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REGULAÇÃO DO SISTEMA PEPTÍDEOS NATRIURÉTICOS NAS CÉLULAS DA GRANULOSA E DO CUMULUS NA FOLICULOGÊNESE EM BOVINOS

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

Orientador: Prof. Paulo Bayard Dias Gonçalves

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RESUMO

REGULAÇÃO DO SISTEMA PEPTÍDEOS NATRIURÉTICOS NAS CÉLULAS DA GRANULOSA E DO CUMULUS NA FOLICULOGÊNESE EM BOVINOS

AUTOR: Matheus Pedrotti De Cesaro
ORIENTADOR: Paulo Bayard Dias Gonçalves

Esta tese tem como objetivo contribuir para o conhecimento da regulação e função do sistema dos peptídeos natriuréticos (NP) durante a dominância folicular, ovulação, retomada da meiose oocitária e expansão das células do cumulus em bovinos. No primeiro estudo, caracterizou-se o sistema NP no CCO de bovino e foi demonstrado que o NPPA e o NPPC aumentam os níveis de cGMP no cumulus e no oócito após 3 horas de cultivo, impedindo o aumento de cAMP no oócito na presença de forskolin. No segundo estudo, utilizando modelos experimentais *in vivo*, os três NPs não foram detectados nas células da granulosa durante a divergência folicular em bovinos. Contudo, demonstrou-se que o NPR-3 apresenta maior expressão no momento esperado da divergência folicular, sendo que após este momento os três receptores NPs apresentavam alta expressão nas células da granulosa do folículo dominante. A administração de FSH manteve a expressão dos três receptores NPs após a divergência folicular no maior e segundo maior folículo. Porém, somente a expressão do NPR-1 diminui após a inibição dos receptores de estradiol pela injeção intrafolicular de fulvestrant. Nas células da granulosa de folículos pré ovulatórios, somente o NPPB não foi detectado. De modo que, após a administração de um análogo de GnRH, a expressão de NPPC aumentou em 3 e 6 horas e a de NPR-3 gradualmente diminuiu após 3 horas. NPPA e NPR-2 não foram regulados pelo GnRH, e o NPR-1 teve a expressão aumentada somente 24 horas após o GnRH. No terceiro estudo, a abundância de mRNA para NPR-1, NPR-2 e NPR-3 nas células da granulosa não foi alterada por LH e/ou por AG1478 (inibidor do EGFr) após 6 horas de cultivo *in vitro*. Também em cultivo *in vitro* de células da granulosa, o LH estimulou a expressão de NPPC, sendo que o AG1478 impediu este aumento. Como forma de comprovar esses resultados *in vitro*, utilizamos um modelo *in vivo* e observamos que a expressão do NPPC aumentou e a do NPR-3 diminuiu após 6 horas do desafio com GnRH, nas células da granulosa. De maneira que, a injeção intrafolicular de AG1478 preveniu o efeito do GnRH sobre a expressão do NPPC e NPR-3. Além disso, foi demonstrado que o ANP em associação com o LH estimulou a expressão de COX2 em comparação com LH isoladamente ou ausência desta gonadotrofina. Também foi demonstrado que o bloqueio do EGFr *in vivo* não foi suficiente para bloquear a ovulação em bovinos. No quarto estudo, foi observado que a expressão do NPR-3 diminui nas células do cumulus pelo tratamento dos CCOs com FSH ou FSH+LH via EGFr em bovinos. Forskolin (estimulador adenilato ciclase) também induziu a diminuição da expressão do NPR-3. A expressão do NPR-1 foi muito baixa no cumulus de bovino e o NPR-2 não apresentou regulação nos tratamentos propostos. Além disso, foi observado que a ativação do NPR-3 por um agonista específico ($cANP^{4-23}$) não interferiu na maturação nuclear oocitária em bovinos, porém, inibiu a completa expansão das células do cumulus estimulada por FSH+LH. De maneira que, a associação do $cANP^{4-23}$ com CNP potencializou a inibição da expansão das células do cumulus.

Palavras-chave: Divergência folicular, ovulação, NPPC, NPR-2, EGFr, $cANP^{4-23}$, granulosa.

ABSTRACT

REGULATION OF THE NATRIURETIC PEPTIDE SYSTEM IN GRANULOSA AND CUMULUS CELLS IN THE FOLLICULOGENESIS IN BOVINE

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ADVISER: Paulo Bayard Dias Gonçalves

This thesis aims to contribute to the knowledge of the regulation and function of the natriuretic peptide (NP) system during follicular dominance, ovulation, oocyte meiosis resumption and cumulus cells expansion in cattle. In the first study, the NP system was characterized in bovine COC. Moreover, NPPA and NPPC increased cGMP levels in cumulus cells and oocyte after 3 hours of culture, preventing the increase of cAMP in oocyte in the presence of forskolin. In the second study, using *in vivo* experimental models, none of the three NPs were detected in the granulosa cells during follicular deviation in cattle. However, NPR-3 is highly expressed at the expected time of follicular deviation, and all three NP receptors are expressed in granulosa cells of the dominant follicle. FSH injection maintained the expression of the three NP receptors after follicular deviation in the largest and second largest follicles. However, only NPR-1 mRNA decreased after inhibition of estradiol receptors by intrafollicular injection of fulvestrant. In the granulosa cells of pre ovulatory follicles, only NPPB mRNA was not detected. Nevertheless, after the administration of a GnRH analog, NPPC mRNA expression increased within 3 and 6 hours and NPR-3 mRNA gradually decreased after 3 hours. NPPA and NPR-2 mRNA was not regulated by GnRH, but NPR-1 mRNA increased at 24 hours after GnRH. In the third study, abundance of mRNA for NPR-1, NPR-2 and NPR-3 was not altered by LH and/or AG1478 (EGFr inhibitor) after 6 hours of culture *in vitro*. Also, LH stimulated NPPC mRNA expression and AG1478 prevented this increase in granulosa cultured *in vitro*. To confirm the *in vitro* results, was used an *in vivo* model and observed that the NPPC mRNA expression increased and NPR-3 mRNA decreased in granulosa cells after 6 hours of GnRH challenge. However, intrafollicular injection of AG1478 prevented the effect of GnRH on NPPC and NPR-3 mRNA expression. In addition, it we obseved that ANP in association with LH stimulated COX2 expression in comparison with LH alone or absence of this gonadotropin. It was observed that the EGFr blockade *in vivo* did not prevent ovulation in cattle. In the fourth study, it was observed that NPR-3 mRNA expression decreases in bovine cumulus cells after treatment of COCs with FSH or FSH+LH via EGFr. Forskolin, an adenylate cyclase stimulator, also decreased NPR-3 mRNA expression in cumulus cells. Expression of NPR-1 mRNA was very low in bovine cumulus cells and NPR-2 mRNA was not regulated in the proposed treatments. In addition, it was observed that the activation of NPR-3 by a specific agonist ($cANP^{4-23}$) did not interfere in bovine oocyte nuclear maturation, but it inhibited the complete expansion of cumulus cells FSH+LH-stimulated. Additionally, the association of $cANP^{4-23}$ with CNP further decreased cumulus cell expansion.

Keywords: Follicular deviation, ovulation, NPPC, NPR-2, EGFr, $cANP^{4-23}$, granulosa cells.

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LISTA DE ABREVIATURAS

AngII: Angiotensina II

ANP: Peptídeo natriurético tipo A / Peptídeo natriurético atrial

AT2 / AGTR2: Receptor tipo 2 da angiotensina II

AI: Anáfase I

BMP15: Proteína morfogenética óssea 15

BNP: Peptídeo natriurético tipo B

cAMP: Adenosina monofosfato cíclico

$cANP^{4-23}$: Agonista seletivo NPR-3

CNP: Peptídeo natriurético tipo C

CC: cumulus cells

CCO / COC: Complexo cumulus-oócito / Cumulus oocyte-complex

cGMP: Guanosina monofosfato cíclico

COX2 / PTGS2: Cicloxigenase 2 / Prostaglandina endoperoxidade sintetase 2

DO: denuded oocytes

eCG: Gonadotrofina coriônica equina

EGF: Fator de crescimento epidermal

EGF-*like growth factors*: Fatores de crescimento semelhante ao EGF

- AREG: Anfirregulina
- BTC: Betacelulina
- EREG: Epirregulina

EGFr: Receptor do fator de crescimento epidermal

FSG10: Fator de crescimento fibroblástico 10

FGF8: Fator de crescimento fibroblástico 8

FSH: Hormônio folículo estimulante

GDF9: Fator de crescimento e diferenciação 9

GVBD: Germinal vesicle breakdown

HA: Ácido hialurônico

HAS2: Hialurona sintase 2

hCG: Gonadotrofina coriônica humana

IRAP: insulin-regulated aminopeptidase / aminopeptidase regulada por insulina

LH: Hormônio luteinizante

LHr: Receptor de LH

MAPK: Proteína quinase ativada por mitógenos

ERK1: Quinase regulada por sinal extracelular-1

ERK2: Quinase regulada por sinal extracelular-2

MPF: Fator promotor da maturação

MI: Metáfase I

MII: Metáfase II

NP: Peptídeos natriuréticos

NPPA: Precursor do peptídeo natriurético tipo-A

NPPB: Precursor do peptídeo natriurético tipo-B

NPPC: Precursor do peptídeo natriurético tipo-C

NPR-1 Receptor peptídeo natriurético 1

NPR-2: Receptor peptídeo natriurético 2

NPR-3: Receptor peptídeo natriurético 3

PDE: Fosfodienterase

PGE2: Prostaglandina E2

PGF2α: Prostaglandina F2α

PKA: Proteína quinase-A

PTX3: Pentraxina 3

TI: Telófase I

TGF-β: Fator de Transformação e Crescimento Beta

TNFP6 / TNF / TSG6 / TNFAIP6: Proteína indutora do fator de necrose tumoral alfa

TZP: Projeções transzonais

VG: Vesícula germinativa

SUMÁRIO

1. INTRODUÇÃO

A foliculogênese é um processo extremamente complexo que começa na vida fetal e tem como objetivo a ovulação de um gameta feminino apto a ser fecundado após a puberdade. Os eventos fisiológicos cíclicos que ocorrem durante a onda folicular são regulados de forma endócrina e, enormemente, pelas vias parácrina e autócrina. A compreensão da regulação de eventos celulares e intracelulares são de grande importância no processo reprodutivo para maximizar a reprodução de animais domésticos, diagnosticar possíveis distúrbios de infertilidade e desenvolver métodos contraceptivos mais seguros. Na construção do conhecimento, aprofundamos os estudos na regulação e função do sistema peptídeos natriuréticos (NP) durante a dominância folicular, ovulação, retomada da meiose oocitária e expansão das células do cumulus em bovinos.

O sistema NP consiste de três distintos peptídeos endógeno: NP tipo-A (ANP), NP tipo-B (BNP) e NP tipo-C (CNP), além de três receptores localizados na superfície da célula alvo com um simples segmento trans-membrana (Gardner *et al.*, 2007): receptor NP-1 (NPR-1), NPR-2 e NPR-3 (Yasoda *et al.*, 2004; Potthast e Potter, 2005; Nishikimi *et al.*, 2011). Nos processos reprodutivos, o CNP, atuando via NPR-2 apresenta papel central. Em camundongos, o CNP que é produzido pelas células murais da granulosa se liga ao NPR-2 do cumulus e bloqueia a retomada da meiose oocitária por manter inativa a enzima fosfodienterases 3 (Zhang *et al.,* 2010). Funções semelhantes também foram descritas em suínos (Hiradate *et al.,* 2013), cabras (Peng *et al.,* 2013; Zhang *et al.,* 2015), bovinos (Franciosi *et al.,* 2014) e gatas (Zhong *et al.,* 2015). Recentemente, na espécie suína, foi demonstrado que o NPR-3, em conjunto com o NPR-2, também participa do bloqueio meiótico (Santiquet et al., 2014). Além disso, foi demonstrado que o CNP tem capacidade de estimular o crescimento de folículos pré-antrais e antrais em camudongos (Sato *et al.,* 2012).

No que diz respeito a regulação do sistema NP, sabe-se que o FSH/eCG, estradiol e o crescimento folicular estimulam a expressão do precursor do CNP (NPPC) e NPR-2 em camundongos (Kawamura *et al.,* 2011; Lee *et al.,* 2013) e suínos (Zhang *et al.,* 2014; Zhang *et al.,* 2015), atingindo os maiores níveis concomitantemente com o pico pré-ovulatório de LH (Kawamura *et al.,* 2011; Lee *et al.,* 2013; Tsuji *et al.,* 2012; Zhang *et al.,* 2014; Zhang *et al.,* 2015). Após o LH, através da ativação do receptor do fator de crescimento epidermal (EGFr), nas espécies multiovular, a expressão de NPPC nas células da granulosa (Kawamura *et al.,* 2011; Robinson *et al.,* 2012; Zhang *et al.,* 2014) e NPR-2 no cumulus diminuem

rapidamente (Robinson *et al.,* 2012; Wang *et al.,* 2013). De forma oposta, o NPR-3 apresenta baixa expressão durante o crescimento folicular, de maneira que, após o LH ocorre aumento da expressão deste receptor nas células da granulosa e do cumulus de camundongos (Lee *et al.,* 2013).

Embora grandes avanços foram realizados para o entendimento da regulação e função do sistema NP no ambiente folicular de espécies multiovular, ainda há uma lacuna muito grande a esse respeito em espécies monovular. Além disso, até o momento, não foi demonstrado efeito da ativação do NPR-3 sobre a expansão das células do cumulus. Dessa forma, com a utilização de consolidados modelos experimentais *in vitro* e *in vivo* em bovinos, objetivou-se caracterizar e compreender a regulação do sistema NP em momentos crucias da foliculogênese nas células da granulosa e do cumulus, além da função do NPR-3 do cumulus.

2. REVISÃO BIBLIOGRÁFICA

2.1 Maturação oocitária

Os oócitos são derivados das células germinativas primordiais, as quais, ainda no desenvolvimento fetal, transformam-se em oogônias que, por sua vez, diferenciam-se das outras células por apresentarem um citoplasma claro, devido à pouca quantidade de organelas e uma alta frequência de divisão mitótica, podendo chegar a 2.700.000 oogônias no dia 110 de gestação em bovinos, entretanto, aproximadamente 95% dos oócitos são perdidos até o nascimento (Erickson, 1966). Segundo Motta *et al.,* (1997), muitos oócitos sofrem processos degenerativos como resultados de erros genéticos ocorridos durante o "*crossing over*" ou devido a distúrbios metabólicos e/ou vasculares. Na fêmea bovina, em torno dos 72-82 dias de gestação, alguns oócitos já iniciam a primeira prófase meiótica, passando então pelos estádios de leptóteno, zigóteno, paquíteno e diplóteno, no qual ocorre o primeiro bloqueio da meiose, também denominado estádio de dictióteno da prófase I ou de vesícula germinativa (VG) (Richards, 1980).

Em muitas espécies de mamíferos, o início da meiose acontece durante a vida fetal, na qual o oócito permanece no estádio de VG até o folículo se tornar atrésico ou se desenvolver e ser capaz de induzir a ovulação (Sirard *et al.*, 1989) pela estimulação da secreção do pico ovulatório do hormônio luteinizante (LH). A maturação do oócito tem por objetivo formar uma célula haplóide (bloqueada em metáfase II - MII), apta a ser fecundada, e que tenha capacidade para suportar os primeiros estádios de desenvolvimento embrionário, até a ativação do seu genoma. Os oócitos bovinos adquirem progressivamente a competência meiótica (capacidade de reiniciarem a meiose), a qual é completa quando atingem 110 – 115 µm, em folículos com 2-3 mm de diâmetro (Fair *et al.*, 1995; Otoi *et al.*, 1997). *In vivo,* o reinício da divisão meiótica ocorre em resposta ao pico ovulatório do LH somente em oócitos inclusos em folículos pré-ovulatórios completamente diferenciados. Porém, *in vitro,* esse processo é desencadeado independentemente de hormônios, simplesmente pela remoção do oócito competente do ambiente folicular (Pincus e Enzmann, 1935). A interação entre oócito, células da granulosa e células da teca, pela liberação de fatores autócrinos e parácrinos, determina o controle do desenvolvimento folicular e maturação oocitária (Eppig, 2001). Esses fatos evidenciam que no folículo são produzidos fatores inibitórios que impedem a maturação

nuclear, e que com o surgimento do LH ovulatório, a maquinaria das células foliculares é alterada, cessando a produção de inibidores e estimulando a síntese de fatores promotores da meiose (Downs, 2010).

O reinício da meiose, tanto *in vivo* como *in vitro*, inicia pela dissolução da membrana nuclear e condensação da cromatina no processo denominado de *germinal vesicle breakdown* (GVBD), progredindo sucessivamente para os estádios de metáfase I (MI), anáfase I (AI), telófase I (TI) e MII, na qual ocorre o segundo bloqueio da meiose (Sirard *et al.*, 1989). O tempo requerido para a maturação nuclear varia entre as espécies. No bovino, a GVBD ocorre de 7 – 12 horas, a MI de 12-15 horas, a AI e a TI de 15-18 horas e a MII 18-22 horas após o pico pré-ovulatório de LH ou remoção do oócito do ambiente folicular (Sirard *et al.*, 1989; Wu *et al.*, 1997).

A regulação da meiose em bovinos é extremamente complexa, incluindo eventos de fosforilação e de desfosforilação. O armazenamento de RNAm, através do encurtamento da cauda poli-A, principalmente para o fator promotor da maturação (MPF), é necessário para conferir competência para o oócito. O MPF é uma proteína de 79kD composta por uma subunidades catalítica (34kD) conhecida como p34Cdc2 kinase, e uma subunidade regulatória (45kD) conhecida como ciclina B (Gautier *et al.*, 1990). Para a ativação do MPF, a treonina-14 e a tirosina-15 da subunidade catalítica devem ser desfosforiladas pela enzima Cdc25 fosfatase (Kumagai e Dunphy, 1992). Os oócitos em fase de crescimento têm níveis baixos de p34Cdc2 e não progridem da fase G2 (intervalo entre síntese de DNA e divisão celular) para a fase M (divisão celular, meiose), entretanto, no final da fase de crescimento há um grande aumento na concentração e atividade da p34Cdc2, o que confere a competência meiótica (Chesnel e Eppig, 1995; De Vant'ery *et al.*, 1996). Durante a GVBD, os níveis de MPF estão baixos, tendo um aumento gradual até atingir níveis máximos no estádio de MI. Após essa fase, o MPF apresenta uma diminuição significativa coincidindo com AI e TI, e um novo aumento é observado em MII, que é mantido por várias horas no oócito, diminuindo gradativamente depois de 30 horas de maturação ou imediatamente após a fecundação e ativação (Wu *et al.*, 1997; Liu e Yang, 1999).

Outro importante fator envolvido na maturação do oócito é a proteína quinase ativada por mitógenos (MAPK). Em oócitos de mamíferos estão presentes duas isoformas da MAPK conhecidas como quinase regulada extracelular-1 (ERK1) e ERK2, sendo a última isoforma mais abundantemente expressa em oócitos bovinos (Trounson *et al.*, 2001). O momento da ativação da MAPK varia nas diferentes espécies, de modo que é ativada em consequência da fosforilação de uma tirosina e uma treonina durante a maturação do oócito (Kosako *et al.*,

1994). Nos bovinos, a MAPK é ativada na GVBD, tendo sua atividade máxima em MI e permanecendo elevada até a formação dos pronúcleos (Fissore *et al.*, 1996), sem diminuir em MII. Durante a maturação do oócito, a atividade da MAPK é essencial para a manutenção do MPF, formação dos fusos meióticos e manutenção do bloqueio meiótico na fase de MII (Colledge *et al.*, 1994; Hashimoto *et al.*, 1994).

Além dos fatores promotores da maturação, o reinício da meiose também é regulado por fatores inibidores produzido pelas células foliculares. Esse fato pode ser evidenciado pela manutenção de oócitos em VG quando co-cultivados com metades foliculares ou em meio condicionado pelas mesmas (Sirard e First, 1988; Richard e Sirard, 1996; Giometti *et al.*, 2005; Stefanello *et al.,* 2006; Barreta *et al.*, 2008). Com esse mesmo modelo, o nosso laboratório demonstrou que a angiotensina II (AngII) é capaz de reverter o efeito inibitório das células foliculares sob a maturação do oócito (Giometti *et al.*, 2005; Stefanello *et al.,* 2006; Barreta *et al.,* 2008). Em trabalhos *in vitro* utilizando metades foliculares como modelo experimental foi comprovado que AngII, progesterona, ocitocina e prostaglandinas (E2 e F2α) são passos sequenciais na via que culmina com a maturação do oócito em bovinos (Barreta *et al.,* 2008; Siqueira *et al.,* 2012; De Cesaro *et al.,* 2013). Além disso, a administração de um antagonista dos receptores da AngII (Barreta et al., 2008) ou antagonista do receptor de progesterona (Siqueira *et al.,* 2012) em folículos pré-ovulatórios *in vivo* foi capaz de bloquear o reinício da meiose induzido por GnRH/LH. Dessa forma, confirmando os resultados *in vitro* e o importante papel da AngII na maturação nuclear de oócitos bovinos.

A adenosina monofosfato cíclica (cAMP) tem sua concentração aumentada em função do estímulo de LH no folículo. Entretanto, níveis altos de cAMP dentro do oócito impedem o reinício da meiose em diversas espécies (Thomas *et al.*, 2002). Uma hipótese para explicar isso, propõem que o cAMP é compartimentalizado dentro do folículo ovariano, sendo diferentemente regulado dentro do oócito (célula germinativa) e nas células somáticas (células da teca, granulosa e cumulus), devido a diferentes localizações de fosfodienterases (PDE) (Tsafriri *et al.*, 1996). Nos bovinos, altos níveis de cAMP no oócito causa um atraso no reinício da meiose, mas não impede a progressão à MII (Sirard e First, 1988). Esse evento é correlacionado com a proteína quinase-A (PKA), a qual é dependente de cAMP (Sirard, 1990). Os níveis de cAMP presentes nas células do cumulus e no oócito são dependentes da síntese por adenilatociclase e da degradação por PDE. Em bovinos, a PDE tipo 3 (PDE3) é a principal enzima envolvida na degradação do cAMP no oócito, e a PDE8 está principalmente envolvida com essa degradação nas células do cumulus (Sasseville *et al.*, 2009), que também influenciam os níveis de cAMP no interior do oócito, via comunicação intercelular pelas

junções tipo *gap*. O cultivo *in vitro* de complexos cumulus-oócito (CCO) bovinos na presença de um inibidor da PDE3 atrasa o reinício da meiose, pela diminuição da degradação e consequente aumento de cAMP (Bilodeau-Goeseels, 2003). Entretanto, quando CCOs de camundongo são cultivados na presença de um inibidor de PDE3 o reinício da meiose é totalmente bloqueado (Romero e Smitz, 2010). Além da comunicação via junções tipo *gap*, existem estreitas projeções das células do cumulus que atravessam a zona pelúcida (projeções transzonais - TZP) e se comunicam com o oócito participando da regulação do crescimento e maturação do oócito (Carabatsos *et al.,* 1998; Albertini *et al.,* 2001; Combelles *et al.,* 2004; Li e Albertini 2013; El-Hayek *et al.,* 2015; Romero *et al.,* 2016).

A regulação dos eventos responsáveis pela retomada da divisão meiótica e completa maturação do oócito ainda não foram totamente elucidadas. *In vivo*, provavelmente o pico de LH seja um sinal comum para ativar o MPF e a MAPK promovendo a maturação dos oócitos (Dekel, 1996). Porém, a ausência de receptores de LH no oócito e no cumulus (Peng *et al.*, 1991) sugere que essa gonadotrofina não atue diretamente no gameta feminino, mas estimule a produção de fatores intrafoliculares que induzem a GVBD. No entanto, os eventos existentes entre o pico de LH e a ativação do MPF e da MAKP no oócito não são bem compreendidos. Alguns fatores foram identificados como possíveis reguladores da maturação nuclear. Fatores de crescimento semelhantes ao fator de crescimento epidermal (EGF-*like* grow factors), como anfiregulina (AREG), epiregulina (EREG) e betacelulina (BTC) foram apontados como principais mediadores dos efeitos do LH sobre a expansão do cumulus, maturação de oócitos e ovulação (Park *et al.*, 2004; Shimada *et al.*, 2006 Romero e Smitz, 2010). Vaccari *et al.*, (2009) demonstraram que guanosina monofosfato cíclica (cGMP) e cAMP estão integrados e envolvidos no bloqueio da meiose em oócitos de camundongos através da regulação da PDE3. De maneira que, o precursor do peptídeo natriurético tipo-C (NPPC), produzido pelas células murais da granulosa, se liga ao receptor peptídeo natriurético 2 (NPR-2) do cumulus e impede o reinício da meiose por inibir a PDE3 do oócito (Zhang *et al.*, 2010).

