institute, Cary, NC, USA). Differences were considered significant when $P<0.05$.

3. Results

Regulation of *FGF2* mRNA levels in CC during IVM: influence of time, FSH and oocyte derived factors

The FSH dose response and time course experiments were performed to assess the regulation of *FGF2* mRNA abundance in CC during IVM. *FGF2* mRNA expression increased at 4 hours of culture and did not change after that (Fig 1A). FSH increased *FGF2* mRNA levels in a dose dependent manner, with the first significant increase induced by 10 ng/mL of FSH (Fig 1B).

Removal of the oocyte did not affect *FGF2* mRNA expression at 4 hours in the presence of either FSH or AREG, but significantly decreased *FGF2* mRNA abundance at 22 hours with both maturation inducers, which was reversed by the addition of denuded oocytes (Fig 2).

Effects of FGF2 on oocyte nuclear maturation, cumulus expansion, matrix cohesiveness and related genes

Addition of FGF2 at 1ng/mL to the IVM medium increased the percentage of oocytes with GVBD at 6 hours, but higher concentrations did not alter germinal vesicle dynamics (Fig 3A). FGF2 did not affect the percentage of oocytes reaching meiosis II at 22 hours of culture (Fig 3B).

FGF2 did not alter the percentage of COC exhibiting full expansion, but increased ECM cohesiveness at all concentrations tested (Fig 4). The effects of FGF2 on expansion related genes were assessed at 6 and 22 hours of culture. FGF2 decreased *PTGS2* mRNA levels at all concentrations and decreased *TNFAIP* at concentration of 100ng/mL at 6 hours of culture (Fig 6). At 22 hours of culture, FGF2 increased *CD44* mRNA abundance at 1 ng/mL, and decreased *HAS2* mRNA abundance at 100 ng/mL. FGF2 had no effect on *VERS* mRNA levels (Fig 6). FGF2 had no influence on mRNA abundance of *NPPC* or *NPR2* (Fig 8).

Effects of FGF2 on cell survival, apoptosis of CC and related genes

Treatment with FGF2 increased CC survival at concentrations of nor percentage of cells staining for Annexin V, but reduced the number of caspase 3/7 positive cells at 10 and 100 ng/mL (Fig 5). The effect of FGF2 on apoptosis genes did not alter *FAS* expression but increased *nPR* mRNA levels at 100 ng/mL and the ratio *BCL2/BAX* at 10 ng/ml (Fig 7).

4. Discussion

Previously studies have shown that *FGF2* mRNA increases after LH surge *in vivo* (Assidi et al. 2010). Also, *FGF2 receptor* mRNA expression is increased by FSH *in vitro* (Caixeta et al. 2013). There are no previous studies exploring the influence of oocyte or FSH on *FGF2* mRNA levels. More, it has been already shown that FGF2 have the ability to induce MAPK/ERK pathway leading to meiosis resumption (Jiang et al. 2011). Here we demonstrate the regulation of *FGF2* mRNA expression on CC is influenced by oocyte and FSH, also the results are in according to (Jiang et al. 2011) but we demonstrate that FGF2 did not influence the maturation stage at 22 hours. Previously studies have demonstrated that FGF2 decrease apoptosis on granulosa cells (Peluso et al. 2001) also it has been shown a protective effect of nPR on rat granulosa cell (Friberg et al. 2010) and a decrease in caspase 3 activity and increase in *BCL2/BAX* ratio in bovine luteal cells (Liszewska et al. 2005) and here we demonstrated the effects of FGF2 decreasing apoptosis on CC through increase

of nPR and *BCL2/BAX* ratio and decrease in caspase 3 activity. These results show an important role of FGF2 on bovine COC maturation.

The pattern of mRNA expression of *FGF2* in CC during IVM is interesting and the present data are consistent with increased *FGF2* mRNA in bovine CC after the LH surge *in vivo* (Assidi et al. 2010). The stimulation of FGF2 signaling by FSH was reinforced by a previous study in which the expression of FGF2 receptors was also stimulated by FSH during IVM in cattle (Caixeta et al. 2013). In addition, we demonstrate that the oocyte regulates *FGF2* mRNA expression in cumulus cells stimulated with FSH and co-culture of oocytectomized COC with DO indicates that this is mediated by oocyte secreted factors.