2.3 Expansão das células do cumulus *oophorus*

Folículos antrais possuem duas sublinhagens de células somáticas, as quais em contato com o líquido folicular. Além da localização, estas duas sublinhagens apresentam responsividade a hormônios e perfil de expressão gênica diferente (Russell e Robker, 2007).

As células que estão próximas a membrana basal, revestindo internamente o folículo, são denominadas células murais da granulosa, enquanto as camadas de células adjacentes ao gameta feminino são denominadas de células do cumulus *oophorus* (Buccione *et al.*, 1990a; Cortvrindt e Smitz, 2001). As células do cumulus *oophorus* são definidas como um grupo de células da granulosa intimamente associadas que envolvem o oócito no folículo antral (Tanghe *et al.*, 2002; Van Soom *et al.*, 2002). De maneira que, se comunicam entre si e com o oócito intimamente, através das *gap junctions*, além das projeções dessas células atravessar a zona pelúcida através das TZPs e se comunicarem intimamente com a membrana plasmática do oócito (Albertini *et al.,* 2001; Li e Albertini, 2013; El-Hayek *et al.,* 2015). O conjunto de células do cumulus e o oócito formam o complexo cumulus-oócito (CCO), e este é extremamente influenciado por fatores parácrinos e autócrinos (Eppig, 2001; Russell e Robker, 2007).

O surgimento do pico pré-ovulatório de LH faz com que a maquinaria celular dos constituintes do folículo se altere, culminando com a retomada da meiose do oócito, expansão do cumulus e ovulação (Li e Albertini, 2013). Porém, os receptores para essa gonadotrofina são predominantemente expressos nas células murais da granulosa (Peng *et al.*, 1991; Cotterill *et al.*, 2012). Com a ativação desses receptores por LH ocorre aumento da expressão de fatores de crescimento semelhantes ao EGF (Park *et al.*, 2004; Panigone *et al.*, 2008), que são apontados como os principais mediadores do efeito desse hormônio no folículo (Park *et al.*, 2004; Romero e Smitz, 2010). Os fatores de crescimento semelhantes ao EGF (AREG, EREG e BTC) são sintetizados como pró-peptídeos e somente desempenham suas funções após atividade proteolítica da enzima ADAM17 (A Disintegrin And Metalloprotease), sendo, portanto, liberados no ambiente folicular e exercendo suas funções ao se ligarem nos receptores (EGFr) presentes nas células da granulosa e nas células do cumulus estimulando o reinicio da meiose, expansão das células do cumulus e ovulação (Park *et al.*, 2004; Shimada *et al.*, 2006 Romero e Smitz, 2010).

Neste contexto, é possível caracterizar a importância das células do cumulus antes, durante e após a ovulação (Tanghe *et al.*, 2002). Segundo Chian *et al.*, (1994) a remoção das células do cumulus antes do cultivo *in vitro* é prejudicial à maturação do oócito em bovinos, pelo fato que essas células tem papel fundamental em manter o oócito bloqueado em VG, participam na indução da meiose e auxílio na maturação citoplasmática (Tanghe *et al.*, 2002; Ferreira *et al.,* 2009). Durante a ovulação, as células do cumulus facilitam a saída do CCO do folículo e a posterior captura pelas células do infundíbulo (fímbrias), sendo que a taxa de ovulação diminui quando ocorre falhas na expansão destas células (Chen *et al.*, 1993; Zhuo e

Kimata, 2001). E para a fecundação, as células do cumulus são benéficas por aumentar o número de espermatozoides viáveis ao redor do oócito, criar um microambiente que facilita a capacitação dos espermatozoides, a reação do acrossoma e a penetração (Tanghe *et al.*, 2002). Além de prevenir a prematura liberação dos grânulos corticais em suínos (Galeati *et al.*, 1991).

Em estudos de expansão do cumulus *in vitro*, o FSH é amplamente utilizado (Hensleigh e Hunter, 1985; Buccione *et al.*, 1990b; Eppig *et al.*, 1993; Calder *et al.*, 2001; Sugiura *et al.*, 2010; Lapointe *et al.*, 2012; Marei *et al.*, 2014). Receptores para FSH são predominantemente expressos na granulosa e nas células do cumulus (Tisdall *et al.*, 1995), sendo esses ligados a proteína G que ativa adenilato ciclase, dessa forma, ativando PKA através do aumento de cAMP (Russell e Robker, 2007). Apesar de fatores derivados do oócito, como fator de crescimento fibroblástico 8 (FGF8) e 10 (FGF10) e proteína morfogenética óssea 15 (BMP15), estimular a expansão das células do cumulus em bovino (Zhang *et al.,* 2010; Caixeta *et al.,* 2013), a ausência do gameta feminino, nesta espécie, não inibe que ocorra este evento em meio com FSH e LH (Lima *et al.,* 2016). Em roedores a expansão do cumulus é dependente do oócito e de fatores derivados do oócito (Buccione *et al.*, 1990b), principalmente os relacionados a superfamília do Fator de Transformação e Crescimento Beta (TGF-β), como o fator de crescimento e diferenciação 9 (GDF9) (Dragovic *et al.*, 2005) e BMP15 (Hussein *et al.*, 2005). Além disso, acredita-se que, em camundongos, o GDF9 e BMP15 regulam a expressão do EGFr no cumulus (Su *et al.*, 2010). *In vitro* o FSH estimula a síntese de AREG, EREG e BTC no cumulus (Su *et al.*, 2002; Downs e Chen, 2008), de maneira que esses estimulam genes essenciais para a expansão do cumulus, como a hialurona sintase 2 (HAS2), proteína indutora do fator de necrose tumoral alfa (TNFP6 ou TNF ou TSG6 ou TNFAIP6), cicloxigenase 2 (COX2, também denominada prostaglandina endoperoxidade sintetase 2 - PTGS2) e a pentraxina 3 (PTX3) (Salustri *et al.*, 2004; Ashkenazi *et al.*, 2005; Conti *et al.*, 2006; Shimada *et al.*, 2006; Su *et al.*, 2010). Ou seja, a capacidade dos EGF-*like growth factors* induzir a expansão do *cumulus* decorre da ativação da transcrição de genes essenciais a esse processo como a HAS2, TNFP6, COX2 e PTX3 (Diaz *et al.*, 2006).

Durante a expansão do cumulus, é secretado por essas células um material sensível a hialuronidade, que é composto por proteoglicanas e glicosaminoglicanas, sendo a principal macromolécula da matrix extracelular o ácido hialurônico (HA - glicosaminoglicanas) (Salustri *et al.*, 1990b; Nagyova, 2012), que é sintetizado pela HAS2. Durante a expansão o HA se liga às células do cumulus e aumenta o espaço entre elas (Eppig, 2001). Em

camundongos, a remoção do oócito do CCO compromete a habilidade das células do cumulus em sintetizar HA e expandir (Buccione *et al.*, 1990b; Salustri *et al.*, 1990a; Vanderhyden, 1993), demonstrando assim a importância do oócito nesse evento. Outro gene de suma importância para a expansão do cumulus e também para a fertilidade é o TNFP6. Fulop *et al.*, (2003) e Ochsner *et al.*, (2003) demonstraram que apesar de ocorrer ovulação em camundongos *knockout* para TNFP6, houve falha na adequada mucificação das células do cumulus e também infertilidade. Da mesma forma, foi demonstrado que a PTX3 é um constituinte estrutural da matrix extracelular das células do cumulus, sendo essencial para fertilidade da fêmea (Varani *et al.*, 2002; Salustri *et al.*, 2004). Além disso, sabemos que as prostaglandinas são essenciais para a maturação do oócito (Barreta *et al.*, 2008) e para a expansão do cumulus (Lim *et al.*, 1997). A produção das prostaglandinas é realizada pela enzima COX2, que em camundongos tem um padrão multifásico de expressão no cumulus, com alta expressão 4 e 12 horas após administração de gonadotrofina coriônica humana (hCG) *in vivo* (Joyce *et al.*, 2001). A ausência de COX2 em camundongos impede que ocorra a expansão das células do cumulus em resposta ao LH (Davis *et al.,* 1999).

2.3 Ovulação

A ovulação em mamíferos envolve um complexo e dinâmico controle, na qual se assemelha a um processo inflamatório que culmina com a luteinização das células foliculares, ruptura do estigma ovulatório e liberação do complexo cumulus-oócito, sendo induzido pelo pico pré-ovulatório de LH (Espey, 1980; Russell e Robker, 2007). As fêmeas bovinas são capazes de ovular após a dominância folicular, quando atingem elevada responsividade ao LH, o que ocorre após o folículo dominante atingir um diâmetro ≥12mm (Sartori *et al.,* 2001). Apesar dos receptores de LH (LHr) serem expressos nas células da granulosa em todos os tamanhos de folículos entrais, somente após a divergência folicular essas células apresentam receptores funcionais para essa gonadotrofina (Robert *et al.,* 2003). Esse controle, através da transcrição de mais uma sequência de RNAm é denominado *splicing* alternativo. O LHr funcional está ligado a uma proteína G e não varia durante o desenvolvimento folicular nas células da teca, porém a ovulação somente ocorre com a presença de LHr funcional e aumento da sua abundância nas células da granulosa (Richards, 1980). Isso já foi demonstrado nas células da granulosa de ratos (Peng *et al.*, 1991), camundongos (Eppig *et al.*, 1997) e bovinos (Robert *et al.*, 2003).

O processo de sinalização da ovulação é iniciado pelo pico pré-ovulatório de LH, via LHr, que induz grandes mudanças na maquinaria celular do folículo pré-ovulatório. A cascata de sinalização inicia-se pelo estímulo da adenilato ciclase, que aumenta as concentrações do segundo mensageiro cAMP nas células da granulosa e consequentemente ativam a via proteína quinase dependente de cAMP (PKA) (Mcfarland *et al.,* 1989; Richards, 2001; Conti, 2002). Com isso, ocorre intensa atividade de transcrição e tradução de genes envolvidos no processo de ovulação e alterações morfológicas e funcionais das células foliculares que culminam com a luteinização e liberação de um oócito apto a ser fecundado (Russell e Robker, 2007; Li *et al.,* 2009). Entre os genes mais importantes no processo de ovulação, e denominados mediadores intrafoliculares da ação do LH, destacam-se os EGF-*like growth factors* (AREG, EREG e BTC) (Park *et al.*, 2004; Panigone *et al.*, 2008; Romero e Smitz, 2010). Entretanto, para que AREG, EREG e BTC possam se ligar ao EGFr, presente nas células da granulosa e do cumulus, é necessário a ação proteolítica de enzimas ADAMs, principalmente ADAM17 (Lee *et al.,* 2003; Richards *et al.,* 2002; Yamashita *et al.,* 2009). A regulação da abundância de RNAm para ADAMs nas células da granulosa podem ser observados pela ativação do receptor de progesterona e prostaglandinas (Fortune *et al.,* 2009; Willis *et al.,* 2016) além da associação de AngII com LH (Portela *et al.,* 2011). Com a estimulação do EGFr ocorre a fosforilação de ERK1/2 (MAPK) nas células do cumulus e da granulosa que é essencial para mediar muitos dos efeitos do LH, incluindo a maturação do ócito, expansão das células do cumulus e ovulação (Conti *et al.,* 2006; Fan *et al.,* 2009; Yamashita and Shimada, 2012). De maneira semelhante, Duggavathi and Murphy (2009) demonstraram, em camundongos, que existe uma via secundária/alternativa para a fosforilação de ERK1/2. Na qual, apesar de utlizar inibidor específico para EGFr (Panigone et al., 2008) ou knockout condicional para este receptor (Hsieh *et al.,* 2011), o LH conseguiu fosforilar parcialmente ERK1/2. Entretanto em bovinos, não se conhece o efeito da inibição do EGFr sobre a ovulação.

Durante a ovulação, também, observa-se alterações na esteroidogênese intrafolicular, na qual os níveis de progesterona aumentam 4-5 vezes após 90 minutos do pico pré-ovulatório de LH (Fortune *et al.,* 2009) e a concentração de estradiol diminui após 3 horas da administração de análogo de GnRH (Dos Santos *et al.,* 2012). Em bovinos, o máxima concentração plasmática de LH é observada em torno de 2 horas após aplicação do análogo de GnRH (Rajamahendran *et al.,* 1998; Komar *et al.,* 2001). Outros hormônios e mediadores dos processos inflamatórios também são fundamentais para que ocorra a ovulação. As prostaglandinas são derivadas do ácido araquidônico através da ação da ciclooxigenase 1 e 2

(COX1 e COX2) (Hinz & Brune, 2002), sendo que apenas COX2 tem sua abundância aumentada nas células da granulosa (Lim *et al.*, 1997), bem como as concentrações das prostaglandinas E2 (PGE2) e F2α (PGF2α) no fluído folicular são elevadas (Sirois, 1994; Lim *et al.*, 1997) em resposta ao estímulo gonadotrófico. Além disso, foi observado aumento de RNAm e proteína para COX2 na granulosa de bovinos cultivada *in vitro* quando tratadas com a associação de AngII e LH (Portela *et al.,* 2011). As principais funções da PGE2 e PGF2α estão relacionadas com aumento da vasodilatação tecidual e degradação da parede folicular, pela ativação de proteinases, antes da ovulação (Sirois *et al.,* 2004), além de participarem do processo de maturação do oócito (Barreta *et al.,* 2008; Siqueira *et al.,* 2012; De Cesaro *et al.,* 2013). A inibição das cicloxigenases ou knockout para COX2 determina falhas na ocorrência do processo ovulatório (Espey *et al.,* 1986; Davis *et al.,* 1999; Peters *et al.,* 2004; Sena & Liu, 2008). Segundo Shimada et al. (2006) e Yamashita e Shimada (2012), a PGE2 e os EGF-*like growth factors* participam de uma sinalização parácrina/autócrina com as células da granulosa e cumulus auxiliando no processo de maturação oocitária, expansão do cumulus e ovulação. Em outro estudo, com células da granulosa de humanos, demonstrou-se que pelo estímulo de LH ocorre a ativação do EGFr (por AREG, EREG e BTC) que induz o aumento da abundância de COX2 e da concentração de PGE2, sendo que esse efeito é dependente da ativação de ERK1/2 (Fang *et al.,* 2013).

Além das alterações supracitadas, ocorre também aumento nas concentrações AngII no ambiente folicular de bovinos após o pico pré-ovulatótio de LH (Acosta *et al.,* 1999). Com base nisso, estudos do nosso grupo demonstraram que o efeito da AngII nas células foliculares são mediados via receptor tipo 2 (AT2) (Ferreira et al., 2007; Portela *et al.,* 2008), diferente do que ocorre no sistema circulatório. De maneira que, a ativação de AT2 pela AngII é fundamental na divergência folicular e síntese de estradiol durante o desenvolvimento do folículo dominante e é indispensável nos momentos iniciais da cascata ovulatória induzida pelo LH (Ferreira *et al.,* 2007; Portela *et al.,* 2008; Ferreira *et al.,* 2011).

2.4 Peptídeos Natriuréticos

Os Peptídeos Natriuréticos (NPs) foram descobertos após observação que a infusão de extrato de tecido atrial em ratos provocava rápida diminuição da pressão arterial, vasodilatação, atividade natriurética (excreção aumentada de sódio na urina) e diurética (De Bold *et al.*, 1981). Após essas observações, purificou-se o peptídeo e foi demonstrado que ele

também possuía capacidade de relaxar a musculatura lisa, passando a ser denominado NP atrial (ANP) (Flynn *et al.*, 1983). O peptídeo natriurético tipo B (BNP), que inicialmente foi denominado peptídeo natriurético cerebral (Sudoh *et al.*, 1988), e o peptídeo natriurético tipo C (CNP) (Sudoh *et al.*, 1990) foram purificados de extratos de cérebro suíno baseado na habilidade para relaxarem a musculatura lisa, porém também apresentam capacidade natriurética. O sistema dos NPs consiste de três distintos peptídeos endógeno: ANP, BNP e CNP, além de três receptores localizados na superfície da célula alvo com um simples segmento trans-membrana (Gardner *et al.*, 2007): receptor NP-1 (NPR-1), NPR-2 e NPR-3 (Yasoda *et al.*, 2004; Potthast e Potter, 2005; Nishikimi *et al.*, 2011). Apesar do hormônio precursor para cada um dos NPs ser codificado por um gene diferente, os NPs apresentam alta homologia, na qual possuem 17 aminoácidos (10 aminoácidos homólogos) em um anel com ligação dissulfeto, necessário para a atividade biológica (Nakao *et al.*, 1992; Potthast e Potter, 2005). Os NPs são expressos nos tecidos como pré-pró-hormônios. O pré-pro-ANP possui 151 aminoácidos, sendo clivado para pro-ANP com 126 aminoácidos (Nakao *et al.*, 1992) e armazenado em grânulos atriais, sendo que esse é clivado pela enzima corin para formar o peptídeo ativo com 28 aminoácidos (ANP-28) (Yan *et al.*, 2000). O pré-pró-BNP humano é sintetizado com 134 aminoácidos, sendo clivado à 108 aminoácidos formando pró-hormônio, e por fim uma nova clivagem origina o peptídeo ativo com 32 aminoácidos (BNP-32) (Potter *et al.*, 2006). O pré-pró-CNP é sintetizado com 126 aminoácidos, com a clivagem de 23 aminoácidos forma o pró-CNP (103 aminoácidos) (Tawaragi *et al.*, 1991), e esse é processado pela endoprotease furin formando o peptídeo ativo com 53 aminoácidos (CNP-53) (Wu *et al.*, 2003). Em alguns tecidos, CNP-53 é clivado formando outro peptídeo ativo com 22 aminoácidos (CNP-22) por uma protease ainda desconhecida (Potter *et al.*, 2006). CNP-53 e CNP-22 apresentam diferentes expressões nos tecidos, mas ativam igualmente o NPR-2 e desencadeiam funções semelhantes (Yeung *et al.*, 1996).

Os ANP e BNP são produzidos principalmente no átrio e no ventrículo cardíaco, respectivamente, influenciando diretamente a pressão e a homeostase dos fluidos corporais (Matsukawa *et al.*, 1999). O CNP, em contraste ao ANP e BNP que atuam como hormônios cardiovasculares, é expresso fortemente no cérebro, mas também em outros tecidos, apresentando níveis normalmente baixos na circulação. Dessa forma, acredita-se que o CNP apresente função autócrina ou parácrina, sendo comumente considerado um hormônio com propriedades anti-mitogênicas, regulador da ossificação endocondral (Matsukawa *et al.*, 1999) e com grandes evidências de estar envolvido em vários processos reprodutivos (Tamura *et al.*, 2004; Walther e Stepan, 2004).

Os NPs exercem seus efeitos fisiológicos através da interação com receptores na célula-alvo. A ligação dos NP a dois de seus receptores (NPR-1 e NPR-2) ativa guanilatociclase, aumenta a concentração intracelular do segundo mensageiro guanosina monofosfato cíclico (cGMP), modulando a atividade biológica desses peptídeos (Kone, 2001). Por exemplo, regulam proteínas kinases dependentes de cGMP e específicas PDE (Potthast e Potter, 2005).

São conhecidos três receptores para os NPs. Os três receptores apresentam um domínio de ligação extracelular (aproximadamente 450 aminoácidos) e uma simples região trans-membrana hidrofóbica (Porter *et al.*, 1990; Gardner *et al.*, 2007). NPR-1 e NPR-2 apresentam domínio intracelular idêntico, consistindo de um domínio tipo kinase, um domínio de dimerização e um domínio carboxi-terminal guanilatociclase, sendo a sinalização desses dois receptores através da síntese de cGMP (Potter et al., 2006; Potter et al., 2009). NPR-1 apresenta alta expressão nos rins, pulmões, adrenal, tecido vascular e adiposo (Lowe *et al.*, 1989), enquanto o NPR-2 apresenta alta expressão no cérebro, pulmões, rins, cartilagem e tecidos reprodutivos (Schulz *et al.*, 1989). Já o NPR-3 contém um domínio intracelular com 37 aminoácidos e não tem atividade guanilato ciclase, dessa forma, controlando as concentrações dos NPs por internalizar e degradar o ligante (Levin *et al.*, 1998), também sendo conhecido como receptor de limpeza e é amplamente expresso em diversos tecidos (Matsukawa *et al.*, 1999).

Os receptores NPs apresentam especificidades diferentes para cada ligante. Segundo Suga *et al.*, (1992), o NPR-1 apresenta afinidade pelo ANP maior ou igual ao BNP e pouca para CNP, já o NPR-2 possui afinidade pelo CNP muito maior que para o ANP, que apresenta maior ou igual afinidade por esse receptor quando comparado ao BNP. A afinidade do CNP pelo NPR-2 é 50 e 500 vezes maior que o ANP e o BNP, respectivamente (Koller *et al.*, 1991). O NPR-3 apresenta afinidade pelo ANP maior que para o CNP, que possui maior ou igual afinidade para o BNP (Suga *et al.*, 1992), de modo que em camundongos *knockout* para o NPR-3 a meia vida plasmática do ANP aumenta (Matsukawa *et al.*, 1999).

Dentre os NPs, o CNP atuando via NPR-2 apresenta numerosas evidências de participação durante os processos reprodutivos em roedores. Sato *et al.*, (2012), demonstraram que o precursor peptídeo natriurético tipo C (NPPC) estimula o desenvolvimento folicular, além disso, Jankowski *et al.*, (1997) e Noubani *et al.*, (2000), observaram que NPPC, NPR-1 e NPR-2 apresentaram variações durante o ciclo estral, possuindo máxima expressão no pró-estro. Gutkowska *et al.*, (1999) demonstraram que o sistema NP no ovário de ratas é regulado por gonadotrofinas, especialmente FSH, e que NPPC e NPR-2 aparecem como um importante

sistema de regulação intraovariano. Em camundongos adultos, a expressão de NPPC é destacada no tecido uterino e nos ovários (Stepan *et al.*, 2000). Nessa mesma espécie, o estradiol induz a expressão gênica de NPPC no útero (Acuff *et al.*, 1997). Camundongos *knockout* para NPR-2 com 90 dias de vida não apresentavam corpo lúteo no ovário, e não possuem indícios de ciclo estral com base nos achados de citologia vaginal (Tamura *et al.*, 2004). Em ratos, a expressão de NPPC e NPR-2, no ovário e útero, é modulada durante o ciclo estral, com máxima expressão no pró-estro (Dos Reis *et al.*, 1995). Em camundongos, Zhang *et al.*, (2010) demonstraram que o NPPC, produzido pelas células murais da granulosa, se liga ao NPR-2 do cumulus e impede o reinício da meiose por inibir a PDE3 do oócito. Em suínos, recentemente, foi observado função semelhante para o NPPC e NPPB (Hiradate *et al.*, 2013; Zhang *et al.*, 2015). Função semelhate foi observada em cabras (Peng *et al.,* 2013; Zhang *et al.,* 2015), bovinos (Franciosi *et al.,* 2014) and gatas (Zhong *et al.,* 2015) para o NPPC *in vitro*. Além disso, também em espécies multiovular, foi demostrado que NPPC estimula o crescimento de folículos pré-antrais e antrais (Sato *et al.*, 2012), e é essencial para manter a meiose bloqueada em folículos iniciando a formação do antro (Tsuji *et al.*, 2012). Em adição, o FSH/eCG, estradiol e o crescimento folicular, estimulam a expressão de NPPC nas células da granulosa de camundongos, atingindo os níveis mais elevados, *in vivo*, concomitantemente com o pico pré-ovulatório do LH (Kawamura *et al.*, 2011; Lee *et al.*, 2013). Em suínos a máxima abundância de mRNA para NPPB, NPPC e NPR-2 tanto na granulosa como no cumulus também é observado próximo ao pico de LH (Zhang *et al.,* 2015). Fatores derivados do oócito, principalmente BMP15, GDF9 e FGF8, juntamente com o estradiol promovem a expressão e manutenção da funcionalidade do NPR-2 no cumulus (Zhang *et al.*, 2010; Zhang *et al.*, 2011; Lee *et al.*, 2013). Contrariamente a isso, o LH/hCG através dos EGF-*like grow factors* (AREG, EREG e BTC) reduzem a expressão do NPPC na granulosa (Kawamura *et al.*, 2011; Tsuji *et al.*, 2012) e a atividade do NPR-2 nas células do cumulus (Robinson *et al.*, 2012; Wang *et al.*, 2013). Diminuição da expressão também é observado em suínos para o NPPB, NPPC e NPR-2 via EGF-like (Zhang *et al.,* 2014). Somando-se a isso, a expressão de NPR-3 aumenta após o pico de LH nas células da granulosa e do cumulus em camundongos, entretanto, a degradação do CNP não foi alterada em animais *knockout* ou não para este receptor (Lee *et al.,* 2013). Sugerindo assim, que no ambiente folicular o NPR-3 pudesse apresentar outra função além de internalizar e degradar o ligante. Em recente estudo, utilizando o suíno como modelo experimental, demonstrou-se que a utilização de um agonista seletivo para o NPR-3 ($cANP^{4-23}$), apesar de não estimular cGMP, auxila o CNP a manter o oócito bloqueado na meiose (Santiquet *et al.,* 2014).