To investigate the actions of FGF2 on COC during IVM, we first tested effects of FGF2 on nuclear maturation at 6 hours of IVM, and observed that oocytes treated with 1 ng/ml FGF2 were more prone to undergo germinal vesicle breaking down (GVBD), but did not affect meiosis progression at 22 hours. NPPC/NPR2 signaling is essential for maintaining meiotic arrest, but FGF2 did not change *NPPC* or *NPR2* mRNA expression. Whether FGF2 regulates NPPC/NPR2 post-transcriptionally remains to be assessed.

A previous study demonstrated that FGF2 stimulates the MAPK/ERK pathway in bovine granulosa cells (Jiang et al. 2011), the same signaling pathway activated by FSH and AREG to induce meiosis resumption and induce CC expansion in mouse, pigs and bovine (Diaz et al. 2006; Gilchrist 2011; Procházka et al. 2011). We can thus infer that FGF2 may stimulate meiosis resumption and regulate CC expansion via ERK1/2 signaling pathways (Dailey et al. 2005; Jiang et al. 2011).

In the present study, FGF2 does not alter CC expansion but FGF2 decreased *HAS2* mRNA expression at 22 hours of IVM, which may be not so important at this point of maturation. Hyaluronic acid (HA) is the product of *HAS2* and is the main component of the ECM and thus fundamental for cumulus cells expansion in mouse (Chen et al. 1993). The ECM is important to facilitate COC to deform and easily pass through the ruptured follicle wall during the ovulation in porcine (Nagyova 2012). FGF2 also decreased *PTGS2* mRNA abundance, which is required for PGE2 production and cumulus expansion. The association of CD44-HA have the ability to diminish the PGE2 by suppressing the PTGS2 (Yasuda 2010). In contrast in this study, *PTGS2* expression was decreased at 6 hours, before the increase of CD44 which infer that reduction of *PTGS2* mRNA expression was not associated with CD44-HA interaction.

Progesterone signaling was demonstrated to be essential for cumulus expansion once PTGS2 signaling lead to PGE2 production during maturation, increasing ability to production progesterone (Nuttinck et al. 2008), that maintain and induce a protease which is required for production of mature forms of AREG and EREG (Yamashita et al. 2011). Thus, progesterone and PTGS2 participate in the EGFR signaling required for CC expansion and oocyte maturation (Fair and Lonergan 2012). In contrast, in this study the reduction of *PTGS2* mRNA expression was not correlated with the increase in the *nPR* mRNA expression.

ECM is constituted by several proteins others than CD44 and HA, such as PTX3, TSG6 also VERS and ADAMTS-1, which are produced by mural granulosa cells, but specifically translocate to and act within the COC. This combined endocrine and paracrine intrafollicular communication enables the co-ordination of maternal receptivity with oocyte maturation (Russell and Robker 2007). All

these proteins interact to form the correct structure of ECM. Any disruption in this structure can affect ECM cohesiveness in the COC (Salustri et al. 2004). In the control of angiogenesis, FGF2 compete with TSG6 to bind to PTX3 (Leali et al. 2012). This represents a possible mechanism by which FGF2 could directly decrease PTX3/TSG6 binding and thus TSG6/HA interaction, altering ECM stability.

The cellular viability is characterized by whole cells and FGF2 increased the number of viable cells, also FGF2 decreased the number of CC marked for caspase 3/7, suggesting a decrease in number of apoptotic cells. This can be correlated with the increase of *nPR* mRNA expression and/or increase *BCL2/BAX* ratio.

Progesterone protects oocyte from onset of apoptosis and lower rates in CC apoptosis are related with better developmental competence of oocytes (Fair and Lonergan 2012). The main anti apoptotic action of progesterone is through nPR once cells treated with nPR antagonist Org 31710 induced increase in activity of caspase 3 (Friberg et al. 2010). Also, treatment of bovine luteal cells with progesterone for 24 hours decreased caspase 3 activity and increased *BCL2/BAX* ratio (Liszewska et al. 2005). Therefore, (Fair and Lonergan 2012) affirm that the anti-apoptotic effects of progesterone are not confined to one pathway, but attributed to several pathways and processes occurring during oocyte maturation. Considering all together, FGF2 protects CC from apoptosis by decreasing caspase 3/7 trough increase *nPR* and *BCL2/BAX* ratio.