Em espécie monovular, como a bovina, poucos trabalhos relacionando os NPs na reprodução foram publicados até o momento. Com um sistema de microdiálise em folículos foi proposto que o ANP estimula a produção de prostaglandinas e AngII, além de modular a esteroidogênes nas células foliculares (Acosta *et al.,* 1999). Em cultivos *in vitro* de granulosa bovina foi sugerido que o NPPA atua diretamente para favorecer a dominância folicular (Montrezor *et al.*, 2015). Em células da granulosa de folículos de 2 a 6 mm de diâmetro, em bovinos, foi mostrado que o LH diminuiu a expressão de NPPC via ativação de EGFr (Yang et al., 2016). Além disso, foi demonstrado que 100nM de NPPC tem capacidade de atrasar a retomada da meiose após 6 ou 8 horas de cultivo *in vitro* e manter a comunicação cumulus-oócito (Franciosi *et al.*, 2014). Nosso grupo, além de caracterizar o sistema NPs no complexo cumulus-oócito de bovino, demonstrou que os três NPs tem capacidade de estimular a retomada da meiose inibida por forskolin *in vitro*, de maneira que, o NPPA e o NPPC aumentam os níveis de cGMP no cumulus e no oócito após 3 horas de cultivo e impedem o aumento de cAMP no oócito na presença de forskolin (De Cesaro *et al.*, 2015). Contudo, ainda não se conhece a cinética e regulação por gonadotrofinas dos receptores NPs nas células da granulosa e do cumulus durante o processo de ovulação, retomada da meiose e expansão das células do cumulus em bovinos.

3. ARTIGO 1

TRABALHO PUBLICADO:

NATRIURETIC PEPTIDES STIMULATE OOCYTE MEIOTIC RESUMPTION IN BOVINE

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Natriuretic Peptides stimulate oocyte meiotic resumption in bovine

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Short title: **NPPC stimulates GVBD in bovine oocyte**

Abstract

The aim of the present study was to evaluate the expression of mRNA encoding natriuretic peptides (NPs) and their receptors in the cumulus-oocyte complex in cattle, a monovular mammalian species, and also to investigate the role of NPs in oocyte meiotic resumption *in vitro*. mRNA was observed for the NP precursor type-A (*NPPA*), type-C (*NPPC*), NP receptor-1 (*NPR-1*), receptor-2 (*NPR-2*) and receptor-3 (*NPR-3*) in bovine cumulus cells, and *NPR-2* mRNA was observed in oocytes. These results are different from those obtained in mouse and pig models. The effects of NPPA, NP precursor type-B (NPPB) and NPPC on the resumption of arrested meiosis maintained by forskolin were studied at three different doses (10, 100 and 1000 nM) with a 12h culture system. The germinal vesicle breakdown rates were greater ($P \leq 0.05$) in oocytes that were cultured in the presence of one or a combination of NPs (from 44% to 73%) than the negative control (from 24% to 27%). Additionally, it was demonstrated that the concentration of cyclic guanosine 3',5'-monophosphate (cGMP) is increased by NPPA and NPPC in oocytes and cumulus cells after 3h of *in vitro* maturation. However, in both groups, the concentration of cyclic adenosine 3',5'-monophosphate (cAMP) in the oocyte did not increase between 3 and 6h of culture, even when forskolin was used. In summary, we observed the presence of mRNA for NPs and their receptors in the bovine cumulus-oocyte complex and demonstrated that, *in vitro*, NPPA, NPPB and NPPC stimulate oocyte meiotic resumption in a monovular species.

Keywords: Germinal vesicle breakdown, forskolin, cumulus-oocyte complex, cattle.

1. Introduction

The natriuretic peptides (NPs) system consists of three endogenous peptides with high homology: NP precursor type-A (NPPA), type-B (NPPB) and type-C (NPPC), as well as three

receptors (NPR-1, -2 and -3) that are located on the target cell surface (Gardner et al., 2007; Misono et al., 2011). These peptides have mainly been studied in the circulatory system and are critical in the development of female reproductive organs (Tamura et al., 2004). NPPA and NPPB have higher affinities for NPR-1, whereas the main receptor for NPPC is NPR-2 (Potter et al., 2009). Both NPR-1 and NPR-2 are bound to guanylate cyclase and, when stimulated, increase the synthesis of cyclic guanosine 3',5'-monophosphate (cGMP) (Hsueh et al., 2015). NPR-3 has no guanylate cyclase activity and is responsible for internalization and degradation of the ligand (Potter et al., 2009; Potter, 2011).

NPPC is essential to block meiosis in mice (Zhang et al., 2010; Tsuji et al., 2012) and pigs (Hiradate et al., 2013; Zhang et al., 2015). Loss-of-function mutations in either NPPC or NPR-2 result in precocious resumption of meiosis in oocytes that are enclosed within antral follicles (Zhang et al., 2010; Tsuji et al., 2012; Geister et al., 2013). NPPC is mainly synthesized in the mural granulosa cells, and its cognate receptor (NPR-2) is present in large amounts in cumulus cells (Zhang et al., 2010; Kawamura et al., 2011; Zhang et al., 2011). Besides stimulating follicular development (Sato et al., 2012), the main reproductive function of NPPC in rodents is preventing resumption of meiosis. In mice, NPPC binds to NPR-2, which stimulates the synthesis of cGMP that diffuses into the oocyte through gap junctions and inhibits phosphodiesterase 3A (PDE3A; also know as oocyte-specific phosphodiesterase), maintaining high levels of cyclic adenosine 3',5'-monophosphate (cAMP) (Vaccari et al., 2009; Norris et al., 2010; Zhang et al., 2010). In addition, FSH/eCG and estradiol increase the expression of NPPC in mouse granulosa cells (Kawamura et al., 2011; Lee et al., 2013), whereas oocyte-derived paracrine factors and estradiol promote the expression of mRNA and maintain the functionality of NPR-2 in cumulus cells (Zhang et al., 2011; Lee et al., 2013). Conversely, through epidermal growth factor receptor (EGFR), LH/hCG reduces the expression of NPPC in granulosa (Kawamura et al., 2011; Tsuji et al., 2012) and NPR-2

activity in cumulus cells in rodents (Robinson et al., 2012; Wang et al., 2013). However, the expression and functional roles of natriuretic peptides in oocyte meiotic resumption are poorly reported in monovular species.

It has been demonstrated that angiotensin II (Ang II), acting through type-2 receptor (AT2), is essential in the early stages of the ovulatory process (Ferreira et al., 2007) and stimulates resumption of meiosis in cattle (Giometti et al., 2005; Stefanello et al., 2006; Barreta et al., 2008). In the circulatory system, Ang II (signaling through AT2) and NPs have similar functions (Paulis and Unger, 2010). Our hypothesis is that the role of NPs in the bovine reproductive system is to stimulate resumption of meiosis, similar to Ang II. For this reason, the objectives of the present study were to evaluate the expression of mRNA encoding NPs and their receptors in the cumulus-oocyte complex (COC) and to investigate their roles in bovine meiosis resumption *in vitro*.

2. Materials and methods

All experimental procedures were approved by the Federal University of Santa Maria Animal Care and Use Committee (23081.013597/2011–66 CCR/UFSM). All chemicals used were purchased from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise indicated.

2.1. Oocyte recovery and nuclear maturation

Bovine ovaries at various stages of the estrous cycle were obtained from an abattoir and transported to the laboratory in 30°C saline solution (0.9% NaCl) containing 100 IU/ml penicillin and 50 μg/ml streptomycin sulfate. The COCs were aspirated from follicles 3-8 mm in diameter. Only COCs grades 1 and 2 were recovered and selected under a stereomicroscope according to Leibfried and First (1979). After selection, COCs were washed three times in

TCM-199 containing Earle's salts and L-glutamine (Gibco Labs, Grand Island, NY, USA) supplemented with 25 mM Hepes, 0.2 mM pyruvic acid, 0.4% fatty acid-free bovine serum albumin (BSA), 100 IU/ml penicillin, and 50 μg/ml streptomycin (TCM wash). Subsequently, the COCs were transferred to four-well culture dishes (Nunc®, Roskilde, Denmark) containing 200 μl of maturation medium with the appropriate treatment. Then, the COCs were cultured at 39°C in an atmosphere containing 5% CO_2 in air, at 95% relative humidity, for 12 h. The basic culture medium was TCM-199 with Earle's salts and L-glutamine (Gibco Labs, Grand Island, NY, USA) supplemented with 25 mM Hepes, 2.2 mg/ml sodium bicarbonate, 0.2 mM pyruvic acid, 0.5 μg/ml of FSH (Folltropin®-V, Bioniche, ON, CA), 100 IU/ml penicillin, 50 μg/ml streptomycin, and 0.4% BSA.

2.2. Analysis of nuclear maturation

At the end of the culture period, the cumulus cells of COCs were removed by repeated pipetting, and denuded oocytes (DO) were fixed in 4% paraformaldehyde for 15 min, followed by permeabilization of the nuclear membranes with 0.5% Triton X-100. For assessment of nuclear maturation, oocytes were exposed to 10 μg/ml of bisbenzimide (Hoescht 33342) for 15 min. After slides were mounted, stained oocytes were classified under UV light (filter cube +A, with a wavelength of 340-380 nm) with a fluorescence microscope (Leica, DMI 4000B) according to the characteristics of the chromatin: germinal vesicle (GV), GV breakdown (GVBD), and metaphase I (MI). Oocytes that resumed meiosis (GVBD or MI) were described and statistically analyzed as GVBD.

2.3. Nucleic acid extraction, RT-PCR and PCR

After selection, cumulus cells (CC) and denuded oocytes (DO) were separated by vortexing for 5 min. The CC were immediately stored in Trizol, and the DO were exposed to

0.5% proteinase K until the zona pellucida was completely removed. Total RNA from a pool of COCs, DO and CC, was extracted using Trizol (Invitrogen) according to the manufacturer's instructions. Total RNA was quantified at 260 nm wavelength using a spectrophotometer (NanoDrop1000, Thermo Scientific). Purity was assessed at an absorption ratio of 260/280 nm, and samples with values below 1.8 were not used.

Total RNA (1 μg) was first treated with 0.2 U DNase (Invitrogen) at 37°C for 5 min to digest any contaminating DNA, followed by heating at 65°C for 3 min. RNA was reverse-transcribed (RT) in the presence of 1 μM oligo (dT), 4 U Omniscript RTase (Omniscript RT Kit; Qiagen, Mississauga, ON, Canada), 0.5 mM dideoxynucleotide triphosphate (dNTP) mix, and 10 U RNase inhibitor (Invitrogen) in a volume of 20 μl at 37°C for 1 h. The reaction was ended by incubation at 93°C for 5 min. PCR was performed using StepOnePlus™ Real-Time PCR (Applied Biosystems, Foster City, CA, USA) with Platinum SYBR Green Master Mix (Applied Biosystems) and specific primers (listed in Table 1). All primers used were designed based on sequences from GenBank, using Primer Express Software v3.0, and were synthesized by Invitrogen. The dissociation curve was examined to verify the identity of the product. The integrity of the sample was assessed by amplification of the GAPDH gene, and the contamination of DO with CC was assessed by amplification of the CYP19 gene.

2.4. Measurement of cGMP and cAMP levels

COCs were collected and maturated as described above. At the end of the culture period (3 or 6 h), cells from 50 COCs were separated by vortexing for 5 min to obtain both cumulus cells and the associated DOs, using TCM wash medium containing 1 mM 3-isobutyl-1-methylxanthine (Sasseville et al., 2009). After washing with PBS, the samples were solubilized in 100 μl of 0.1M HCl on ice for 10 min, and then snap-frozen in liquid nitrogen and stored in a freezer at -80°C until required for use in cAMP and/or cGMP assays. For the

cAMP and cGMP assays, samples were thawed and centrifuged at 12,000 *g* for 5 min, after which the supernatant was collected in a tube and dried in an oven at 60°C (Zhang et al., 2010; Wang et al., 2013). The samples were then analyzed using cAMP-EIA and cGMP-EIA kits (Cayman Chemicals; Ann Arbor, MI, USA) according to the manufacturer's instructions.

2.5. Experimental design

2.5.1. Experiment 1: mRNA expression of natriuretic peptides and their receptors in bovine COCs

This experiment was designed to assess the presence of mRNA encoding NPs and their receptors in bovine COCs. RT-PCR was performed on the pool of COCs, DO and CC from follicles 3 to 8 mm in diameter. The amplicons were subjected to 2.5% agarose gel electrophoresis, stained with ethidium bromide and observed under UV light.

2.5.2. Experiment 2: Effect of natriuretic peptides in preventing the oocyte meiotic resumption in cattle

To evaluate the effect of NPs in preventing meiosis resumption of the bovine oocyte, firstly, we cultivate COCs in the presence of NPPA, NPPB and NPPC, separately, at three different concentrations (10, 100 and 1000 nM). Secondly, considering that mouse and bovine oocytes differ in several aspects, including the pattern of *NPPA*, *NPPC*, *NPR-1*, *NPR*-2 and *NPR*-3 mRNA expression in cumulus cells, COCs were cultured in the presence of a combination of the three NPs at the greatest concentration tested (1000 nM). Both parts of this experiment were performed three times and used COCs that were cultured without treatments as a control group.

2.5.3. Experiment 3: Effect of natriuretic peptides in stimulating oocyte meiotic resumption in bovine

Based on the earlier experiment that NPs alone or combined were not efficient at blocking bovine oocyte meiotic resumption, this experiment was subdivided into four parts to better evaluate the effect of NPs on the bovine oocyte arrested by forskolin. All parts of this experiment were performed in triplicate and had a positive control group (without forskolin and treatment) that was used to validate the oocyte maturation system and a negative control group (with 100 μM forskolin) that was used to validate inhibition of meiotic resumption. In the first part of this experiment, the effect of NPPA and NPPC on oocyte meiotic resumption was tested at three different concentrations (10, 100 and 1000 nM). In the second part, the hypothesis that the combination of NPPA and NPPC induces an increase in the percentage of oocytes that resume meiosis was tested. The third part of the experiment was similar to the first part, except that in this case NPPB was tested at three different concentrations (10, 100 and 1000 nM) to induce meiotic resumption. Finally, the fourth part of this experiment was conducted to determine the different combinations of NPs required to induce higher rates of GVBD; therefore, the COCs were divided into eight groups (positive and negative controls and six treatments). In the treatment groups, the COCs were cultured in the presence of forskolin and NPPA, NPPB, NPPC, NPPA+NPPB, NPPB+NPPC or NPPA+NPPB+NPPC.

2.5.4. Experiment 4: Effects of NPPA and NPPC on the concentrations of cAMP and cGMP in bovine oocytes and cumulus cells

To evaluate the effects of NPPA and NPPC on concentrations of cGMP and cAMP in oocytes and cumulus cells, COCs were cultured for 3 or 6 h. For each time point, there was a positive control group (without forskolin), a negative control group (with 100 μM forskolin), a group with 1000 nM NPPA and another group with 1000 nM NPPC; both treatments were

in the presence of forskolin. A total of 1200 COCs were used to quantify concentrations of cAMP and cGMP at 3 and 6 h, using 50 COCs per replicate in each group. Also, as a replicate control, 20 COCs in each group were kept in culture for 12 h for subsequent evaluation of nuclear maturation (supplementary data). Three replicates were used.

2.6. Statistical analysis

The analyses were performed with SAS Software (SAS; SAS Institute, Inc., Cary, NC, USA). The proportion of GVBD in the various treatments was evaluated using a statistical model for categorical data (PROC CATMOD). The LSMeans Differences Student's T method was used to evaluate the concentrations of cAMP and cGMP. The concentrations of cAMP and cGMP are represented as the mean ± the standard error of the mean, and the rates of nuclear maturation are represented as percentages. All experiments were performed in triplicate, and P-values less than 0.05 were considered to represent significant differences.

3. Results

3.1. mRNA expression of natriuretic peptides and their receptors in bovine COCs

Initially, we examined the presence of mRNA coding for NPs and their receptors using Real-Time PCR in bovine COCs, DO and CC from follicles of 3 to 8 mm in diameter (Fig. 1). Real-Time PCR amplicons were run in agarose gel, resulting in single bands for *NPPA*, *NPPC*, *NPR-1*, *NPR-2* and *NPR-3* that were consistent with the expected size for each of these targets in cumulus cells. However, in oocytes, mRNA was only observed for NPR-2 (Fig. 1b).

3.2 Effect of natriuretic peptides on oocyte meiotic resumption in bovine

First, COCs were cultured either in the presence of 10, 100 and 1000 nM of NPPA, NPPB and NPPC, or in the absence of NPs (control group). Oocyte meiotic resumption was unaffected by the presence of NPs at different concentrations (supplementary data). Similar to the first part of this experiment, NPs had no effects on meiotic resumption, and about 100% of oocytes reached GVBD at 12 h maturation (supplementary data).

3.3. Do natriuretic peptides stimulate oocyte meiotic resumption in bovine?

In the first part of this experiment, COCs were cultured in the presence of 10, 100 or 1000 nM NPPA or NPPC. Oocytes were partially stimulated to undergo GVBD when cultured in the presence of NP, independent of the concentration used. All oocytes from the positive control resumed meiosis, whereas only 24% of oocytes from the negative control underwent GVBD. When oocytes were cultured in the presence of forskolin, with concentrations of NPPA and NPPC as before, rates from 44% to 73% of GVBD were observed (Fig. 2b). In the second part of the experiment, the effect of the interaction of NPPA and NPPC on oocyte meiotic resumption was evaluated. The rate of GVBD in the NPPA+NPPC (70%) was not different from the rates observed when COCs were incubated with NPPA (63%) or NPPC (57%) (Fig. 2c). The resumption of meiosis was also partially stimulated by NPPA+NPPC compared to the positive and negative control groups. Although expression of *NPPB* mRNA in COCs was not seen it is well known that NPPB has a high binding affinity for NPR-1 (Potter et al., 2009). For this reason, we verified the effect of NPPB at concentrations of 10, 100 and 1000 nM on oocyte meiotic resumption. In this part of this experiment, the rates of GVBD were similar to those observed with NPPA or NPPC (Fig. 3a). Finally, we investigated the interaction of all NPs to induce GVBD (Fig. 3b). The pattern of meiotic resumption was similar to that previously noted, in which each NP or combination of NPs partially induced the resumption of meiosis in bovine oocytes (Fig. 2c and 3b).

3.4. Cyclic GMP and AMP measurement

Knowing that NPs are able to stimulate meiosis resumption inhibited by forskolin and that NPPA and NPPC are expressed in the COC and in granulosa cells (unpublished observations), we evaluated the concentrations of cAMP and cGMP in oocyte and cumulus cells after 3 and 6 h of maturation, with the same model that was used in the previous experiment. Initially, we observed that NPPA and NPPC increased concentrations of cGMP after 3 h of maturation in both oocyte (Fig. 4a) and cumulus cells (Fig. 4b) compared to the negative control. After 6 h of maturation, only oocytes from the NPPA group remained, and had greater concentrations of cGMP compared to the positive control (Fig. 4a). When comparing different time points within the same group, it was observed that NPPA- and NPPC-treated cumulus cells had greater concentrations of cGMP at 3 h of maturation compared to 6 h (Fig. 4b).

A low concentration of cAMP within the oocyte is essential for resumption of meiosis. Results showed that the positive control had the lowest cAMP levels compared to the other experimental groups, except at 6 h in cumulus cells (Fig. 5). Furthermore, we noted that even with the increased concentrations of cGMP that were stimulated by NPPA and NPPC after 3 h of culture, these NPs were not effective in reducing cAMP in the presence of forskolin. However, there was not difference in cAMP concentration within the oocyte between the positive control and NPPA at 6 h of maturation (Fig. 5a). Moreover, even with forskolin, the concentration of cAMP in the oocytes in the NPPA and NPPC groups did not increase from 3 to 6 h of culture (Fig. 5a). However, an increase was observed in the negative control (Fig. 5a).

4. Discussion

In the present study, the presence of mRNA for natriuretic peptides and their receptors in bovine cumulus-oocyte complex was recorded, as was the ability of NPPA, NPPB and NPPC to stimulate resumption of bovine oocyte meiosis in the presence of forskolin. Our main findings were as follows. 1) mRNA for *NPPA*, *NPPC*, *NPR-1*, *NPR-2* and *NPR-3* are present in cumulus cells, whereas oocytes express only *NPR-2* mRNA. 2) Natriuretic peptides were not able to maintain meiotic arrest in bovine oocytes. 3) NPPA, NPPB and NPPC partially stimulated resumption of meiosis inhibited by forskolin in bovine oocytes. 4) Combinations of two or three NPs did not increase the rate of meiosis resumption in cattle. 5) NPPA and NPPC stimulated cGMP production mainly in the cumulus cells after 3 h of maturation. 6) The increase in the concentration of cAMP within the oocyte was inhibited by NPPA and NPPC, even in the presence of forskolin. These results demonstrate that NPs in cattle promote resumption of meiosis; presumably, stimulating degradation mechanisms or inhibition of cAMP production within the oocyte may have functions other than those described so far in meiosis control. It appears that this is the first study showing that these three peptides have similar functions in same species. However, this contrasts with the effects of NPPA that have been reported in rats (Tornell et al., 1990), and of NPPC in mice (Zhang et al., 2010), as well as the effects of NPPB and NPPC that have been recently reported in pigs (Hiradate et al., 2013; Zhang et al., 2015), in which these peptides are essential for maintaining oocyte meiotic arrest.

Initially, we observed that mRNA for *NPPA*, *NPPC* and their receptors (*NPR-1, NPR-2* and *NPR-3*) were present in cumulus cells, and that in the bovine oocyte only mRNA for *NPR-2* was present (Fig. 1). The characterization and function of this system have been demonstrated in rodents, in which mRNA expression for the three NP receptors were observed in rat ovary homogenates (Nagase et al., 1997). With greater predominance of

mRNA for *NPR-2*, these receptors have been demonstrated in granulosa (Robinson et al., 2012) and in cumulus cells from mice (Zhang et al., 2010; Zhang et al., 2014) and pigs (Hiradate et al., 2013), but not in the oocytes (Zhang et al., 2010; Tsuji et al., 2012; Zhang et al., 2015). Consequently, this is the first report of the presence of *NPR-2* in oocytes. However, it is interesting to note that forskolin does not inhibit the resumption of meiosis in denuded bovine oocytes (Sasseville et al., 2009). In addition, the regulation of *NPR-2* in cumulus cells by estradiol, testosterone, dihydrotestosterone and progesterone (Zhang et al., 2011) or oocyte-derived paracrine factors (Zhang et al., 2010), especially BMP15, GDF9, and FGF8, is still unknown in cattle. The presence and regulation of NPPC in mouse granulosa cells is well established (Kawamura et al., 2011; Lee et al., 2013). However, regulation of NPPA mRNA by eCG and hCG was not observed, despite its presence in mouse ovary cells (Kawamura et al., 2011).

In contrast to our results, Franciosi et al. (2014) demonstrated that 100 nM of NPPC delayed oocyte meiosis resumption after 6 or 8 h of culture, maintaining communication via gap junctions in bovine COCs. However, as observed by Bilodeau-Goeseels (2007) for NPPA in bovine, we similarly demonstrated that none of the NPs (NPPA, NPPB and NPPC), individually or combined, inhibit the rate of meiosis resumption in bovine oocytes (supplementary data). Based on the fact that NPs and Ang II, acting through AT2, have similar functions in the circulatory system (Paulis and Unger, 2010), our next step was to investigate whether NPs would stimulate the resumption of meiosis in bovine oocytes, similar to that was demonstrated with Ang II through the AT2 receptor by our group (Giometti et al., 2005; Stefanello et al., 2006; Barreta et al., 2008). To test this hypothesis, we used a well-established model using bovine oocytes cultured in the presence of 100 μM of forskolin to prevent the spontaneous resumption of maturation during the 12 h of culture (Thomas et al., 2002; Sasseville et al., 2009).

When three different concentrations (10, 100 and 100 nM) of each NP were used, we observed that NPPA, NPPB or NPPC stimulated resumption of meiosis in the presence of forskolin (Fig. 2b and 3a), but at lower rates compared to the positive control. A similar effect was observed when two or three NPs were combined (Fig. 2c and 3b), explained by the greater complexity in the mechanisms involved in meiotic arrest in bovine oocytes (Thomas et al., 2002; Sasseville et al., 2009), in combination with the sub-optimal conditions in the *in vitro* process. However, previous results from our group suggest that NPPC is upregulated by LH, using an *in vivo* model to study ovulation in cattle (Tonellotto dos Santos et al., 2012). Furthermore, studies with Ang II (Giometti et al., 2005; Stefanello et al., 2006) and progesterone (Siqueira et al., 2012; De Cesaro et al., 2013) have shown that resumption of meiosis in bovine oocytes was partially stimulated *in vitro.* These results were successfully confirmed *in vivo* (Barreta et al., 2008; Siqueira et al., 2012).

In the present study, it was observed that three NPs were efficient in stimulating resumption of meiosis in a monovular species, using the bovine as a model. These results are the opposite of those observed in polyovular species. In the mouse, Zhang et al. (2010) showed that NPPC binds to NPR-2 in cumulus cells, increasing the concentration of cGMP that reaches the oocyte through gap junctions and thus inhibiting phosphodiesterase 3A (PDE3A) to maintain elevated concentration of cAMP in the oocyte, which prevents resumption of meiosis. We have demonstrated that both NPPA and NPPC induced a significant increase in concentrations of cGMP in bovine cumulus cells and oocytes after 3 h of culture (Fig. 4). Bilodeau-Goeseels (2007) demonstrated that NPPA (1000 nM) stimulates cGMP in bovine COCs after 3 h of maturation, but inhibition of oocyte meiosis was not obtained as stated herein. Conversely, NPPA induced meiotic resumption by stimulating accumulation of cGMP and activating cAMP-phosphodiesterase in hamster and xenopus oocytes (Hubbard and Price, 1988; Sandberg et al., 1993).

Additionally, these two peptides (NPPA and NPPC) prevented the increase of cAMP within the oocyte after 6 h of culture, which is essential for meiotic resumption, even in the presence of forskolin (Fig. 5a). Based on previous studies of PDEs in bovine oocytes (Sasseville et al., 2009) and these *in vitro* results, we may suggest that depending on the model and/or the culture conditions, the cGMP level can interfere with the activity of some of the major PDEs (PDE3, PDE4 and PD8) that degrade cAMP in the bovine oocyte. Furthermore, we cannot rule out a direct action of NPs on the adenylate cyclase.

Although humans are also monovular species, NPPC protein levels in ovarian follicular fluid decrease 36 h after treatment with LH/hCG (Kawamura et al., 2011). In addition, in human oocytes, cilostamide (a PDE3-specific inhibitor) reversibly inhibits the resumption of meiosis (Shu et al., 2008), and PDE3 is the enzyme primarily responsible for cAMP degradation (Conti et al., 2012; Conti, 2013). Thus, although *in vitro* results suggest that NPs will stimulate resumption of meiosis in cattle, it is likely that the control of meiosis in human (also a monovular species) is similar to that in mice oocytes (Hsueh and Kawamura, 2013).

In summary, mRNA encoding natriuretic peptides and their receptors are present in the bovine cumulus-oocyte complex, and *in vitro* oocyte maturation has demonstrated that the natriuretic peptides stimulated resumption of meiosis in cattle, possibly through the modulation of cGMP and cAMP.

Acknowledgements

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Table 1 – Primers used in the expression analysis for system natriuretic peptide characterization experiment.

Gene		Sequence	Ref. or accession no.
NPPA	F	GGAGCAAATCCCGTGTATGG	NM_174124.1
	R	CCTCATCTTCTAAAGGCATCTTGTC	
NPPB	F	GCAACGTGCTGAGGAGGTACT	NM_001166570.1
	R	GAGAAGAACCATCTTATATAAAACAACCAA	
NPPC	F	CAACGCGCGCAAATACAA	NM_174125.2
	R	TCAGCAAAACGCAGCAAGTC	
NPR-1	F	AATTATGGCTCCCTACTAACCACAGA	ENSBTAT00000008184
	R	TCCGGTTCACACGTTTCACA	
NPR-2	F	TCTGCTCCTAAGCTGGGTGAGT	ENSBTAT00000015204
	R	CGGTCATCTGTGCGAGCAT	/ NM_174126
NPR-3	F	TTTGAAGCTAAGCAAGCGTACTCA	NM_174127.2
	R	CAGAACTTTTCACCTCCATGGAA	
GAPDH	F	GATTGTCAGCAATGCCTCCT	NM_001034034.1
	R	GGTCATAAGTCCCTCCACGA	
CYP19	F	GTGTCCGAAGTTGTGCCTATT	(Luo e Wiltbank, 2006)
	R	GGAACCTGCAGTGGGAAATGA	

F: Forward primer; R: Reverse primer

Figure 1 – Expression of mRNA for Natriuretic Peptides and their receptors in bovine cumulus oocyte-complexes (COCs), oocytes and cumulus cells from follicles 3-8 mm in diameter. (a) Natriuretic peptide precursor type-A (NPPA; 100 pb), type-B (NPPB; 150 pb), type-C (NPPC; 150 pb) and (b) natriuretic peptide receptor-1 (NPR-1; 100 pb), receptor-2 (NPR-2; 101 pb) and receptor-3 (NPR-3; 100 pb) were amplified by Real-Time PCR and visualized in agarose gel. The identity of the amplified product was performed based on a DNA ladder (Invitrogen). GAPDH was amplified to validate sample integrity, and water was used as a negative control for each gene.

Figure 2 – Effect of NPPA and/or NPPC to stimulate resumption of meiosis inhibited by forskolin (100 μM) in bovine oocytes. (a) Percentage of GV-arrested oocytes during 12 h of *in vitro* maturation. (b) Dose-response effect (10, 100 and 1000 nM) of NPPA and NPPC, and (c) effect of the combination of NPPA (1000 nM) and NPPC (1000 nM) to stimulate meiosis resumption. C+: positive control; C-: negative control. For each time point in the graph (a), at least 10 COCs per replicate were used (total n = 217). We used at least 15 COCs per treatment/replicate (total COCs used for graph (b) = 402 and (c) = 264). The experiments were performed in triplicate. Different letters indicate statistically significant differences ($P \leq 0.05$) between groups.

Figure 3 – Effect of NPPB and its combinations with NPPA and NPPC in stimulating meiotic resumption in bovine oocytes inhibited by forskolin (100 μM). (a) Dose-response effect (10, 100 and 1000 nM) of NPPB, and (b) combination of NPPA (1000 nM), NPPB (10 nM) and NPPC (1000 nM) to stimulate the meiosis resumption. C+: positive control; C-: negative control; A: NPPA; B: NPPB; C: NPPC. We used at least 15 COCS per treatment/replicate (total COCs used to graph A = 264 and B = 423). The experiments were

performed in triplicate. Different letters indicate statistically significant differences (P≤0.05) between groups.

Figure 4 – cGMP levels in bovine oocyte and cumulus cells in the presence of NPPA and NPPC after 3 and 6 h of maturation. COCs were cultured in the presence of 100 μM forskolin supplemented with NPPA (1000 nM) or NPPC (1000 nM). After 3 or 6 h of culture, the COCs were denuded to measure cGMP in oocyte (a) and cumulus cells (b). C+: positive control; C-: negative control. The experiments were performed in triplicate. Different letters indicate statistically significant differences between groups, and the hash (#) indicates the difference between the hours in the same group (P ≤ 0,05).

Figure 5 – cAMP levels in bovine oocyte and cumulus cells in the presence of NPPA and NPPC after 3 and 6 h of maturation. COCs were cultured in the presence of 100 μM forskolin, supplemented with NPPA (1000 nM) or NPPC (1000 nM). After 3 or 6 h of culture, the COCs were denuded to measure cAMP in oocyte (a) and cumulus cells (b). C+: positive control; C-: negative control. The experiments were performed in triplicate. Different letters indicate statistically significant differences between groups, and the hash (#) indicates the difference between the hours in the same group (P ≤ 0,05).

Figure 1

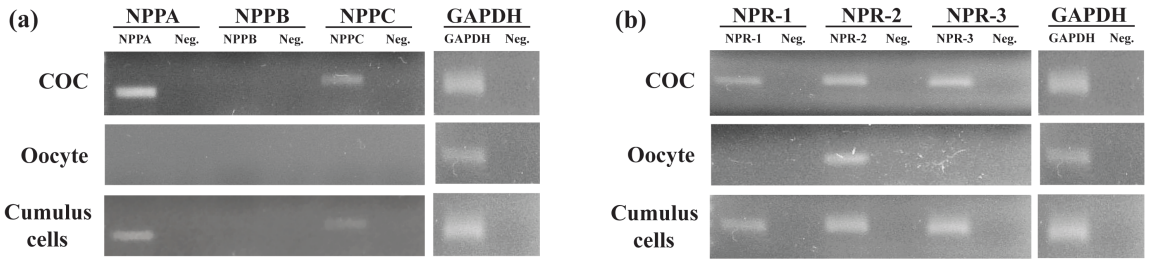

(a)
NPPA
NPPA Neg.
NPPB
NPPB Neg.
NPPC
NPPC Neg.
GAPDH
GAPDH Neg.
COC
Oocyte
Cumulus cells
(b)
NPR-1
NPR-1 Neg.
NPR-2
NPR-2 Neg.
NPR-3
NPR-3 Neg.
GAPDH
GAPDH Neg.
COC
Oocyte
Cumulus cells

Figure 2

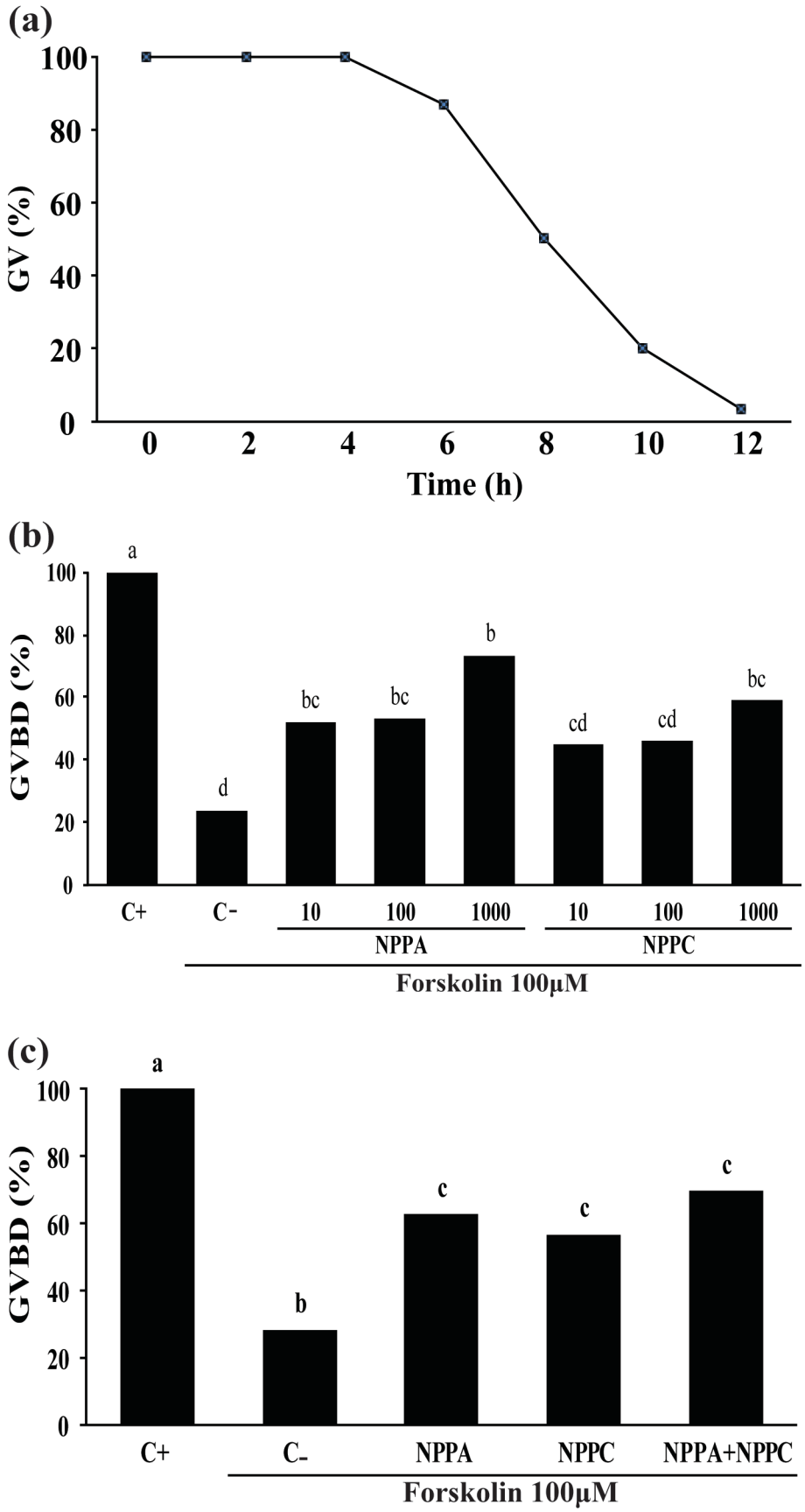

(a)
GV (%)
100
80
60
40
20
0
0
2
4
6
8
10
12
Time (h)
(b)
GVBD (%)
a
d
bc
bc
b
cd
cd
bc
C+
C-
10
100
1000
10
100
1000
NPPA
NPPC
Forskolin 100µM
(c)
GVBD (%)
a
b
c
c
c
C+
C-
NPPA
NPPC
NPPA+NPPC
Forskolin 100µM

Figure 3

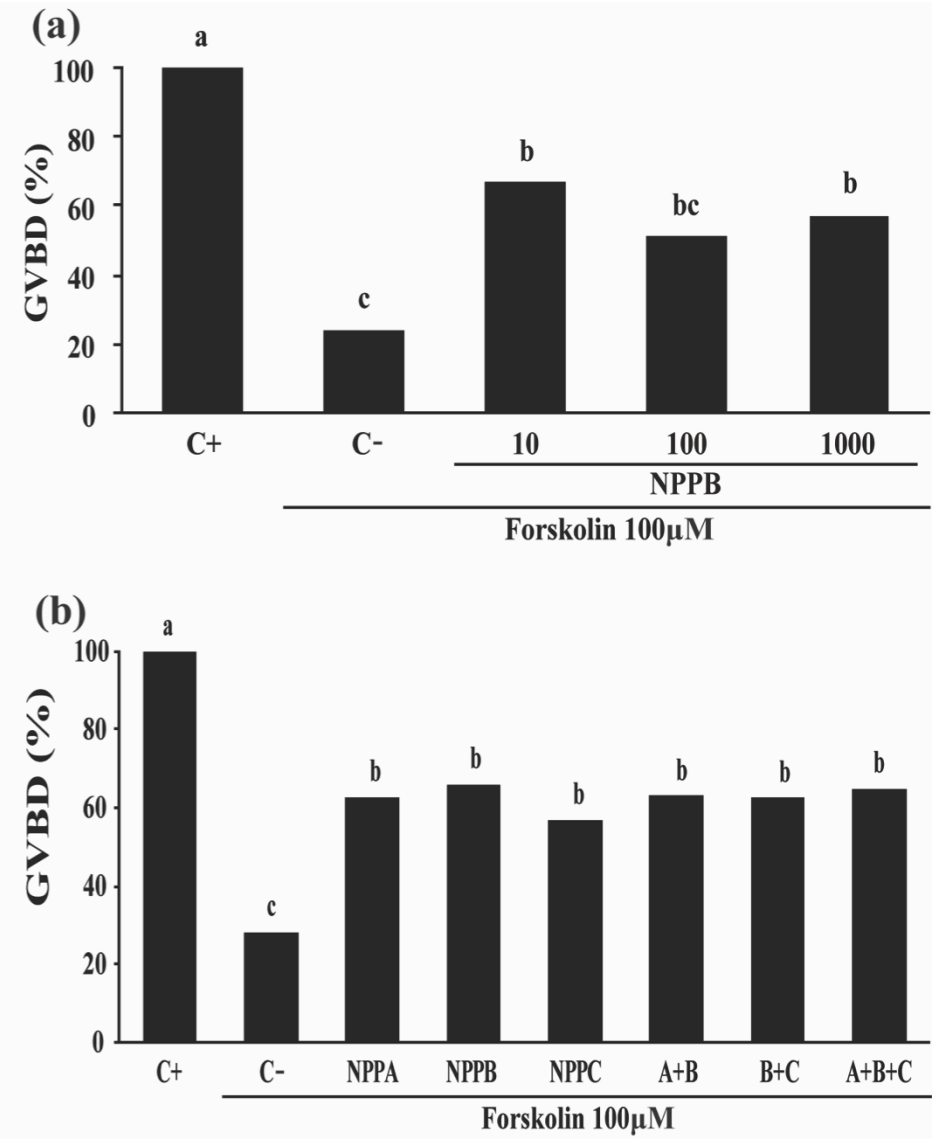

(a)
GVBD (%)
100
80
60
40
20
0
a
c
b
bc
b
C+
C-
10
100
1000
NPPB
Forskolin 100µM
(b)
GVBD (%)
100
80
60
40
20
0
a
c
b
b
b
b
b
b
C+
C-
NPPA
NPPB
NPPC
A+B
B+C
A+B+C
Forskolin 100µM

Figure 4

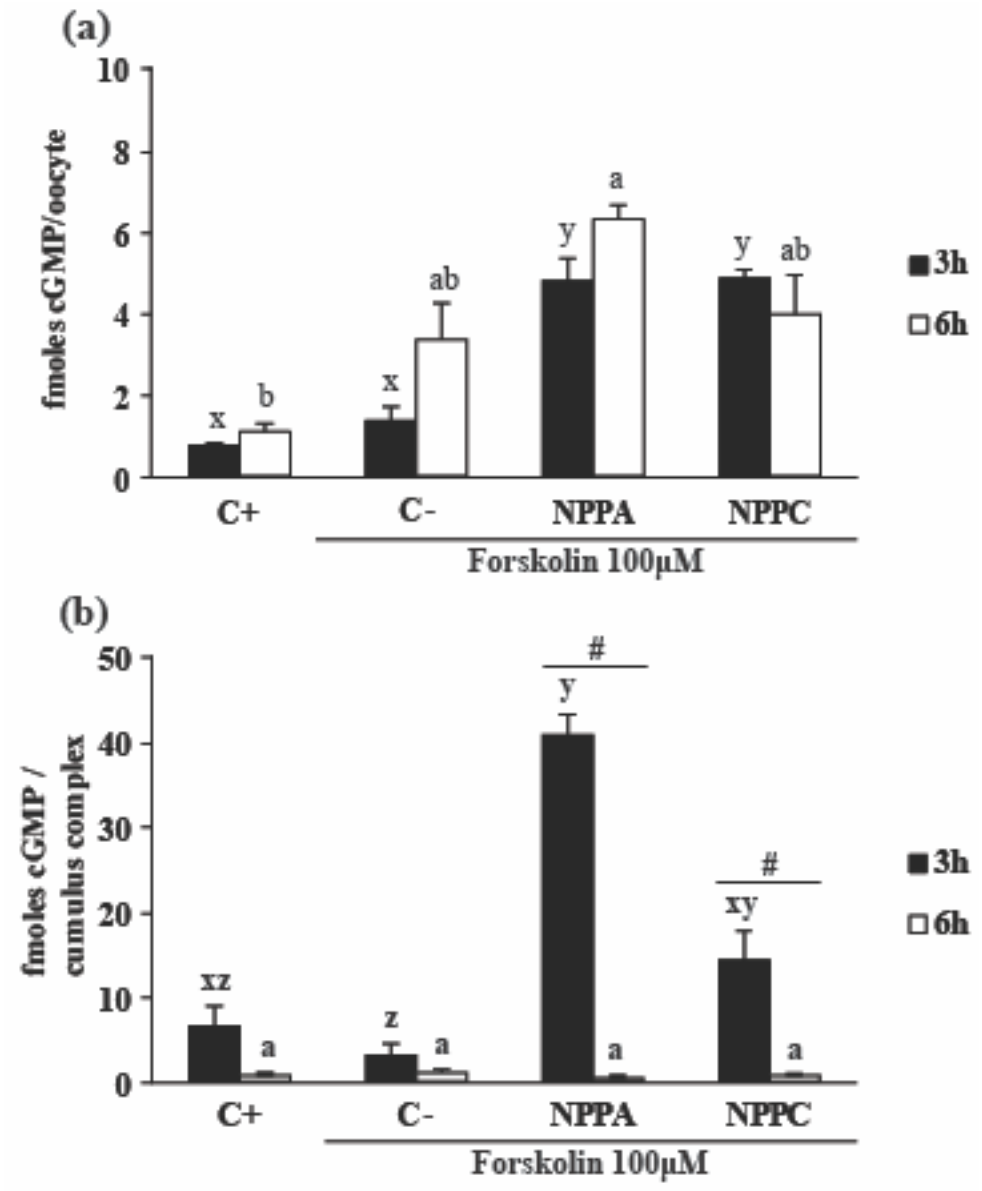
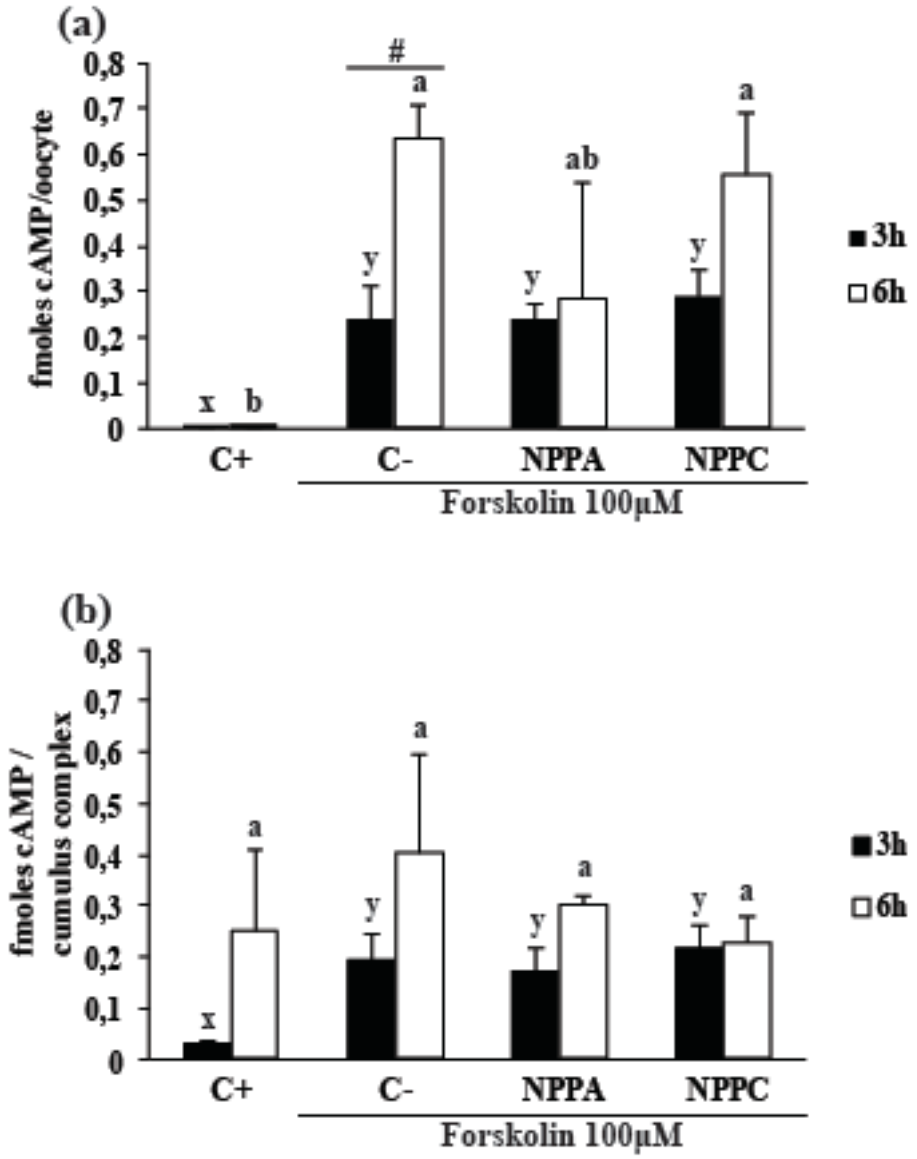