In this study the oocyte stimulates *FGF2* mRNA expression in cumulus cells and co-culture of oocytectomized with DO indicates that this is mediated by oocyte secreted factors and treatment with FGF2 decreased the number of CC

marked for caspase 3/7. According to (Hussein et al. 2005) OSF can decrease CC apoptosis, considering this the oocyte can regulate apoptosis maybe by stimulation of FGF2 expression on CC.

In conclusion, the present data suggest that FGF2 is involved in bovine COC maturation and it is mRNA expression in CC is influenced by oocyte which induces meiosis resumption, alteration of ECM and decrease of caspase activity.

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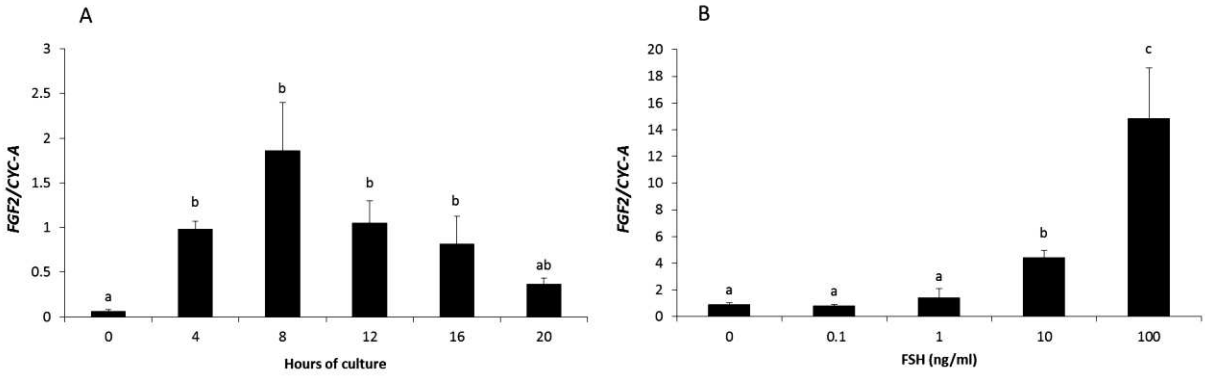

Figure 1. Effects of time in culture (A) and FSH dose (B) on *FGF2* mRNA abundance in bovine CC undergoing IVM. To assess the effects of FSH dose, COC were cultured for 12 hours (n=3-4 replicates per time or FSH dose). Data are presented as mean values (±S.E.M.) relative to a calibrator sample calculated by the ΔΔCt method with efficiency correction. Bars with different letters are significantly different (P<0.05).