(a)
fmoles cGMP/oocyte
10
8
6
4
2
0
x
b
x
ab
y
a
y
ab
3h
6h
C+
C-
NPPA
NPPC
Forskolin 100µM
(b)
fmoles cGMP / cumulus complex
50
40
30
20
10
0
xz
a
z
a
#
y
a
#
xy
a
3h
6h
C+
C-
NPPA
NPPC
Forskolin 100µM

Figure 5


(a)
fmoles cAMP/oocyte
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
#
a
ab
a
y
y
y
x
b
3h
6h
C+
C-
NPPA
NPPC
Forskolin 100µM
(b)
fmoles cAMP / cumulus complex
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
a
a
a
y
y
y
a
x
3h
6h
C+
C-
NPPA
NPPC
Forskolin 100µM

4. ARTIGO 2

TRABALHO SUBMETIDO PARA PUBLICAÇÃO:

CHARACTERIZATION OF NATRIURETIC PEPTIDE SYSTEM IN GRANULOSA CELLS DURING FOLLICLE DEVIATION AND OVULATION IN CATTLE

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CHARACTERIZATION OF NATRIURETIC PEPTIDE SYSTEM IN GRANULOSA CELLS DURING FOLLICLE DEVIATION AND OVULATION IN CATTLE

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Abridged title: **Natriuretic peptide system in folliculogenesis**

Abstract

Using a well-established *in vivo* model we demonstrate, in bovine granulosa cells, the mRNA for the 3 natriuretic peptide (NP) receptors, whereas none of NP precursors were detected around follicle deviation. The abundance of NPR-3 mRNA was higher in dominant compared to subordinate follicles at the expected time of follicular deviation. After deviation, mRNA for all NP receptors was significantly more abundant in the dominant follicle. FSH treatment maintained mRNA expression of all NP receptors in the second largest follicles and intrafollicular inhibition of estrogen receptors downregulated NPR-1 mRNA of dominant follicles. In granulosa cells of preovulatory follicles collected post GnRH treatment revealed that NPPC mRNA increased at 3 and 6 h after GnRH treatment, but decreased at 12 and 24 h to similar levels observed in samples collected at 0 h. While NPR-2 mRNA was not regulated by GnRH, NPR-3 mRNA gradually decreased at 3 h post GnRH treatment. The mRNA expression of the enzyme Furin increased after 24 h of GnRH challenge. In summary, this study revealed that mRNA encoding important components of the NP system is regulated in bovine granulosa cells during follicular deviation and in response to GnRH treatment.

Keywords: Bovine, follicle development, follicle dominance; granulosa cells, natriuretic peptides.

1. Introduction

In cattle, endocrine and paracrine/autocrine factors control follicle selection, dominance and ovulation, and granulosa cells are essential in these processes. The NP system comprises three small polypeptides, atrial NP (ANP), B-type NP (BNP) and C-type NP (CNP), with highly conserved 17-member ring structure, as well as three receptors, NPR-1, -2 and -3, expressed on the target cell surface (Levin *et al.* 1998; Gardner *et al.* 2007; Misono *et*

al. 2011; Potter 2011). Expression of natriuretic peptide precursors, NPPA, NPPB and NPPC, can be assessed at the mRNA level (Nakao *et al.* 1992; Potthast and Potter 2005; Potter *et al.* 2006). Beyond peptides and receptors, the convertase enzymes corin and furin are involved in NP regulation, processing NPPA (Yan *et al.* 2000) and NPPC (Wu *et al.* 2003) into mature peptides.

In the reproductive system, CNP and NPR-2 are known to have pivotal roles. In rodents, oocyte meiotic resumption is blocked when CNP binds to NPR-2, which stimulates the synthesis of cyclic guanosine 3,5-monophosphate (cGMP) that diffuses into the oocyte through gap junctions and inhibits phosphodiesterase 3A, maintaining high levels of cyclic adenosine 3,5-monophosphate (cAMP) (Vaccari *et al.* 2009; Norris *et al.* 2010; Zhang *et al.* 2010). It has also been shown that both CNP and BNP bind to NPR-2 and inhibit the resumption of oocyte meiosis in pigs (Hiradate *et al.* 2013; Zhang *et al.* 2014; Zhang *et al.* 2015). In mice, loss-of-function mutations in either CNP or NPR-2 result in precocious resumption of oocyte meiosis (Zhang *et al.* 2010; Tsuji *et al.* 2012; Geister *et al.* 2013). Furthermore, NPR-2 knockout mice had defective corpus luteum formation (Tamura *et al.* 2004). On the other hand, CNP can stimulate preantral and antral follicular growth in mice, since the expression of key genes controlling follicle maturation, steroidogenesis, and ovulation were similarly induced by CNP compared to eCG treatment (Sato *et al.* 2012).

It has been reported that FSH/eCG, estradiol and follicular development stimulate NPPC and NPR-2 expression in cumulus and granulosa cells of mice (Kawamura *et al.* 2011; Lee *et al.* 2013) and pigs (Zhang *et al.* 2014; Zhang *et al.* 2015). The highest expression of NPPC and NPR-2 occur simultaneously to the preovulatory LH surge (Kawamura *et al.* 2011; Tsuji *et al.* 2012; Lee *et al.* 2013; Zhang *et al.* 2014; Zhang *et al.* 2015). Similar regulation was observed for NPPB in swine granulosa cells (Zhang *et al.* 2015). Factors involved in follicular dominance in monovular species, such as BMP15, GDF9, FGF8 and estradiol,

promote the expression of NPR-2 in cumulus cells (Juengel *et al.* 2009; Zhang *et al.* 2010; Zhang *et al.* 2011; Hiradate *et al.* 2013; Jiang *et al.* 2013; Lee *et al.* 2013; Gasperin *et al.* 2014).

The expression of NPPC in granulosa cells (Kawamura *et al.* 2011; Robinson *et al.* 2012; Zhang *et al.* 2014), and NPR-2 activity in cumulus cells (Robinson *et al.* 2012; Wang *et al.* 2013) decrease quickly after the LH/hCG surge in multiovular species. In addition, Lee *et al.* (2013) showed that NPR-3 expression in cumulus and granulosa cells increases after the LH peak in mice, and that CNP clearance in the follicle is similar between control and knockout animals for this receptor. These results suggest that NPR-3 may have other functions, besides promoting clearance of other peptides. It was showed that CNP may inhibit the resumption of oocyte meiosis in pigs by acting on both NPR-2 and NPR-3 (Santiquet *et al.* 2014).

In mice, NPR-1 is expressed in granulosa cells, but it is not regulated by FSH/eCG or LH/hCG (Kawamura *et al.* 2011). However, it main ligand, ANP, is expressed in granulosa cells of rodents (Russinova *et al.* 2001) and pigs (Ivanova *et al.* 2003). It was demonstrated that low ANP levels in the ovary may contribute to the abnormal steroid hormone balance in rodents with polycystic ovaries (Pereira *et al.* 2014), and may have a role in the pathogenesis of polycystic ovary syndrome in women (Lauria *et al.* 2013).

So far, few studies have been conducted to investigate the role of the NP system in the regulation of reproductive functions in monovular species, including cattle. Using a microdialysis system, it was proposed that ANP increases prostaglandins and angiotensin II production in mature follicles, and modulates steroidogenesis in follicular cells (Acosta *et al.* 1999). It was also suggested that ANP may participate in the regulation of follicular dominance in cattle (Montrezor *et al.* 2015). Moreover, Franciosi *et al.* (2014) demonstrated that CNP has the ability to delay oocyte meiotic resumption and maintain cumulus-oocyte

communication in bovine cumulus-oocyte complexes (COCs). In addition, our previous studies revealed that two NPs (NPPA and NPPC) and all three receptors (NPR-1, -2 and -3) are expressed in bovine COCs, and demonstrated that NPPA and NPPC increased cGMP levels in cumulus cells after 3 h of culture (De Cesaro *et al.* 2015). However, it remains unclear how the NP system is regulated during follicular selection, dominance and ovulation in monovular species. Therefore, the aim of this study was to evaluate the mRNA expression of NP precursors, receptors and key convertases enzymes in bovine granulosa cells during follicular deviation and after GnRH/LH-induced ovulation.

2. Material and methods

All experimental procedures were approved by the Federal University of Santa Maria Animal Care and Use Committee. Adult beef cows (predominantly Hereford and Angus), cycling and non-lactating with a body condition score between 3-4 [scale from 1 (thin) to 5 (obese)] were used in this study.

2.1 Collection of granulosa cells

Cows were ovariectomized by colpotomy (Drost *et al.* 1992) and granulosa cells were harvested from the desired follicles, washed in PBS and immediately stored in liquid nitrogen. After arriving at the laboratory, the samples were stored at -80°C for future analyzes.

2.2 RNA extraction, reverse transcription and real-time PCR

Total RNA was extracted from granulosa cells using silica-based protocol (Qiagen, Mississauga, ON, Canada) according to the manufacturer's instructions. Quantity and RNA purity were measured using the NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA; absorbance ratio at 260/280 nm). Ratios above 1.8 were considered pure, and all

samples used in the present study were above this threshold. To generate the complementary DNA (cDNA), 500 ng RNA was first treated with 0.1U DNAse (Invitrogen, Carlsbad, CA, USA) at 37°C for 5 minutes). After DNAse inactivation at 65°C for 10 minutes, samples were incubated in a final volume of 20 µL with iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) following the manufacturer's protocols.

Real time quantitative PCR (qPCR) reactions were run in the CFX384 real-time PCR detection system (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad), and bovine-specific primers (Table 1) taken from the literature or designed using Primer-Blast, and specificity was confirmed using BLAST (NCBI). Standard two-step qPCR was performed to amplify each transcript with an initial denaturation at 95°C for 5 minutes followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 58°C for 30 seconds and melting-curve analysis was used to verify the specificity of reaction products. Samples were run in duplicates, standard curve method was used to determine the abundance of mRNA for each gene, and expression was normalized to the abundance of the housekeeping genes *cyclophilin*, *RPL19*, *RPLP0* and/or *GAPDH*. To test cross contamination with theca cells, presence of mRNA that encode CYP17A1 was assessed and all samples were free from contamination by theca cells after 30 PCR cycles.

2.3 Ultrasound-guided intrafollicular injection

Epidural anesthesia (3-6 ml of lidocaine 1%) was administered and perineal cleaning was performed before intrafollicular injections. Injections were guided by ultrasound equipped with a 7.5 MHz vaginal probe (Aquila Vet, Pie Medical Equipment BV, Netherlands) coupled to a biopsy guide. A system with two sterile needles was used and the dose of each treatment was estimated by the linear regression equation as previously described by Ferreira *et al.* (2007).

2.4 Experimental design

Experiment 1. NP system expression in granulosa cells around follicle deviation

This experiment was conducted to investigate the mRNA expression of NP system before, during, and after follicular deviation. Thirty-two cyclic cows were synchronized with two injections of a PGF2α analogue (sodium cloprostenol; Ciosin; Intervet/Schering–Plough; 250 mg; i.m.) given eleven days apart (Gasperin *et al.* 2014). Fifteen cows that were detected in estrus 3 to 5 days after the second PGF2α administration were included in the experiment. Ovaries were examined once a day by transrectal ultrasonography. All follicles larger than 5 mm in diameter were drawn using 3-5 virtual slices of the ovary, allowing a three-dimensional localization of follicles and monitoring individual follicles development during the first follicular wave of the cycle (Jaiswal *et al.* 2004). The day of the follicular emergence was designated as Day 0 of the wave and was retrospectively identified as the last day on which the dominant follicle was 4 to 5 mm in diameter (Rivera and Fortune 2001). Cows were randomly assigned to be ovariectomized when the size of the largest and second largest follicle did not have a significant difference (day 2 of the follicular wave; n = 4), had slight difference (day 3; n = 4) or marked difference (day 4; n = 7).

Experiment 2. NP receptors expression after FSH treatment

Based on the results of the first experiment, which revealed that only NP receptors are presents in granulosa cells during the deviation process in bovine, this experiment was conducted to compare mRNA levels of NP receptors between the largest and the second-largest follicles collected from FSH (Folltropin-V, Bioniche Animal Health, ON, Canada) treated cows (n = 3). After estrus synchronization, as described in the first experiment, two doses of 30 and 20 mg FSH were i.m. administered 12 h apart on the second and third days of

the follicular wave, respectively (Rovani *et al.* 2014; Ilha *et al.* 2015). Ovaries were collected 12 h after the last FSH treatment and granulosa cells from the dominant largest follicle (DF1) and the second dominant largest follicle (DF2) were harvested as described above.

Experiment 3. Effect of estrogen receptors inhibition on NP receptors expression in granulosa cells

This experiment was performed to evaluate whether the mRNA expression of NP receptors is regulated by estrogen receptors during follicular deviation. For this, cows were synchronized with a progesterone releasing intravaginal device (progesterone, 1 g; DIB®, Intervet/Schering-Plough, Brazil), an i.m. injection of 2 mg estradiol benzoate (Genix, Anápolis, GO, Brazil) to induce follicular regression and emergence of a new follicular wave, and two (12 h apart) i.m. injections of PGF2α. Four days later, the progesterone devices were removed and the ovaries were monitored daily until the largest follicle of the growing cohort reached a diameter of 7–8 mm. Only cows without a corpus luteum in an ultrasound exam were included in the study. Fulvestrant (Sigma–Aldrich, Brazil), an inhibitor of estrogen receptors, was intrafollicularly injected at the final concentration of 100 μM (n = 3), and a similar volume of saline was injected in the follicles of control animals (n = 3). The fulvestrant dose was determined based on previous studies (Ferreira *et al.* 2011b; Barreta *et al.* 2013; Rovani *et al.* 2014). The cows were ovariectomized 12 h after intrafollicular injection to harvest granulosa cells.

Experiment 4. mRNA expression of the NP system in granulosa cells after GnRH/LH surge

To evaluate the effect of GnRH/LH in the mRNA expression of the NP system and two convertase enzymes (corin and furin) 27 cyclic beef cows were pre-synchronized to

obtain a LH responsive follicle (≥12 mm) (Sartori *et al.* 2001) according to previous studies (Santos *et al.* 2012; Siqueira *et al.* 2013). Briefly, cows received two doses of a PGF2α, 12 h apart, 2 mg of estradiol benzoate, and an intravaginal progesterone device on day 0 (progesterone, 1 g; DIB®, Intervet/Schering-Plough, Brazil). Intravaginal devices were removed on day 9, ovaries were examined by transrectal ultrasonography, and cows that had GnRH-responsive preovulatory follicles (≥12 mm) were challenged with 100 μg of gonadorelin acetate (Profertil®, Tortuga, Brazil) i.m. 12 h after removal of the intravaginal progesterone device. The ovaries were removed by colpotomy performed at 0, 3, 6, 12 or 24 h (5/6 animals per group) after GnRH treatment. Follicular fluid and granulosa cells were collected and stored in liquid nitrogen. Follicular fluid was used to assess the steroid concentration, and granulosa cells were used to assess mRNA expression. Estradiol and progesterone levels in follicular fluid were quantified at private laboratories using competitive immunoassay and direct chemiluminescent technologies (kits ADVIA Centaur: Estradiol-6 III (E2-6 III) and Progesterone (PRGE)).

Experiment 5. DNA sequencing and analysis

A pool of granulosa cells cDNA (used in experiment 4) was used to test the homology of the primers for NPPA, NPPB, NPPC, NPR-1, NPR-2 and NPR-3. For this, PCR was performed in a PTC-100 Programmable Thermal Controller thermocycler (MJ Research) using the enzyme Platinum Taq DNA polymerase (Invitrogen) in a final volume of 25 μl reaction in a total of 40 cycles of amplification. After PCR, 60 ng of each reaction and 4.5 pmol of each primer (forward or reverse) was added to a new tube. Ultrapure water was added to a final volume of 6 μl, and then dried at 37°C. DNA sequencing was performed in a private laboratory using an automated capillary sequencer ABI-Prism 3500 Genetic Analyzer (Applied Biosystems). The electropherogram quality analysis was performed with Chromas

lite 2.01 DNA sequencing software (Technelysium Pty Ltd) and sequences were compared for homologies in Genbank with EMBOSS Needle using the Needleman-Wunsch alignment algorithm (http://www.ebi.ac.uk). Analysis of amplicons confirmed high homology with the gene of interest: NPPA, NPPC, NPR-1, NPR-2 and NPR-3.

2.5 Statistical analysis

All continuous data were tested for normal distribution using Shapiro–Wilk test, normalized when necessary and submitted to ANOVA using JMP software (SAS Institute Inc., Cary, NC, USA). The differences between the two largest follicles in each day were assessed by paired Student's T test using cow as subject. Results are presented as means ± S.E.M. and $P \leq 0.05$ was considered statistically significant.

3. Results

3.1 mRNA expression of the NP system in granulosa cells during follicular deviation

The *in vivo* model of follicular deviation and dominance used in this study has been well characterized in previous studies by our group regarding a number of parameters, including follicular diameter, aromatase (CYP19A1) and LH receptor (LHCGR) expression in granulosa cells, and estradiol concentration in follicular fluid (Ferreira *et al.* 2011a; Gasperin *et al.* 2014; Rovani *et al.* 2014).

We observed in this study that mRNA for NP receptors (NPR-1, NPR-2 and NPR-3) but not NPs (NPPA, NPPB and NPPC) is expressed in granulosa cells around the follicle deviation in cattle. Before follicular deviation (day 2), the abundance of NPR-1, NPR-2 and NPR-3 mRNA did not differ between the largest (F1) and second largest follicles (F2) ($P \geq 0.05$; Fig. 1A). At the expected time of follicular deviation (day 3), NPR-3 mRNA abundance was higher in F1 compared to F2 follicles ($P \leq 0.05$; Fig. 1B). After follicular

deviation (day 4), mRNA for all NP receptors was significantly more abundant in dominant compared to subordinate follicles (P≤0.05; Fig. 1C).

3.2 Effect of FSH treatment on NP receptors expression in granulosa cells

Based on previous studies confirming that the largest and the second largest follicle from FSH-treated cows are healthy and similar to dominant follicles of non-treated cows (Rovani *et al.* 2014; Ilha *et al.* 2015), we hypothesized that FSH is required to maintain NP receptors expression in granulosa cells. As expected, there was no difference in NPR-1, NPR-2 or NPR-3 mRNA levels between the dominant largest (DF1) and the second dominant largest follicle (DF2) collected from FSH-treated animals beyond the follicular deviation stage (P≥0.05; Fig. 2).

3.3 Effect of intrafollicular inhibition of estrogen receptors on NP receptors expression in granulosa cells

To investigate if the expression of NP receptors is affected by estradiol, animals were intrafollicularly injected with 100 μM Fulvestrant, an inhibitor of estrogen receptors. This treatment was previously shown to decrease CYP19A1 expression and induce atresia of the dominant follicle (Rovani *et al.* 2014). The mRNA abundance of NPR-1 was decreased in Fulvestrant-treated compared to control follicles. However, NPR-2 and NPR-3 mRNA abundance did not differ between Fulvestrant- and saline-treated follicles at 12 h after intrafollicular treatment (P≤0.05; Fig. 3).

3.4 mRNA expression of the NP system and convertase enzymes after GnRH/LH surge in granulosa cells of preovulatory follicles

Ultrasound evaluations revealed no differences in follicular diameter at the different time points before ovariectomy (data not shown). Estradiol concentration in follicular fluid increased at 3 h after GnRH treatment, the time when the endogenous LH surge is expected to occur, and gradually decreased thereafter ($P \leq 0.05$; Fig. 4A). Progesterone concentration increased at 3 h after GnRH, declined from 3 to 12 h, and increased again at 24 h, indicating luteinization of the follicular cells (Fig. 4B).

In granulosa cells, qPCR analyses revealed the presence of mRNA encoding NPPA, NPPC, NPR-1, NPR-2, NPR-3, corin and furin, whereas NPPB mRNA was not detected. There was no significant differences in NPPA mRNA abundance between samples collected at the different time points after GnRH treatment (Fig. 5A). On the other hand, NPPC mRNA increased at 3 and 6 h after GnRH treatment ($P \leq 0.05$), but decreased at 12 and 24 h to similar levels of samples collected at 0 h (Fig. 5B). The mRNA abundance of furin increased at 24 h after GnRH treatment ($P \leq 0.05$; Fig. 5D), but corin mRNA was not regulated by GnRH treatment ($P \leq 0.05$; Fig. 5C). NPR-1 mRNA increased at 24 h after GnRH treatment compared to 0 h ($P \leq 0.05$; Fig. 6A), but NPR-2 mRNA did not change after GnRH treatment (Fig. 6B). The abundance of NPR-3 mRNA gradually decreased from 3 to 12 h and remained low at 24 h after GnRH ($P \leq 0.05$; Fig. 6C).

4. Discussion

To gain additional insights into the role of the NP system in the regulation of ovarian cell functions in monovular species, we used cattle as an *in vivo* model to investigate the regulation of mRNA expression of NP components in granulosa cells obtained during follicular deviation, after FSH-treatment, after intrafollicular inhibition of estrogen receptors, and at different time points after GnRH treatment. Experiments in this study were conducted using a well-established *in vivo* model that has been previously validated by our group in

studies investigating follicular deviation, follicular co-dominance and response to GnRH treatment (Ferreira *et al.* 2011a; Ferreira *et al.* 2011b; Santos *et al.* 2012; Siqueira *et al.* 2013; Gasperin *et al.* 2014; Rovani *et al.* 2014; Ilha *et al.* 2015). In addition, identity of amplicons was confirmed by DNA sequencing, and the ovulation model was validated by assessing the steroid levels in follicular fluid. Our results were similar to those observed in previous studies for both estradiol (Santos *et al.* 2012) and progesterone (Fortune *et al.* 2009) profiles in preovulatory follicles. It is well established that LH surge is induced 2 hours after the administration of GnRH agonist (Quirk *et al.* 2004). The shift in the steroidogenesis profile in the follicular fluid demonstrated the physiological process, in which the estradiol levels decreased 3 h after GnRH administration and progesterone levels increased just before ovulation (Bridges *et al.* 2006; Fortune *et al.* 2009). The main advantage of using cattle as a model resides in the fact that samples can be collected from individual follicles and the interaction between different cell types is preserved, being all follicular cells under the same physiological endocrine environment.