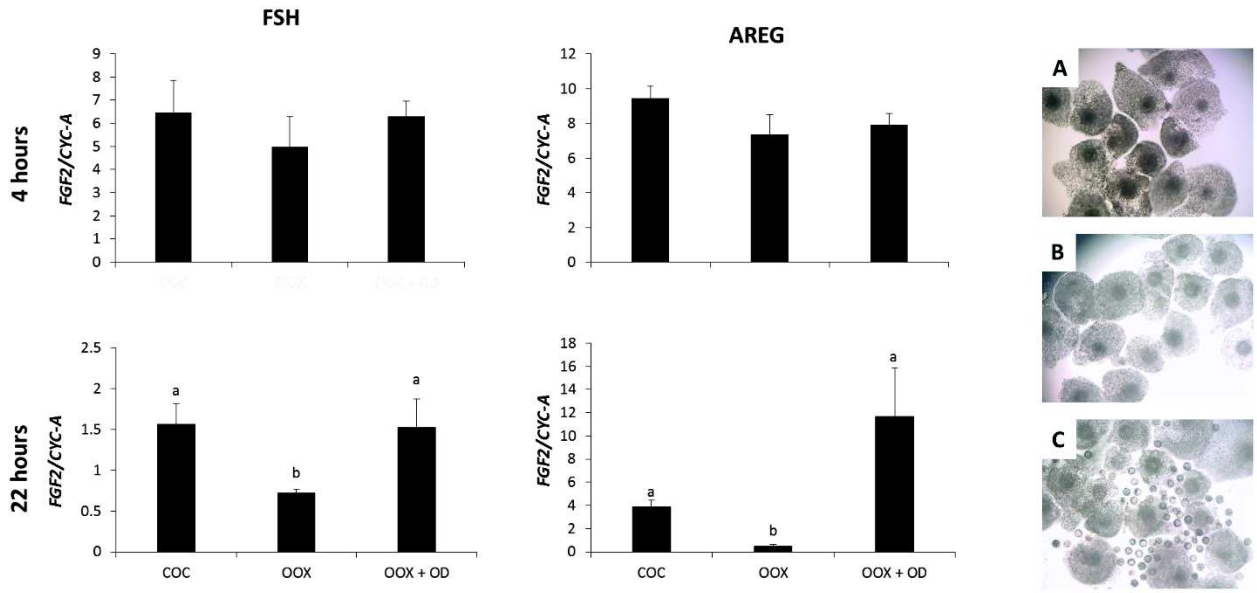

Figure 2. Effects of oocyte removal and oocyte secreted factors on *FGF2* mRNA expression. Intact (COC; A), oocytectomized (OOX; B) and OOX plus denuded oocytes (OOX + OD; 1 oocyte/µL; C) were cultured for 4 9(n= 4 replicates) and 22 hours (n= 5 replicates) with FSH (1 µg/mL) or AREG (100 ng/mL). Data are presented as mean values (±S.E.M.) relative to a calibrator sample calculated by the ΔΔCt method with efficiency correction. Bars with different letters are significantly different (P<0.05).

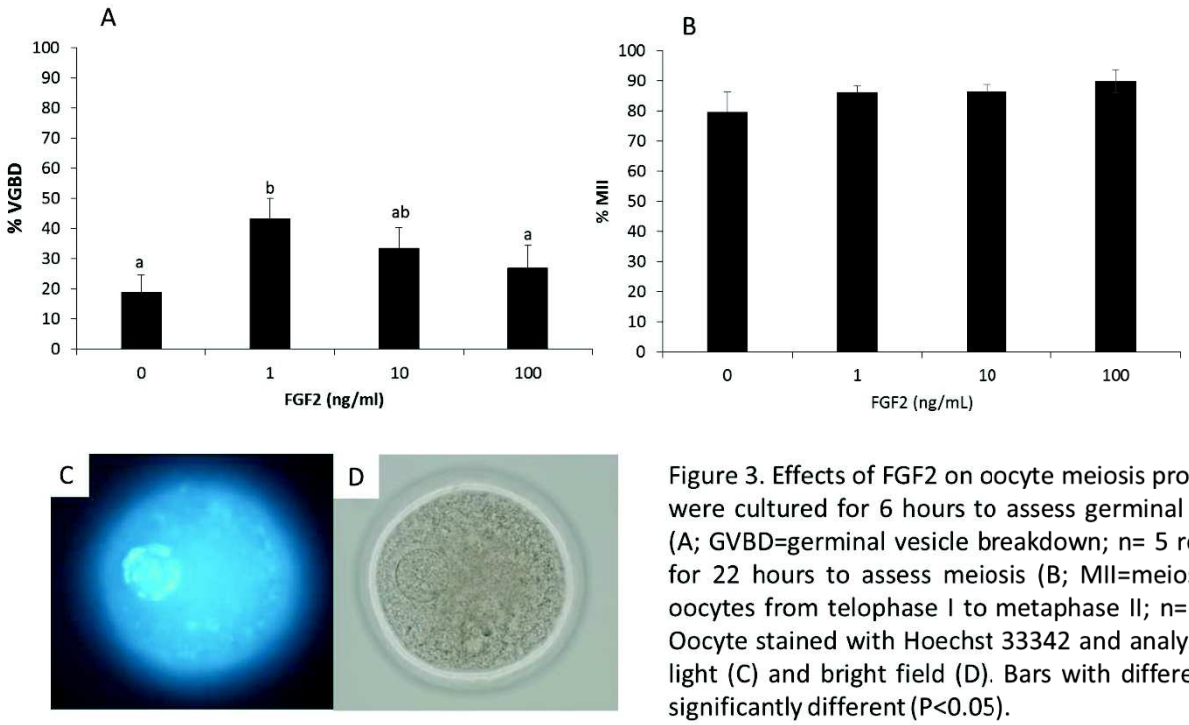

Figure 3. Effects of FGF2 on oocyte meiosis progression. COC were cultured for 6 hours to assess germinal vesicle status (A; GVBD=germinal vesicle breakdown; n= 5 replicates) and for 22 hours to assess meiosis (B; MII=meiosis II/includes oocytes from telophase I to metaphase II; n= 5 replicates). Oocyte stained with Hoechst 33342 and analysed under UV light (C) and bright field (D). Bars with different letters are significantly different ($P<0.05$).