We first observed in this study that mRNA for NPR-1, NPR-2 and NPR-3 is expressed by granulosa cells during follicular deviation and preovulatory follicles. However, none of NP precursors were detected in bovine granulosa cells around follicular deviation. This suggests that NPs may not have a fundamental role in the regulation of follicular deviation and dominance in cattle. However, unlike our results, NPPA and NPPC mRNA expression was detected in granulosa cells during the follicular growth in rodents (Gutkowska *et al.* 1999; Zhang *et al.* 2010; Kawamura *et al.* 2011), NPPB and NPPC in pigs (Zhang *et al.* 2015), and NPPA, NPPB and NPPC in goats (Peng *et al.* 2013). Moreover, Sato *et al.* (2012) demonstrated that CNP stimulated preantral and antral follicle growth, and had similar effects of eCG on expression of genes involved in follicle maturation, steroidogenesis and ovulation.

Based on this knowledge and the results of this study, we suggest that during the follicular wave, the CNP has not fundamental role before ovulation stimulation in cattle.

We have also observed that mRNA expression for the three NP receptors (NPR-1, 2 and 3) is maintained in dominant follicles but decreases in subordinate follicles after deviation. This similar regulation of all three NPRs has not been reported in previous studies in other species. Indeed, only the NPR-2 was shown to be upregulated by FSH and estradiol in polyovulatory species (Kawamura *et al.* 2011; Zhang *et al.* 2011; Zhang *et al.* 2015). Our findings revealed that NPRs expression during follicular deviation is FSH-dependent, since both co-dominant follicles collected beyond the deviation stage from FSH-treated cows have similar mRNA abundance for the three receptors. However, the intrafollicular inhibition of estrogen receptors revealed that only NPR-1 was downregulated after 12 h from treatment. This suggests that, at least for NPR-2 and 3, the positive effect of FSH on NPRs mRNA expression is not mediated via estrogen receptors. Nonetheless, it is possible that the time between treatment and recovery of granulosa cells was not sufficient, given that Fulvestrant is known to induce follicular atresia (Barreta *et al.* 2013; Rovani *et al.* 2014).

The present study also revealed that GnRH/LH triggers NPPC mRNA expression in bovine granulosa cells of preovulatory follicles. This observation contrasts with previous studies that reported downregulation of NPPC mRNA by LH in granulosa cells of mice (Kawamura *et al.* 2011; Lee *et al.* 2013), goats (Peng *et al.* 2013) and pigs (Zhang *et al.* 2014; Zhang *et al.* 2015). Another study *in vitro*, using bovine granulosa cells from follicles with 2-6 mm in diameter, proposed a dose- and time-dependent decrease in NPPC mRNA induced by LH stimulation (Yang *et al.* 2016). However, it is known that bovine follicles with less than 8.5 mm in diameter do not ovulate is response to LH, even using a 10-fold greater dose required to ovulate ≥12 mm follicles (Sartori *et al.* 2001). It is also important to highlight that most *in vitro* studies do not allow the interaction between different cell types and that both LH

and FSH increase concomitantly after GnRH treatment in cattle (Dufour *et al.* 1999). It is therefore possible that the NP system is differently regulated in bovine granulosa cells compared to other species. Consistent with this, it was observed in rodents and pigs that NPPC downregulation occurs through epidermal growth factor receptor (EGFr) in granulosa cells (Tsuji *et al.* 2012; Zhang *et al.* 2014). On the other hand, unpublished data by our group indicate that the NPPC is upregulated by EGF-L (like growth factors) in bovine ganulosa cells.

The increase in the NPR-1 mRNA abundance near ovulation observed in this study corroborates with previous studies that demonstrated an effect of ANP on steroidogenesis and corpus luteum formation (Acosta *et al.* 1999; Kobayashi *et al.* 2002; Montrezor *et al.* 2015). However, we did not observe changes in NPR-2 mRNA expression after GnRH injection, which differs from previous findings in polyovulatory species showing that LH signaling decreases NPR-2 expression in cumulus and granulosa cells (Kawamura *et al.* 2011; Tsuji *et al.* 2012; Lee *et al.* 2013; Zhang *et al.* 2014; Zhang *et al.* 2015). These results provide futrher evidence of species-specific regulation of CNP/NPR-2 during the ovulatory process.

This study also revealed NPPC mRNA is upregulated and NPR-3 mRNA is downregulated in response to GnRH/LH treatment. These results contrast with previous reports for the same NP members in polyovulatory species (Kawamura *et al.* 2011; Tsuji *et al.* 2012; Lee *et al.* 2013; Zhang *et al.* 2014). Recent studies by Santiquet *et al.* (2014) demonstrated other functions of NPR-3 besides clearance of NP. Although we demonstrate the regulation of NPR-3 mRNA in cattle, the reproductive function for this receptor in monovular species is not yet known.

It is known that NEP enzymatic activity decreases after eCG treatment (Pereira *et al.* 2009) and increases in polycystic ovaries of rats (Pereira *et al.* 2014). In our experimental model, the NEP mRNA pattern increased 12 h after GnRH (Santos *et al.* 2012). This suggests

that CNP may have a more relevant role than ANP during the ovulatory process, since NPPA and corin mRNA expression are not regulated by GnRH/LH, and ANP levels depend on the balance between the peptide synthesis and its degradation that can be mediated by NEP or by NPR-3.

In summary, this study characterized the mRNA expression profile of the NP system in bovine granulosa cells during follicular deviation and ovulation. The higher mRNA abundance of NP receptors in the dominant compared to the subordinate follicle may be used as an indicator of follicle health. The increased mRNA expression of NPPC and furin and the decreased mRNA for NPR-3 after GnRH/LH in preovulatory follicle suggest that CNP may be involved in ovulation and luteinization in cattle. Functional studies are necessary to elucidate the signaling pathways and dissect the exact role of the NP system during follicular selection in monovular species.

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Table 1 - Primers used in the expression analysis of *Bos taurus taurus* genes.

Gene	Sequence	Reference or accession nº
Cyclophilin	F GGTCATCGGTCTCTTTGGAA R TCCTTGATCACACGATGGAA	(Ledoux *et al.* 2006)
GAPDH	F ACCCAGAAGACTGTGGATGG R CAACAGACACGTTGGGAGTG	NM_001034034.2
RPL19	F GCCAACTCCCGTCAGCAGA R TGGCTGTACCCTTCCGCTT	NM_001040516.1
RPLP0	F GGCGACCTGGAAGTCCAACT R CCATCAGCACCACAGCCTTC	NM_001012682.1
NPPA	F GGAGCAAATCCCGTGTATGG R CCTCATCTTCTAAAGGCATCTTGTC	(De Cesaro *et al.* 2015)
NPPB	F GCAACGTGCTGAGGAGGTACT R GAGAAGAACCATCTTATATAAAACAACCAA	(De Cesaro *et al.* 2015)
NPPC	F CAACGCGCGCAAATACAA R TCAGCAAAACGCAGCAAGTC	(De Cesaro *et al*, 2015)
NPR-1	F AATTATGGCTCCCTACTAACCACAGA R TCCGGTTCACACGTTTCACA	(De Cesaro *et al.* 2015)
NPR-2	F TCTGCTCCTAAGCTGGGTGAGT R CGGTCATCTGTGCGAGCAT	(De Cesaro *et al.* 2015)
NPR3	F TTTGAAGCTAAGCAAGCGTACTCA R CAGAACTTTTCACCTCCATGGAA	(De Cesaro *et al.* 2015)
Corin	F TTGGAACTCTGCTTGAATTTGC R GAGAAGACTCCCAGCTGATGGA	XM_002688245.1
Furin	F CCTGCGGCAGAAGTGTACAG R CCCGCCAAGTGAGGTTCTTA	NM_174136.2

F, Forward primer; R, Reverse primer.

Figure 1 – Relative mRNA abundance (mean ± standard error of mean) of NPR-1, NPR-2 and NPR-3 in granulosa cells before (A), during (B), and after (C) follicular deviation. The largest (F1; black bar) and second largest (F2; open bar) follicle from each cow was collected of the first follicular wave. Asterisk (*) indicates statistical difference ($P \leq 0.05$) between F1 and F2.

Figure 2 – Relative mRNA abundance (mean ± standard error of mean) of NPR-1 (A), NPR-2 (B) and NPR-3 (C) in granulosa cells of the dominant largest (DF1; black bar) and the second dominant largest (DF2; open bar) follicle collected from FSH-treated animals beyond the follicular deviation. No statistical differences ($P \geq 0.05$) were observed between the DF1 and DF2.

Figure 3 – Relative mRNA abundance (mean ± standard error of mean) of NPR-1 (A), NPR-2 (B) and NPR-3 (C) in granulosa cells of the largest follicle after intrafollicular injection of saline (CONT; black bar) or 100μM fulvestrant (FULV; open bar). Intrafollicular injection was performed when the largest follicle reached a diameter between 7 to 8mm and granulosa cells were recovered after 12 h. Asterisk (*) indicates statistical difference ($P \leq 0.05$) between the CONT and FULV.

Figure 4 – Concentration of Estradiol (A) and Progesterone (P4- B; ng/ml mean ± standard error of mean; n= at least 5-6/time point) in follicular fluid obtained from preovulatory follicles at 0, 3, 6, 12, or 24 h after GnRH analogue challenge. Different letters represent statistical difference ($P \leq 0.05$).

Figure 5 – Relative mRNA abundance (mean ± standard error of mean) of NPPA (A), NPPC (B), CORIN (C) and FURIN (D) in granulosa cells at different times after application of GnRH. Different letters represent statistical difference (P≤0.05).

Figure 6 – Relative mRNA abundance (mean ± standard error of mean) of NPR-1 (A), NPR-2 (B) and NPR-3 (C) in granulosa cells at different times after application of GnRH. Different letters represent statistical difference (P≤0.05).

Figure 1

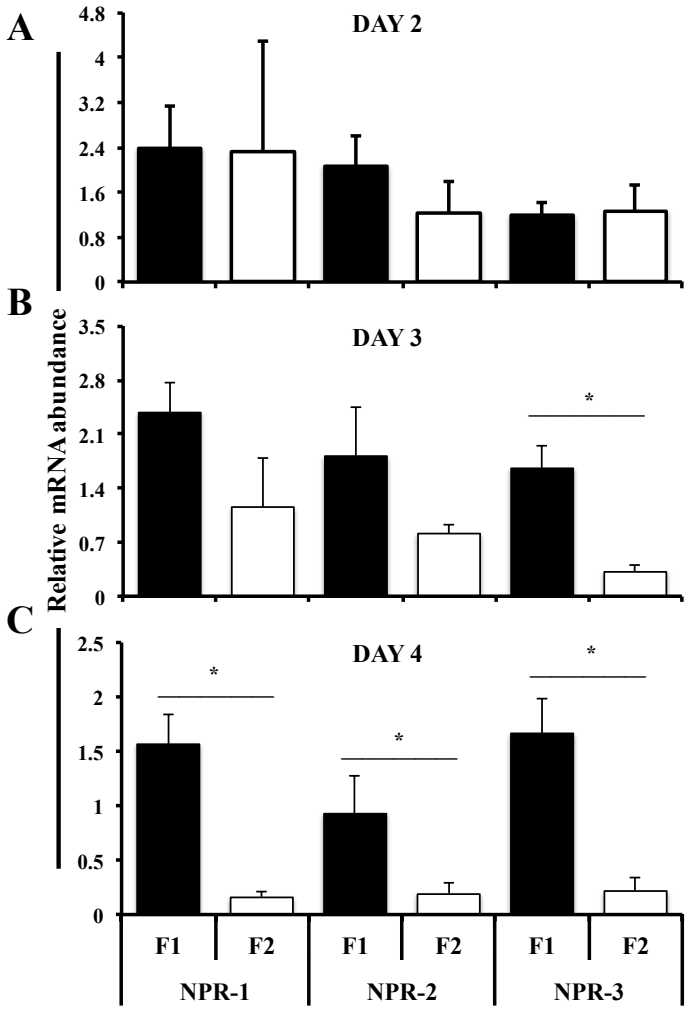

A
DAY 2
4.8
4
3.2
2.4
1.6
0.8
0
B
DAY 3
3.5
2.8
2.1
1.4
0.7
0
Relative mRNA abundance
*
C
DAY 4
2.5
2
1.5
1
0.5
0
*
*
*
F1
F2
F1
F2
F1
F2
NPR-1
NPR-2
NPR-3

Figure 2

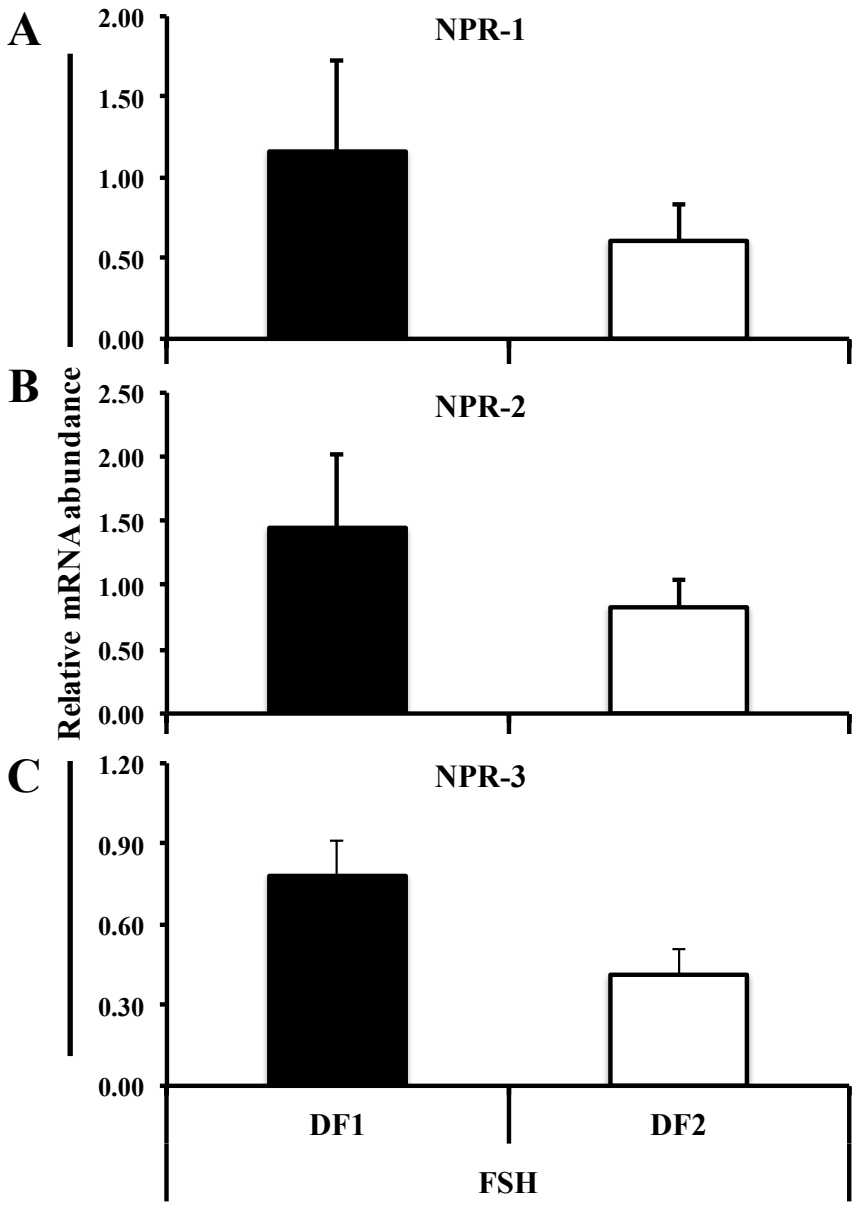

A
NPR-1
2.00
1.50
1.00
0.50
0.00
B
NPR-2
2.50
2.00
1.50
1.00
0.50
0.00
Relative mRNA abundance
C
NPR-3
1.20
0.90
0.60
0.30
0.00
DF1
DF2
FSH

Figure 3

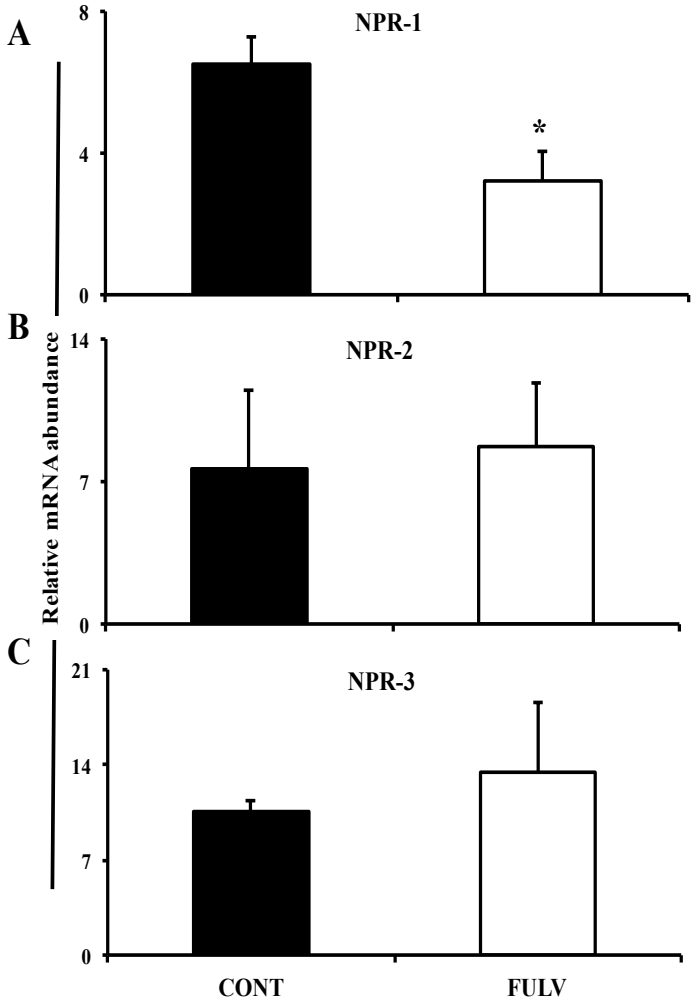

A
NPR-1
8
4
0
*
B
NPR-2
14
7
0
Relative mRNA abundance
C
NPR-3
21
14
7
0
CONT
FULV

Figure 4

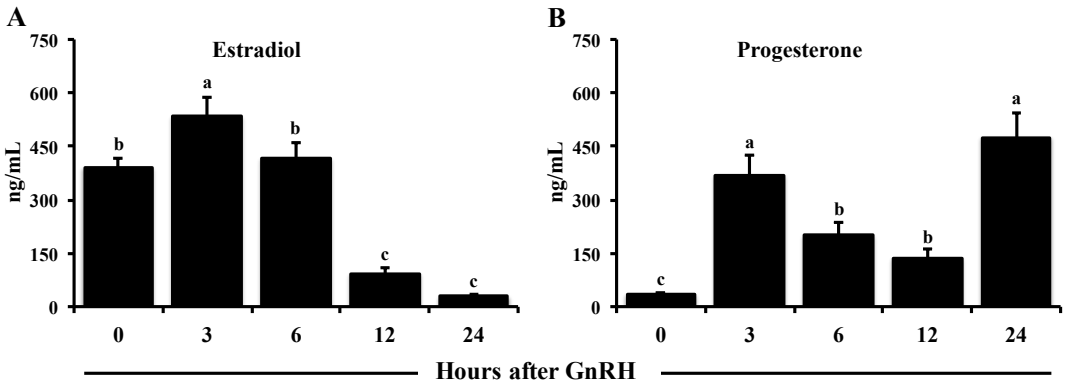

A
Estradiol
750
600
450
300
150
0
ng/mL
a
b
b
c
c
0
3
6
12
24
B
Progesterone
750
600
450
300
150
0
ng/mL
c
a
b
b
a
0
3
6
12
24
Hours after GnRH

Figure 5

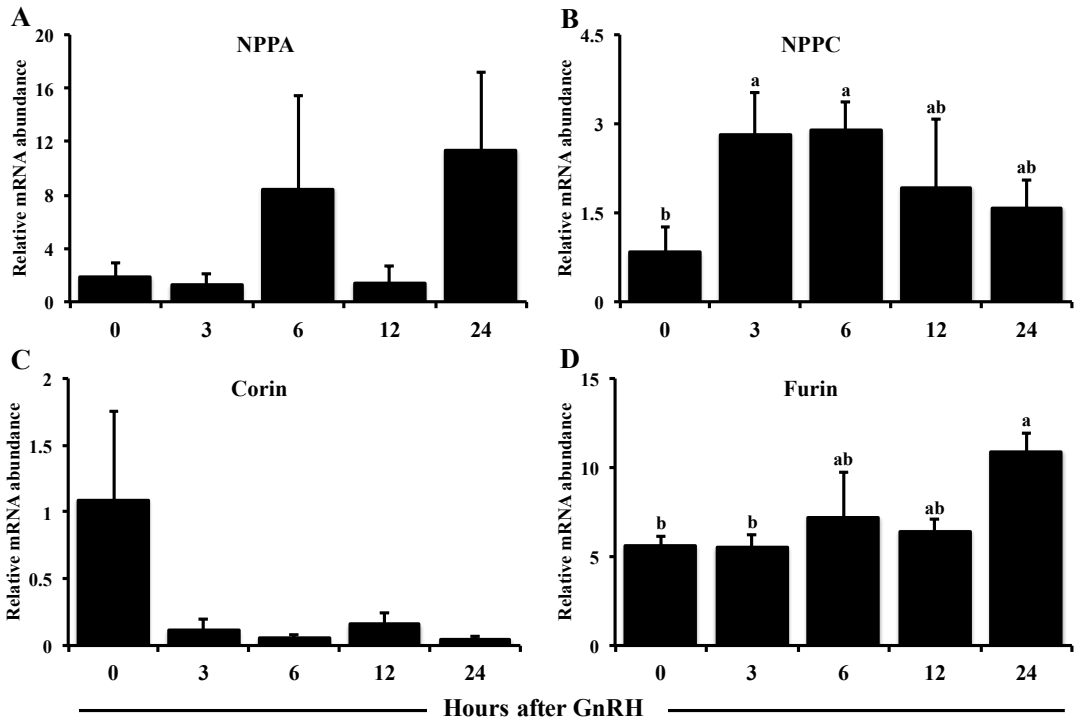
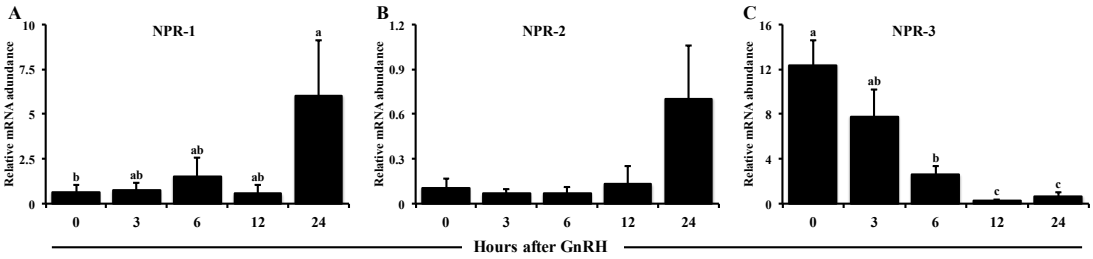

A
NPPA
Relative mRNA abundance
20
16
12
8
4
0
0
3
6
12
24
B
NPPC
Relative mRNA abundance
4.5
3
1.5
0
b
a
a
ab
ab
0
3
6
12
24
C
Corin
Relative mRNA abundance
2
1.5
1
0.5
0
0
3
6
12
24
D
Furin
Relative mRNA abundance
15
10
5
0
b
b
ab
ab
a
0
3
6
12
24
Hours after GnRH

Figure 6


A
NPR-1
Relative mRNA adundance
10
7.5
5
2.5
0
b
ab
ab
ab
a
0
3
6
12
24
B
NPR-2
Relative mRNA abundance
1.2
0.9
0.6
0.3
0
0
3
6
12
24
C
NPR-3
Relative mRNA abundance
16
12
8
4
0
a
ab
b
c
c
0
3
6
12
24
Hours after GnRH

5. ARTIGO 3

TRABALHO A SER SUBMETIDO PARA PUBLICAÇÃO:

NPPC AND NPR-3 mRNA IS REGULATED THROUGH EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) AND ANP IS A LH COFACTOR FOR STIMULATES PTGS2 mRNA IN BOVINE GRANULOSA CELLS

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THERIOGENOLOGY, 2017

NPPC and NPR-3 mRNA is regulated through epidermal growth factor receptor (EGFr) and ANP is a LH cofactor for stimulates Ptgs2 mRNA in bovine granulosa cells

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Abstract

The LH triggers a dynamic and complex event in granulosa cells that culminate with ovulation. The aim of this study was to evaluate whether the epidermal growth factor receptor (EGFr) regulates the expression of natriuretic peptides (NP) system after LH stimulation *in vitro* and *in vivo*, and whether EGFr is required for ovulation in cattle. In addition, we evaluated the association of NP type-A (ANP) and/or type-C (CNP) or with LH modify mRNA expression of genes present in granulosa cells involved in ovulation. Using well-established *in vivo* models and granulosa cell culture *in vitro*, we demonstrate that LH stimulate mRNA for AREG and EREG at 6 hours, and that ANP and CNP do not influence the expression of these genes *in vitro*. The abundance in granulosa cells for NP receptor 1 (NPR-1), receptor 2 (NPR-2) and receptor 3 (NPR-3) mRNA was not altered by LH and/or EGFr inhibition (AG1478) after 6 hours of *in vitro* culture. However, in the same conditions, the mRNA for natriuretic peptide precursor C (NPPC) was upregulated by LH, being that AG1478 (0.5 and 5μM) inhibited the LH stimulus. In order to confirm theses results, the preovulatory follicles were intrafollicularly injected with 5μM AG1478 or 0.9% NaCL and challenged with GnRH agonist *in vivo*. Cows were ovariectomized and granulosa cells harvested 6 hours after GnRH injection. The expression of NPPC and NPR-3 mRNA was up and downregulated by LH, respectively; however, AG1478 prevented the effect of LH. Evaluating whether ANP and/or CNP assisting LH to stimulate known genes involved in ovulation, we observed that, in granulosa cell culture for 6 hours, the ANP associated with LH increased prostaglandin-endoperoxide synthase 2 (Ptgs2) mRNA expression in ~5.5- and ~2.2-fold compared to the negative control group (without LH) and LH alone, respectively. Lastly, we showed that the intrafollicular injection of AG1478 was not able to block bovine ovulation although regulate NPPC and NPR-3 mRNA expression induced by LH *in vivo*. In conclusion, ovulation in cattle may occur independently of EGFr activation. However, we

observed that LH modulates NPPC and NPR-3 mRNA expression through EGFr during the preovulatory process. In addition, the ANP seems to be a LH cofactor to stimulate Ptgs2 mRNA in bovine granulosa cells.