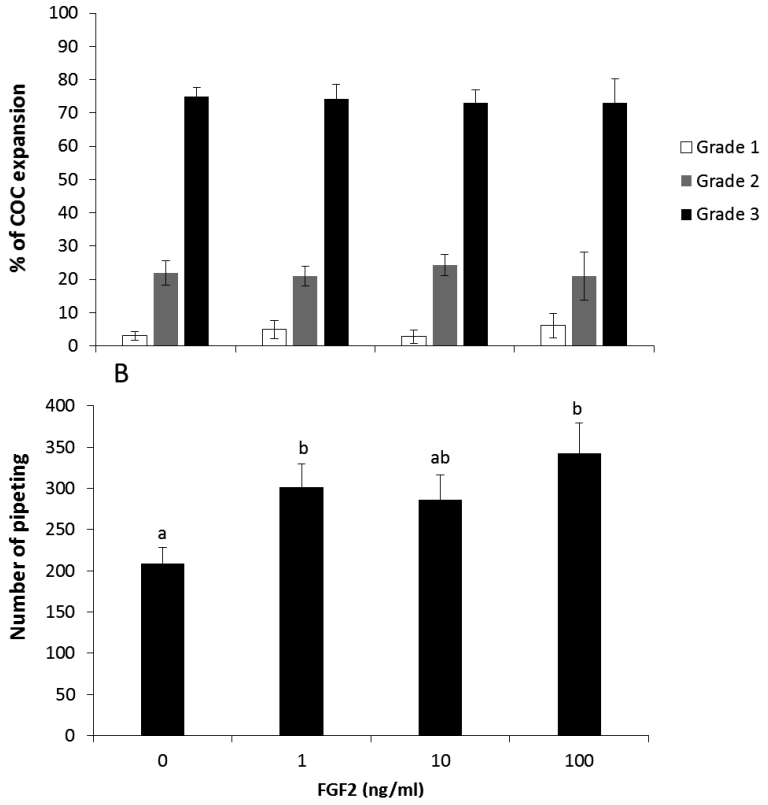

Figure 4. Effects of grading doses of FGF2 on the percentage of COCs exhibiting different degrees of expansion (A; n= 5 replicates) and on ECM cohesiveness estimated by the number of pippeting movements to completely denude the oocytes (B; n= 5 replicates). COC were cultured for 22 hours in the presence of FSH (100 µg/mL). Bars with different letters are significantly different ($P<0.05$).

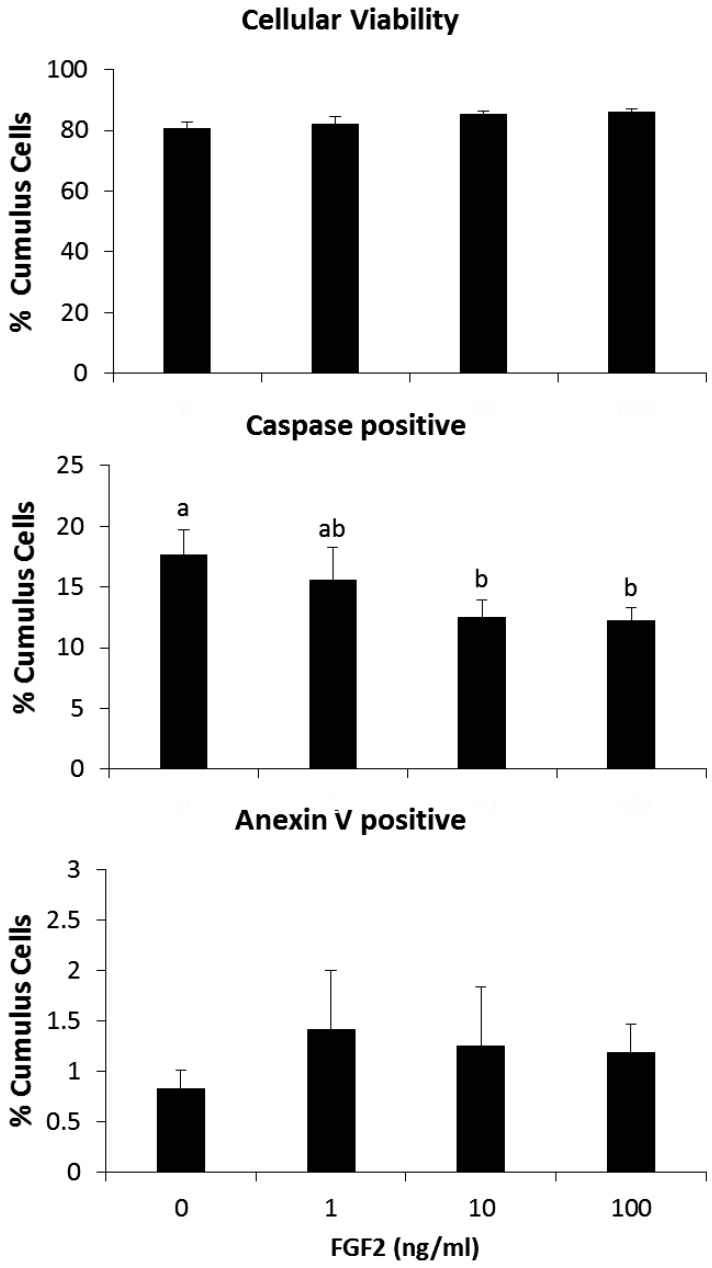

Figure 5. Effects of graded doses of FGF2 on cumulus cells viability as assessed by PI staining, and percentage of cells positive for caspase 3/7 and anexin V. COCs were cultured with increasing doses of FGF2 for 22 h in the presence of FSH (100 μg/mL). Numbers of total and stained cells were determined by flow cytometry (n= 5 replicates). Data are presented as mean values (±S.E.M.) and bars with different letters are significantly different (P<0.05).