Key words: Natriuretic peptides system, ovulation, monovular species, granulosa cells, Ptgs2, EGFr.

1. Introduction

Ovulation in cattle is a dynamic and complex event that resembles an inflammatory process and results in the rupture of ovulatory stigma with releasing of the oocyte able to be fertilized. This biological event is initiated at the time that follicle tissue is stimulated by the preovulatory peak of LH. Among the signaling pathways involved in the cascade of cellular events that occur after the peak of LH *in vivo*, the epidermal growth factor (EGF) system plays a central role that triggering ovulation and the resumption of meiosis [1-3]. In cattle granulosa cells cultured *in vitro*, the mRNA expression for EGF-*like growth factors* amphiregulin (AREG) and epiregulin (EREG) is increased after treatment with LH [4, 5]. Likewise, an increase of AREG and EREG in granulosa cells was demonstrated with 6 hours both after induction of ovulation with hCG *in vivo* and in granulosa cells cultured *in vitro* with forskolin [6]. Nonetheless, is necessary to evaluate these markers (AREG and EREG) in a well-established *in vivo* ovulation model for bovines [7, 8] and in the first hours after the LH peak. AREG and EREG bind to EGF receptor (EGFr) and the ERK1/2 (MAPK) is phosphorylated in granulosa and cumulus cells that are essential to mediate many of LH effects, including oocyte maturation, cumulus cell expansion and ovulation [2, 9, 10]. However, Duggavathi and Murphy [11] demonstrated, in mice, that there is an alternative pathway for ERK1/2 phosphorylation. In which, despite the use of a specific EGFr inhibitor

[3] or conditional knockout for this receptor [12], the LH partially phosphorylated ERK1/2. However, the effect of EGFr inhibition on ovulation is still unknown in cattle.

Besides AREG and EREG, other LH mediators are important during the cascade of events leading to ovulation in cattle. Angiotensin II (AngII) through AngII receptor subtype 2 (AT2) is indispensable in the early stage [13] and acts as cofactor of LH in the stimulation of prostaglandin-endoperoxide synthase 2 (Ptgs2) [4]. The Ptgs2, which is required for synthetizing prostaglandins, is regulated by progesterone (P4) [14] and plays a central role in suitable ovulation [15, 16]. After the LH peak, prostaglandin E2 (PGE2) and EGF-*like growth factors* participate in paracrine and autocrine signaling in granulosa and cumulus cells supporting the oocyte maturation, cumulus expansion and ovulation [10, 17]. With a microdialysis system, it was proposed that natriuretic peptide (NP) type-A (ANP) increases prostaglandins and AngII production in mature bovine follicles, and modulates steroidogenesis in follicular cells [18]. Furthermore, knockout for NP type-C (CNP) or NP receptor 2 (NPR-2) induces early resumption of oocyte meiosis and prevents ovulation in rodents [19-21].

The natriuretic peptides (NP) system comprise three peptides: ANP, NP type-B (BNP) and CNP, that may have the mRNA levels assessed by natriuretic peptide precursors A (NPPA), B (NPPB) and C (NPPC), respectively [22-24]; and three receptors: NP receptor 1 (NPR-1), NPR-2 and NP receptor 3 (NPR-3). In reproductive processes was demonstrated, in mice, that CNP is synthesized by granulosa cells and binds to NPR-2 from cumulus cells to block the meiosis resumption of oocyte [20, 25]. Similar function was observed in goat [26, 27], bovine [28] and cat [29] for CNP *in vitro*. In pigs, both CNP and BNP bind to NPR-2 and holds the oocyte meiosis resumption blocked [30-32]. During the folliculogenesis, the expression of NPPC in mouse granulosa cells reaches the highest levels, *in vivo*, concomitantly with the preovulatory LH surge [25, 33]. In pigs, the maximum abundance of

mRNA for NPPB, NPPC and NPR-2 in both granulosa and cumulus cells is also observed near the LH peak [32]. However, the expression of mRNA for NPPC in granulosa [21, 25] and NPR-2 in cumulus cells [34, 35] is reduced after the LH/hCG, through the stimulation of EGF-*like growth factors* and activation of EGFr. Similarly, NPPB, NPPC and NPR-2 mRNA expression is suppressed by EGFr-mediated signaling in preovulatory follicles in pigs [31]. Conversely, NPR-3 expression in mouse granulosa cells is basal until LH, when it increases the mRNA abundance in ~35- and ~500-fold at 1 and 3 hours after gonadotropin release, respectively [33]. In cattle, it was characterized the expression of three NP receptors and NPPA and NPPC in cumulus and granulosa cells [36, De Cesaro et al., submitted for publication]. We recently observed positive regulation for NPPC and negative for NPR-3 in granulosa cells after LH *in vivo* [De Cesaro et al., submitted for publication]. However, the effect of EGFr inhibition about the NP system in bovine granulosa cells, both *in vivo* and *in vitro*, is still unknown.

Given the importance of NP system and the difference observed between multiovular species and bovine, we hypothesize that LH act through EGFr, both *in vitro* as *in vivo*, to up and downregulate NPPC and NPR-3, respectively. Additionally, ANP and/or CNP in association or not with LH modify mRNA expression of genes present in granulosa cells involved in ovulation. In addition, we hypothesize that the activation of EGFr is dispensable for ovulation in cattle. Along with previous data, our results provide insights to better understand the complex mechanism of ovulation in monovular specie.

2. Material and methods

All experimental procedures were approved by the Federal University of Santa Maria Animal Care and Use Committee (23081.004717/2010-53 and 115/2014). All chemicals used

were purchased from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise indicated.

Ovariectomy and granulosa cells harvested

Cows were ovariectomized by colpotomy under caudal epidural anesthesia [37]. Granulosa cells were harvested from desired follicle by repeated flushing with PBS and stored appropriately for further analysis.

Animals and preovulatory follicles

All *in vivo* experiments were performed with adult beef cows (predominantly Hereford and Angus breeds), cycling, nonlactating and with a body condition score of 3 or 4 (scale from 1-thin to 5-obese). The cows were pre-synchronized to obtain a LH responsive follicle (≥12mm) [38] at the beginning of the experiment according to a previous study [7, 8]. Briefly, cows received two doses of a PGF2α analogue (Sincrocio, Ouro Fino, Ribeirão Preto, Brazil; 250μg i.m.) 12 hours apart, 2mg of estradiol benzoate (Genix, Anápolis, Brazil) and an intravaginal progesterone device on day 0 (progesterone, 1g; DIB®, Intervet/Schering-Plough, Brazil). After intravaginal device removal (day 9), ovaries were examined by transrectal ultrasonography, and cows that had gonadotropin-releasing hormone (GnRH)-responsive preovulatory follicles (≥12 mm) were challenged with 100μg of gonadorelin acetate (GnRH agonist; Profertil®, Tortuga, Brazil) i.m. 12 hours after removal of the intravaginal progesterone device. Depending of the experiment, the animals were submitted to intrafollicular procedure and/or ovariectomy, or also, the injected follicle was monitored by ultrasound examination until ovulation or atresia.

Ultrasound-guided intrafollicular injection procedures

Epidural anesthesia was administered and perineal cleaning was performed before injections. The intrafollicular injections were guided by ultrasound equipped with a 7.5MHz vaginal probe (Aquila Vet, Pie Medical Equipment BV, Netherlands) coupled to a biopsy guide. A system with two sterile needles and the dose of each treatment was estimated by the linear regression equation as previously described by Ferreira et al. [13].

Granulosa cell culture

To obtain granulosa cells *in vitro*, pairs of bovine ovaries without corpus luteum and contained a single large follicle (≥12mm) were collected from abattoir and transported to the laboratory in 30°C saline solution (0.9% NaCl) containing 100IU/ml penicillin and 50µg/ml streptomycin sulfate. At the laboratory the ovaries were rinsed three times with same solution used in the transport. Granulosa cells from transparent follicle (≥12mm) and with yellow follicular fluid were harvested through repeated flushing with PBS, pooled and washed three times by centrifugation at 200xg for 10min in PBS. After the end of last centrifugation the granulosa cells were filtrated and diluted in Dulbecco modified Eagle medium-Ham F12 (DMEM-F12; Invitrogen), supplemented with 10nM sodium bicarbonate, 0.1% bovine serum albumin (BSA), 10ng/mL insulin, 100UI/mL penicillin (Invitrogen), 100µg/mL de streptomycin (Invitrogen) and 2.5µg/mL amphotericin (basic culture medium). Cell viability was estimated with 0.4% trypan blue stain. The granulosa cells density of $1x10^6$ viable cells/mL of DMEM-F12 was seeded into four-well culture dishes (Nunc®, Roskilde, Denmark) and cultured at 37°C in an atmosphere containing 5% CO_2 in air, at 95% relative humidity, for 6 hours. At the end of the *in vitro* culture, cell samples were immediately stored for further analyses. Experiments using granulosa cells *in vitro* cultures were performed in four replicates.

RNA extraction, reverse transcription and real-time PCR

Total RNA was extracted from granulosa cells using Trizol (*in vitro* experiments; Invitrogen, São Paulo, SP, Brazil) or silica-based protocol (*in vivo* experiments; Qiagen, Mississauga, ON, Canada) according to the manufacturer's instructions. Quantity and RNA purity were measured using the NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA; absorbance ratio at 260/280 nm). Ratios above 1.8 were considered pure, and all samples used in the present study were above this threshold. To generate the complementary DNA (cDNA), 500ng RNA was first treated with 0.1U DNAse (Invitrogen; 37°C for 5min). After DNAse inactivation at 65°C for 10 min, samples were incubated in a final volume of 20µL with iScript cDNA Synthesis Kit (Bio-Rad) following the manufacturer's protocols.

Real time quantitative PCR (qPCR) reactions were run in the CFX384 real-time PCR detection system (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad), and bovine-specific primers (Table 1) taken from literature and specificity was confirmed using BLAST (NCBI). Standard two-step qPCR was performed to amplify each transcript with an initial denaturation at 95°C for 5 minutes followed by 40 cycles of denaturation at 95°C for 15sec and annealing/extension at 58°C for 30sec and melting-curve analysis was used to verify the specificity of reaction products. Samples were run in duplicates, standard curve method was used to determine the abundance of mRNA for each gene, and expression was normalized to the abundance of the housekeeping gene *cyclophilin*. To test cross contamination with theca cells, PCR for detection of the mRNA that encode CYP17A1 in granulosa cells was performed in each sample and all samples used were free from contamination by theca cells (after 30 PCR cycles).

Experimental design

Experiment 1- EGF-Like growth factor as early marker of ovulation process in bovine granulosa cells

To evaluate whether amphiregulin (AREG) and epiregulin (EREG) could be used as markers of the bovine ovulatory process *in vivo*, thirty cyclic beef cows were synchronized as detailed above. The cows that had preovulatory follicles (≥12 mm), by ultrasound visualization, were challenged with 100μg of gonadorelin acetate and ovariectomized at 0, 3, 6, 12 and 24 h after the treatment (n= 5/6 animals per time). The granulosa cells from desired follicle were adequately harvested and stored for further analyses.

Experiment 2 – Effect of ANP and CNP on mRNA expression of AREG and EREG in bovine granulosa cell culture

Based on the previous *in vivo* experiment and that LH upregulates NPPC mRNA expression [De Cesaro et al., submitted for publication] and that ANP modulates steroidogenesis in bovine follicular cells [18], culture of granulosa cells during 6 hours was performed to evaluate whether ANP (1μM) and CNP (1μM) affect the mRNA expression of AREG and EREG. The effect of each NP separately and your association (ANP plus CNP) with or without LH (400ng/mL) was tested. Granulosa cells were cultured as described above on following groups: CNP (C); LH+C; ANP (A); LH+A; A+C; LH+A+C. For every part of this experiment had a negative control (C-; only granulosa cells) and a positive control (granulosa cells with LH).

Experiment 3 – Activation of EGF receptor (EGFr), after LH, is required to regulate the mRNA expression of NP member in granulosa cell culture

Results submitted for publications of our laboratory demonstrate that NPPC and NPR-3 were up and downregulated by LH *in vivo*, respectively. With the aim of assess whether LH *in vitro* have de same effect and this regulation occurs through EGFr we evaluated the mRNA expression of NPPC, NPR-1, NPR-2 and NPR-3 in granulosa cell culture with or without LH (400ng/mL) in association or not of selective tyrosine kinase inhibitor of the EGFr (AG1478). For this, granulosa cells were cultured for 6 hours as described above on four groups: negative control (C-; only granulosa cells); positive control (LH); LH + 0.5μM AG1478 (AG0.5); LH + 5μM AG1478 (AG5). Also, to validate the granulosa cell culture we evaluated the mRNA expression of AREG and EREG in C- and LH groups.

Experiment 4 – Expression of members of NP system after intrafollicular blocking of the EGFr signaling pathway in preovulatory follicle in vivo

Based on the *in vitro* experiment (experiment 3) that showed regulation of mRNA expression of NP member by LH through EGFr, we aim confirm this effect in granulosa cells from preovulatory follicle *in vivo*. Thus, twenty cyclic cows were synchronized as described above, and when follicle reached 12mm in diameter, or more, in the ovary the animals were randomly separated and received an intrafollicular injection of 0.9% NaCl solution (Cont.) or 5μM AG1478 (AG). Immediately after the intrafollicular injections, the cows received 100μg gonadorelin acetate (GnRH agonist). Six hours after challenging with the GnRH agonist, the animals were ovariectomized for recovery granulosa cells from intrafollicular injected follicle.

Experiment 5 – Effect of ANP and CNP on mRNA expression of genes present in granulosa cells involved in ovulation

Although ANP and CNP have not regulated AREG and EREG in granulosa cell culture (experiment 2), it is known that LH upregulates NPPC mRNA expression [De Cesaro et al., submitted for publication] and that ANP modulates steroidogenesis in follicular cells

[18]. Therefore, we evaluated whether ANP (1µg/ml) and/or CNP (1µg/ml) affect the mRNA expression of Angiotensin II receptor subtype 2 (AT2), progesterone nuclear receptor (PGr) and prostaglandin-endoperoxide synthase 2 (Ptgs2) in granulosa cells after 6 hours of *in vitro* culture with the same experimental design used in experiment 2 (C-; LH; C; LH+C; A; LH+A; A+C; LH+A+C).

Experiment 6 – Effect of intrafollicular blocking of the EGFr signaling pathway on bovine ovulation

To evaluate the effect of intrafollicular blocking of the EGFr in preventing bovine ovulation, thirty adult cyclic cows were synchronized as described above, and when follicle reached 12mm in diameter, or more, in the ovary the animals were randomly separated and received an intrafollicular injection of 0.9% NaCl solution (Control) or 0.5µM AG1478 or 5µM AG1478. Immediately after the intrafollicular injections, the cows received 100µg gonadorelin acetate (GnRH agonist) and the injected follicle was monitored by ultrasound examination daily until ovulation or atresia.

Statistical analysis

All continuous data were tested for normal distribution using Shapiro–Wilk test, normalized when necessary and submitted to ANOVA using JMP software (SAS Institute Inc., Cary, NC, USA). When a treatment effect occurred, the average among groups was compared using the LSM (*least squares means*). Results are presented as means ± S.E.M. and $P \leq 0.05$ was considered statistically significant.

3. Results

3.1 AREG and EREG are early marker of ovulation process in bovine granulosa cells

Initially, with a strong and well-established experimental *in vivo* model [7, 8] was evaluated AREG and EREG gene expression profile at different time point (0, 3, 6, 12 and 24 hours) after GnRH agonist. Analysis of abundance for AREG (Fig. 1A) and EREG (Fig. 1B) mRNA in granulosa cells revealed a similar regulation for these two genes by GnRH. Significant increase already at 3 hours after GnRH, with a expression peak at 6 hours and a marked decrease at 12 and 24 hours after GnRH (Fig. 1A and 1B).

3.2 Effect of ANP and CNP on mRNA expression of AREG and EREG in bovine granulosa cell culture

Similar to what was observed *in vivo* and validating the granulosa cell culture *in vitro,* we noted that LH stimulated the expression of AREG and EREG mRNA after 6 hours (Fig. 2). Additionally, the three NPs receptors are present in granulosa cells from preovulatory follicle and NPPC mRNA expression is upregulate *in vivo* 3-6 hours after LH (De Cesaro et al., submitted for publication). Then, we hypothesized that ANP and/or CNP with or without LH affect the mRNA expression of AREG and EREG in granulosa cell culture for 6 hours. However, ANP or CNP or ANP+CNP with or without LH did not affect the mRNA expression of AREG and EREG in granulosa cells (Fig. 2A-F).

3.3 LH regulates mRNA expression of NPPC in granulosa cell culture in vitro through EGFr

Based in results of our laboratory which NPPC and NPR-3 were up and downregulated by LH *in vivo*, respectively [De Cesaro et al., submitted for publication], we performed a granulosa cell culture using two doses of AG1478 (selective tyrosine kinase inhibitor of the EGFr) to understand whether LH regulation in member of NP system occurs through EGFr. Initially, we demonstrated the increase in mRNA abundance for both AREG

and EREG after 6 hours of culture by LH (Fig. 3A and B) compared with C-, similar *in vivo* (Fig. 1) and *in vitro* (Fig. 2) experiment, validate the granulosa cell culture. Among the NP system member evaluated (NPPC, NPR-1, -2 and -3) was observed that EGF modulates only NPPC mRNA expression (Fig. 3F). The granulosa cells cultured in the presence of AG1478 did not respond to the LH action as in the positive control group. In the other hand, none of NPs receptors (NPR-1, -2 and -3) evaluated were regulated by LH in association or not with AG1478 (Fig. 3C, D and E).

3.4 Intrafollicular blocking of the EGFr signaling pathway alter mRNA expression of NPPC and NPR-3 in preovulatory follicle in vivo

To confirm the *in vitro* results (previous result - Fig. 3), twenty cyclic cows were synchronized to receive intrafollicularly 5μM AG1478 (AG) or 0.9% NaCl solution (Cont.) plus GnRH agonist i.m., and 6 hours later the animals were ovariectomized to recovery granulosa cells as described above. Nine animals were discarded of this study because the follicular diameter was <12mm in diameter at the end of the synchronization period or by decreased follicular diameter after the intrafollicular injection procedure. Among the eleven remaining animals in this study, more three were discarded because follicle leakage during ovariectomy or by detection of cross contamination between granulosa and theca cells. Therefore, four animals were randomized allocated each group (Cont. or AG) and was observed that NPR-1 and NPR-2 were not regulated (Fig. 4A and B). However, LH up and downregulated mRNA expression of NPR-3 and NPPC, respectively, and the intrafollicular injection of AG1478 prevented the LH effect (Fig. 4C and D).

3.5 *Effect of ANP and CNP on mRNA expression of genes present in granulosa cells involved in ovulation*

Using a granulosa cell culture for 6 hours, the association of LH+ANP increased the mRNA expression for AT2 compared with negative control (C-), but it was not different for LH group (Fig. 5B). However, it was not observe mRNA regulation in PGr (Fig. 5D-F) by ANP and CNP or your association with or without LH. The same result were observed for Ptgs2 mRNA expression in granulosa cells cultured with CNP (Fig. 5G). Nonetheless, Ptgs2 mRNA had the greatest expression in the group with LH+ANP compared with C-, LH or ANP alone (Fig. 5H). Similarly, the association of LH+ANP+CNP also upregulate mRNA expression for Ptgs2, but it was not different of LH (Fig. 5I).

3.*6 Intrafollicular blocking of the EGFr signaling pathway did not inhibits bovine ovulation*

Thirty cyclic cows were synchronized to receive intrafollicularly 0.9% saline solution (Control), 0.5µM or 5µM AG1478 and immediately after the cows received 100µg GnRH agonist i.m. Six animals were discarded of this study because the diameter follicular was <12mm in diameter at the end of the synchronization period or by decreased follicular diameter after the intrafollicular injection procedure. The twenty-four remaining animals in this study were randomized distributed in the 3 experimental groups: Control (n=7); 0.5µM AG1478 (n=7); 5µM AG1478 (n=8). All animals of the three groups ovulated between 24 and 48 hours after intrafollicular injection procedure.

4. Discussion

The significant findings of this study are: 1) LH increased the expression of mRNA for AREG and EREG in bovine granulosa cells at 6 hours *in vitro* and *in vivo*; 2) ANP and CNP did not regulate mRNA expression for AREG and EREG in granulosa cell culture *in vitro*; 3) LH upregulated mRNA expression of NPPC in granulosa cells *in vitro* and *in vivo* through the

EGFr; 4) LH downregulated NPR-3 mRNA expression through the EGFr *in vivo*; 5) ANP potentialized LH to stimulate Ptgs2 mRNA during the preovulatory cascade; 5) The EGFr inhibitor was not able to prevent ovulation in bovines. These results suggest that the NP system in cattle are related, at least in part, to pro-ovulation events, with regulation of mRNA abundance, which differ those observed in multiovular species. Moreover, this is the first study showing regulation of NPR-3 by LH through EGFr *in vivo*.