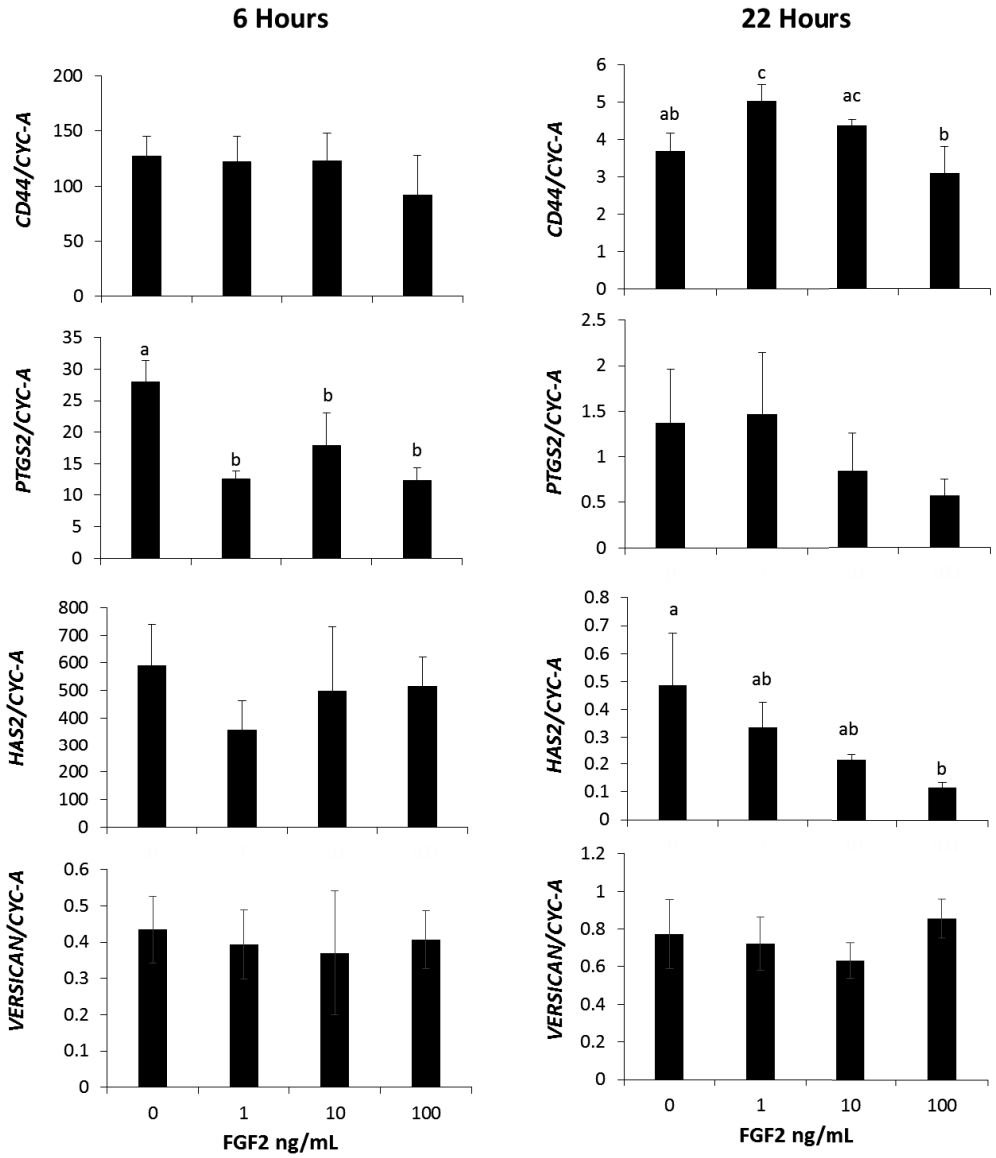

Figure 6. Effects of FGF2 on mRNA abundance of genes regulating cumulus expansion. COCs were cultured with increasing doses of FGF2 for 6 (n= 5 replicates) and 22h (n= 5 replicates). Messenger RNA abundance was measured by real-time PCR. Data are presented as mean values (±S.E.M.) relative to a calibrator sample by the ΔΔCt method with efficiency correction. Bars with different letters are significantly different (P<0.05).

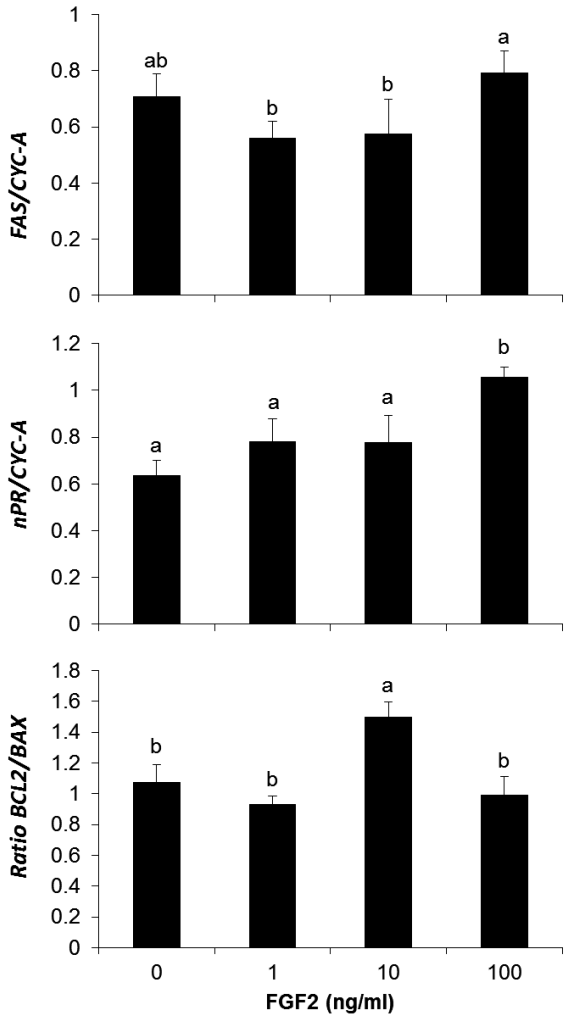

Figure 7. Effects of FGF2 on mRNA abundance of genes regulating apoptosis. COCs were cultured with increasing doses of FGF2 for 22h (n= 5 replicates). Messenger RNA abundance was measured by real-time PCR. Data are presented as mean values (±S.E.M.) relative to a calibrator sample by the ΔΔCt method with efficiency correction. Bars with different letters are significantly different ($P<0.05$).

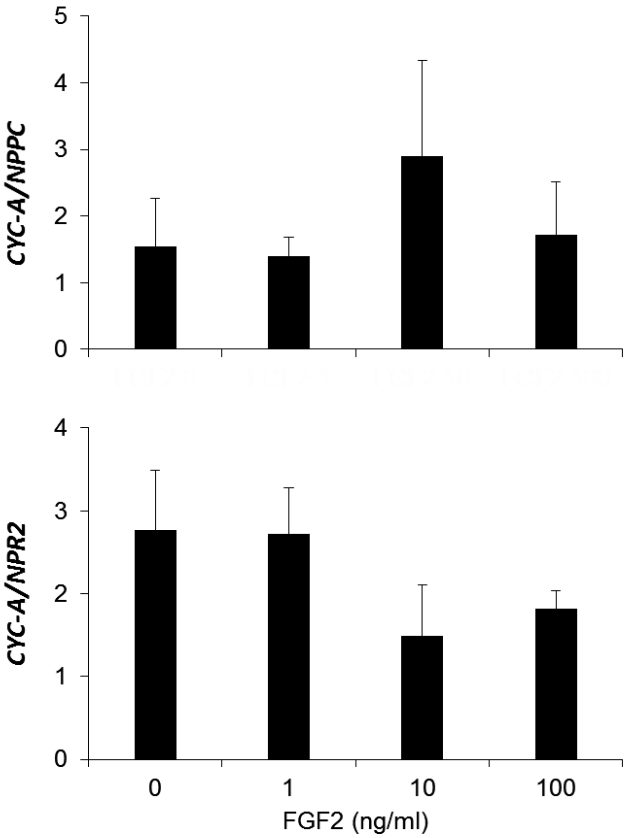

Figure 8. Effects of FGF2 on mRNA abundance of genes regulating oocyte maturation. COCs were cultured with increasing doses of FGF2 for 6 (n= 5 replicates). Messenger RNA abundance was measured by real-time PCR. Data are presented as mean values (±S.E.M.) relative to a calibrator sample by the ΔΔCt method with efficiency correction. Bars with different letters are significantly different (P<0.05).

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

Genes	Primer sequence	Fragment size (bp)	Annealing temperature (°C)	Reference
CYC-A	F: GCCATGGAGCGCTTTGG R: CCACAGTCAGCAATGGTGATCT	65	60	Machado *et al.,* 2009
FGF2	F: GCAAACCGTTACCTTGCTATG R: ACCACCTGGAGTATTTCCTTGA	136	60	NM_174056.4
NPPC	F:TCAGCCTCCTCGCATCT R: ACAGCTGGTGTTGTGTATCC	101	60	de Lima *et al.,*2016
NPR2	F: ATGACAGCATCAACCTGGACTGGA R: AGCACGAAACGACTATCCACCACA	145	60	de Lima *et al.,*2016
HAS2	F: ACACAGACAGGCTGAGGACAACTT R: AAGCAGCTGTGATTCCAAGGAGGA	133	60	Caixeta *et al.,* 2013
CD44	F: GACCCTCAATCTTCCTCTTCAC R: TTTCCTACCAGGTGCCAATC	94	60	NM_174013.3
PTGS2	F: AAGCCTAGCACTTTCGGTGGAGAA R: TCCAGAGTGGGAAGAGCTTGCATT	168	60	Caixeta *et al.,* 2013
VERS	F: TTTGAGAACCAGACAGGCTTCCCT R: TTGGTGTCAATTCTGTCCCAGTCCCA	172	60	Machado *et al.,* 2015
FAS	F: GCAACTCTGCAGCCTCAAATG R:CATCTATTTTGGCTTCCTCCATACC	153	59	NM_174662.2
nPR	F: AGAACTCATCAAGGCAATTGG R: CACATCCCTGCCAATATCTTG	108	60	Machado *et al.,* 2015
BAX	F: TGTTTTCTGACGGCAACTTCA R: CGAAGGAAGTCCAATGTCCAG	139	60	Sartor, 2014
BCL2	F:TTCGCCGAGATGTCCAGTCAGC R: TTGACGCTCTCCACACACATGACC	155	60	Sartor, 2014

F, forward primer; R, reverse primer.