Despite the increase in the expression of AREG and EREG by the action of LH verified in bovine granulosa cells *in vitro* [4, 5] and *in vivo* [6], the expression of these genes prior to 6 hours after GnRH was not known. Initially, we observed that in the few hours (3 hours) after treatment with GnRH, there was an increase in expression of AREG and EREG, with the expression peak after 6 hours (Fig. 1A and B). This result allied to the studies cited above using similar models, gave us the basis for choosing the best time point for analyzing the expression of different genes evaluated in the next *in vitro* and *in vivo* experiments.

We demonstrated that NPPC is upregulated by LH *in vivo* after 3 and 6 hours in bovine granulosa cells [De Cesaro et al., submitted for publication]. In addition, ANP modulates steroidogenesis in bovine follicular cells [18] but it was not regulated by LH [De Cesaro et al., submitted for publication]. Thereby, we hypothesized that ANP and CNP could act as a LH cofactor of EGF-*like growth factors* regulation, similar to AngII [4]. However, no effect was observed on the mRNA expression for AREG and EREG (Figure 2) in the groups treated with ANP and/or CNP. Perhaps, a lower dose of LH might be able to detect a possible cofactor effect of these NPs for LH, similar to the design used by Portela et al. [4]. Another possibility is suggesting that the ANP and CNP are downstream the signaling cascade of the EGFr during ovulation or do not interfere with the regulation of mRNA for AREG and AREG in cattle.

We aim to understand the regulation of the NP system in the granulosa cells derived from preovulatory follicles (diameter ≥12 mm) in cattle. With this objective, primers (NPPA, NPPC, NPR-1, NPR-2 and NPR-3) with high homology to the gene of interest [36, De Cesaro et al., submitted for publication] were used. *In vitro*, the stimulation of AREG and EREG mRNA expression by LH action on granulosa cells validated the culture (Fig. 3A and B). Then, we observed that LH stimulated NPPC mRNA expression, however, the presence of AG1478 prevented this action (a selective tyrosine kinase inhibitor of the EGFr - 0.5 or 5μM) *in vitro* (Fig. 3F) and *in vivo* (Fig. 4D). These results were obtained in different experiments in our laboratory (De Cesaro et al., submitted for publication). However, they are contrary to the downregulation induced by LH on NPPC mRNA expression in granulosa cells from mouse [21, 25, 33], goat [26, 27] and pig [30-32]. In the bovine, it was proposed that LH induce a decrease in NPPC mRNA expression using granulosa cells *in vitro* [39]. However, these authors used granulosa cells derived from follicles with 2-6mm in diameter and did not perform studies *in vivo*. It is important to emphasize that ovarian follicles from bovine with diameter ≤8.5mm did not ovulate even in response to 10-fold greater dose required to ovulate all large follicles (≥12 mm) [38].

Although BNP has similar effect to CNP in inhibiting the oocyte meiosis resumption in pigs [32], the NPPB is not expressed in bovine follicular cells [36, [De Cesaro et al., submitted for publication]. Furthermore, the NPPA mRNA is detected in granulosa cells, but its expression has been very low both *in vitro* and *in vivo* (data not shown), and is not regulated by gonadotropins in mice [25] nor in cattle [De Cesaro et al., submitted for publication]. Among the NPs receptors, we demonstrate that NPR-1 and NPR-2 are not regulated by LH or AG-1478 in bovine granulosa cells *in vitro* or *in vivo* after 6 hours. NPR-2 is expressed in granulosa cells and it is donwregulated by LH in mice [21] and pigs [31, 32]. NPR-2 seems to be extremely important for fertility in multiovular species and its expression

is greater in cumulus cells than in granulosa cells [20, 40]. In addition, it is thought that, at least in mice, LH rapidly (20 min) induces modification on NPR-2 protein and decreases your activity [34], decreasing the mRNA expression in 2-4 hours after LH surge [21, 35]. However, no change in mRNA expression for NPR-2 was observed in bovine granulosa cells *in vivo* during 24 hours after LH [De Cesaro et al., submitted for publication].

In a recent study using an NPR-3-specific agonist ($cANP^{[4-23]}$), it was observed that NPR-3 participates in the inhibition of porcine oocyte meiotic resumption [41]. Thereby, suggesting some other function besides clearance of NPs for this receptor. Our results demonstrated that mRNA expression for NPR-3 was not altered by LH or AG1478 in granulosa cells *in vitro* (Fig. 3E) but the LH donwregulated NPR-3 mRNA expression and AG1478 prevented this effect *in vivo* (Fig. 4C). In the contrary, LH stimulated the increase of NPR-3 mRNA expression in murine granulosa and cumulus cells *in vivo* [33]. In light of these results, it is possible to direct future studies to understand the function of NPR-3. In cattle, the action of NPR-3 seems to be before the LH surge.

Our findings conflict with other studies, which challenged us to investigate the possibility of ANP and CNP to be a cofactor for LH to stimulate known genes involved in ovulation. The ovulatory cascade triggered by LH is complex and involves several genes. It is well established that AngII is required for ovulation, acting throughout its AT2 receptor at least in the first 6 hours after LH surge in cattle [13]. Previously, we have demonstrated that LH upregulates AT2 in granulosa cell culture *in vitro* at 6 hours of culture [4]. The dose of LH and time of cell culture was the same used in the present experiment. However, the ANP alone or associated with CNP plus LH did not influence the expression of AT2 mRNA, compared to the LH group. Similarly, PGr mRNA expression did not change significantly in relation to the control cells although this receptor is required for the ovulatory cascade [14-16].

Interestingly, in the LH+ANP group there was an increase in Ptgs2 mRNA expression in ~5.5- and ~2.2-fold compared to the negative control group (C-) and LH alone, respectively (Fig. 5H). It is important to highlight that the increase of Ptgs2 mRNA is directly related to the increase of the protein of this enzyme [4]. The production of prostanoids after the expression of Ptgs2 is a crucial step in the ovulatory process. After preovulatory LH surge, the main functions of PGE2 and PGF2α are related to increase vasodilation and follicular wall degradation by the activation of proteinases [42]. Moreover, the prostaglandins have important role in the oocyte maturation process [43-45]. The inhibition of cycloxigenases or knockout for Ptgs2 determines failures in the occurrence of the ovulatory process [46-48]. According to Shimada et al. [17] and Yamashita and Shimada [10], PGE2 and EGF-*like growth factors* participate in a paracrine/autocrine signaling with granulosa and cumulus cells, being involved in oocyte maturation, cumulus expansion and ovulation. In another study with human granulosa cells, it has been showed that LH stimulates EGFr activation (AREG, EREG and BTC) and, consequently, an increase in Ptgs2 and PGE2 levels, which are dependent of ERK1/2 activation [49]. Our results demonstrated that ANP alone did not influence Ptgs2 mRNA expression; however, this NP seems to modulate the LH effect to induce Ptgs2, acting as a cofactor.

The EGFr plays a central role in the signaling pathways that triggering ovulation [1-3]. Here, we showed that the intrafollicular injection of AG1478 did not prevent ovulation. On the other hand, the NPR-3 and NPPC mRNA expression induced by LH was regulated by the EGFr inhibitor *in vivo* (Fig. 4C and D). With the same *in vivo* model but using saralasin (AngII receptor antagonist) or PD123 319 (AT2 receptor antagonist), the ovulation was inhibited [13]. It is well known that EGF-*like growth factors* phosphorylates ERK1/2 to mediate the effects of LH on the ovarian follicle [2, 9, 10]. However, ERK1/2 appears to be also phosphorylated by an EGFr-independent pathway in mice [3, 11, 12]. The results

demonstrated here indicate that there is an EGFr-independent ERK1/2 phosphorylation in bovine ovulation. These finds are important for future understanding of the ovulation process, to serve as a tool base to better explore the reproductive potential of females and/or to develop contraceptives with fewer side effects.

In conclusion, the LH surge induces AREG and EREG mRNA expression in three hours, heaving a peak at 6 hours, which modulates NPPC and NPR-3 mRNA expression through EGFr during the preovulatory process. However, the ovulation in cattle may occur independent of activation of the EGFr. In addition, the ANP seems to be a LH cofactor to stimulate Ptgs2 mRNA in bovine granulosa cells. These findings provide new insight into the mechanisms regulating the ovulation process in monovular species.

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Competing interests

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

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Tabela 1 - Primers used in the expression analysis of candidate genes.

Gene		Sequence	Reference or accession nº
Cyclophilin	F	GGTCATCGGTCTCTTTGGAA	[50]
	R	TCCTTGATCACACGATGGAA	
AREG	F	CCATTTTCTTGTCGAAGTTTCTTTC	[51]
	R	TGTTTTTATTACAATCCTGCTTCGAA	
EREG	F	ACTGCACAGCATTAGTTCAAACTGA	[5]
	R	TGTCCATGCAAACAGTAGCCATT	
NPPC	F	CAACGCGCGCAAATACAA	[36]
	R	TCAGCAAAACGCAGCAAGTC	
NPR-1	F	AATTATGGCTCCCTACTAACCACAGA	[36]
	R	TCCGGTTCACACGTTTCACA	
NPR-2	F	TCTGCTCCTAAGCTGGGTGAGT	[36]
	R	CGGTCATCTGTGCGAGCAT	
NPR-3	F	TTTGAAGCTAAGCAAGCGTACTCA	[36]
	R	CAGAACTTTTCACCTCCATGGAA	
AT2	F	GACCTGGCACTTCCTTTTGC	[4]
	R	GGAGCTTCTGCTGGAACCTATTC	
PGr	F	CAGAGCCCACAGTACAGCTT	[5]
	R	ACTTTCGGCCTCCAAGAACC	
Ptgs2	F	CCCTTCTGCCTGACGTCTTT	[5]
	R	GGAAGATTCCTACCGCCAGC	

F: Forward primer; R: Reverse primer.

Figure 1 – Relative mRNA abundance (mean ± standard error of the mean) of AREG (A) and AREG (B) in granulosa cells obtained at 0, 3, 6, 12 and 24 hours (n= 5/6 animals per time) after treatment with GnRH. Different letters represent statistical difference (P≤0.05).

Figure 2 – Relative mRNA abundance (mean ± standard error of the mean) of AREG (A, B and C) and AREG (D, E and F) in granulosa cells cultured *in vitro* for 6 hours with ANP (A; 1μM) and/or CNP (C; 1μM) with or without LH (400ng/mL). The negative control (C-) had no treatment. Different letters represent statistical difference (P≤0.05).

Figure 3 – Effect of LH (400ng/mL) and 0.5μM (AG0.5) or 5μM (AG5) EGFr inhibitor (AG1478) on natriuretic peptide system after 6 hours of granulosa cell culture *in vitro*. After validation of the granulosa cell culture by AREG (A) and EREG (B) mRNA expression stimulated by LH, the relative mRNA abundance (mean ± standard error of the mean) of (C) NPR-1, (D) NPR-2, (E) NPR-3 and (F) NPPC were evaluated. Different letters represent statistical difference (P≤0.05).

Figure 4 – Relative mRNA abundance (mean ± standard error of the mean) of NPR-1 (A), NPR-2 (B), NPR-3 (C) and NPPC (D) in granulosa cells 6 hours after intrafolliclar infection of 0.9% NaCl (Cont.) or 5μM AG1478 (AG). Different letters represent statistical difference (P≤0.05).

Figure 5 – Relative mRNA abundance (mean ± standard error of the mean) of Angiotensin II receptor subtype 2 (AT2; A, B and C), progesterone nuclear receptor (PGr; D, E and F) and prostaglandin-endoperoxide synthase 2 (Ptgs2; G, H and I) in granulosa cells cultured *in vitro* for 6 hours with ANP (A; 1μM) and/or CNP (C; 1μM) with or without LH

(400ng/mL). The negative control (C-) had no treatment. Different letters represent statistical difference ($P \leq 0.05$).

Figure 1

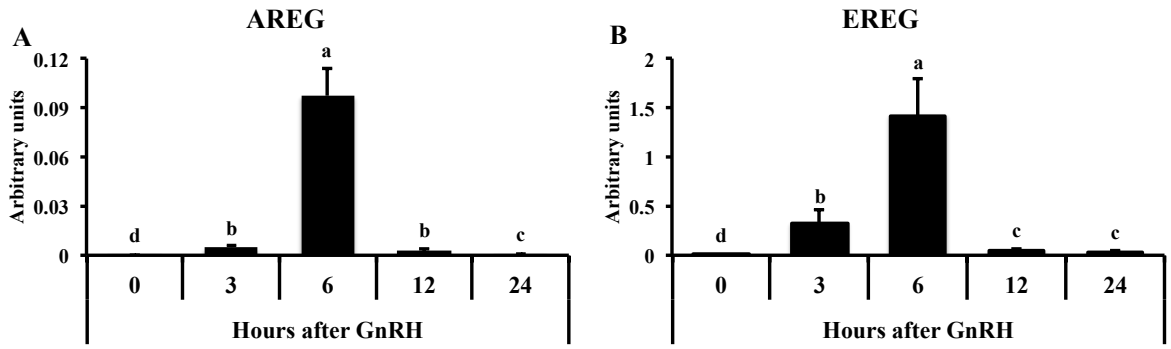

A
AREG
Arbitrary units
0.12
0.09
0.06
0.03
0
a
b
d
b
c
0
3
6
12
24
Hours after GnRH
B
EREG
Arbitrary units
2
1.5
1
0.5
0
a
b
d
c
c
0
3
6
12
24
Hours after GnRH

Figure 2

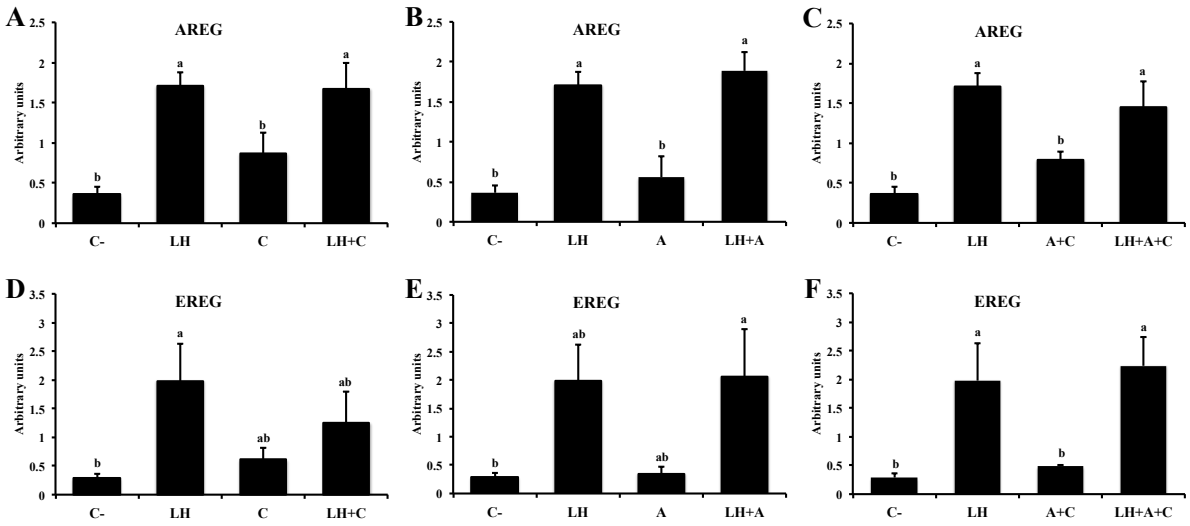

A
AREG
Arbitrary units
2.5
2
1.5
1
0.5
0
b
a
b
a
C-
LH
C
LH+C
B
AREG
Arbitrary units
b
a
b
a
C-
LH
A
LH+A
C
AREG
Arbitrary units
b
a
b
a
C-
LH
A+C
LH+A+C
D
EREG
Arbitrary units
3.5
3
2.5
2
1.5
1
0.5
0
b
a
ab
ab
C-
LH
C
LH+C
E
EREG
Arbitrary units
b
ab
ab
a
C-
LH
A
LH+A
F
EREG
Arbitrary units
b
a
b
a
C-
LH
A+C
LH+A+C

Figure 3

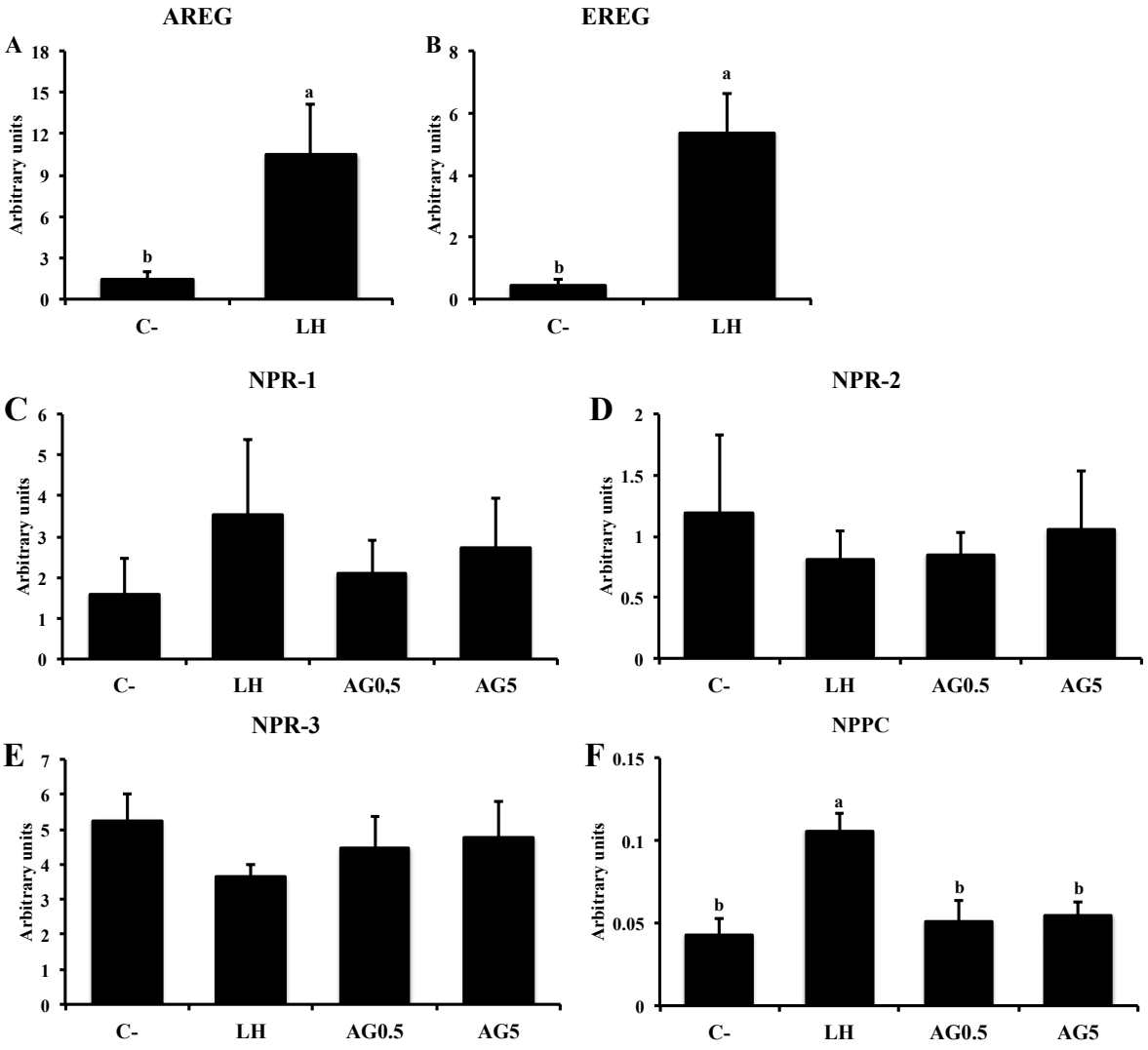

AREG
A
Arbitrary units
18
15
12
9
6
3
0
a
b
C-
LH
EREG
B
Arbitrary units
8
6
4
2
0
a
b
C-
LH
NPR-1
C
Arbitrary units
6
5
4
3
2
1
0
C-
LH
AG0,5
AG5
NPR-2
D
Arbitrary units
2
1.5
1
0.5
0
C-
LH
AG0.5
AG5
NPR-3
E
Arbitrary units
7
6
5
4
3
2
1
0
C-
LH
AG0.5
AG5
NPPC
F
Arbitrary units
0.15
0.1
0.05
0
a
b
b
b
C-
LH
AG0.5
AG5

Figure 4

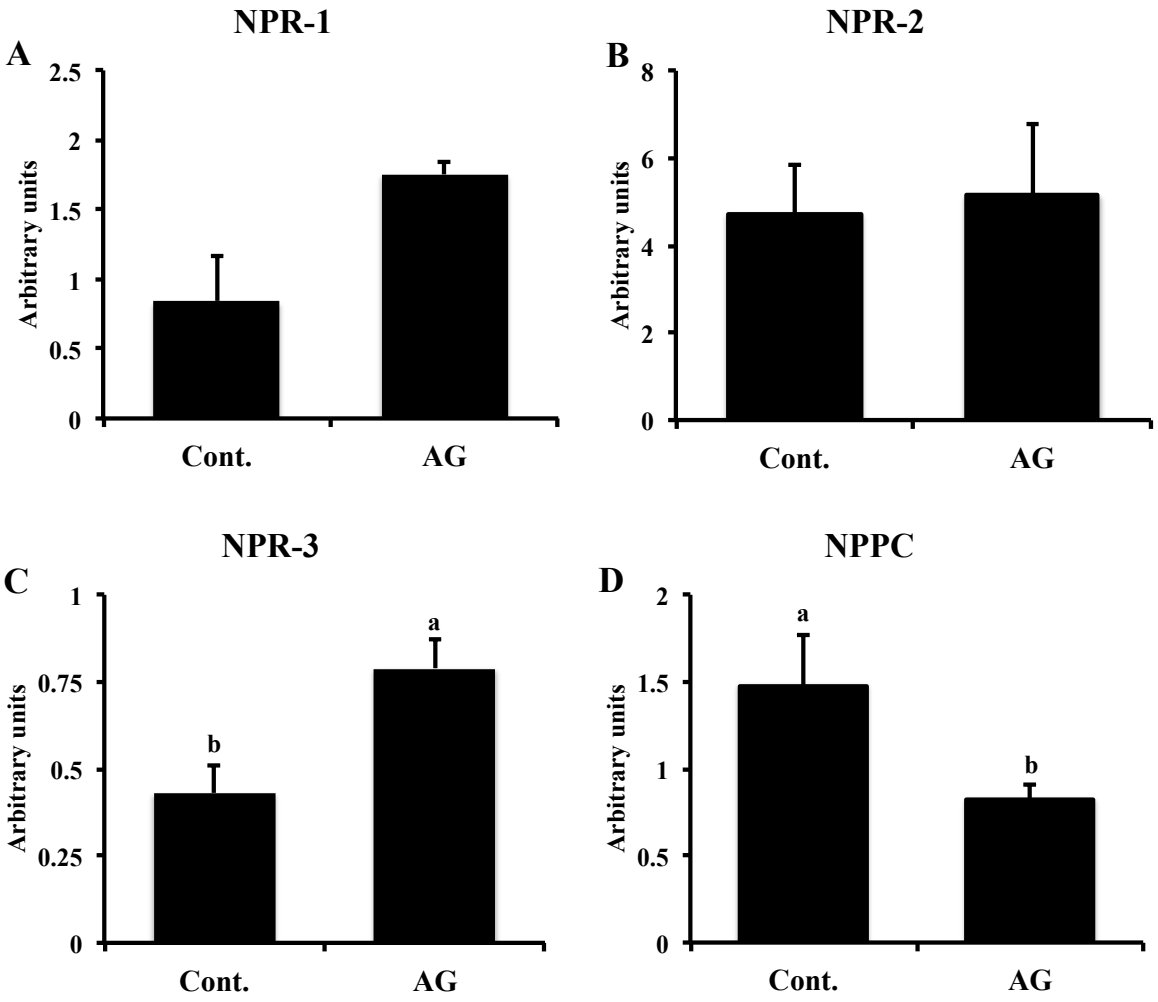
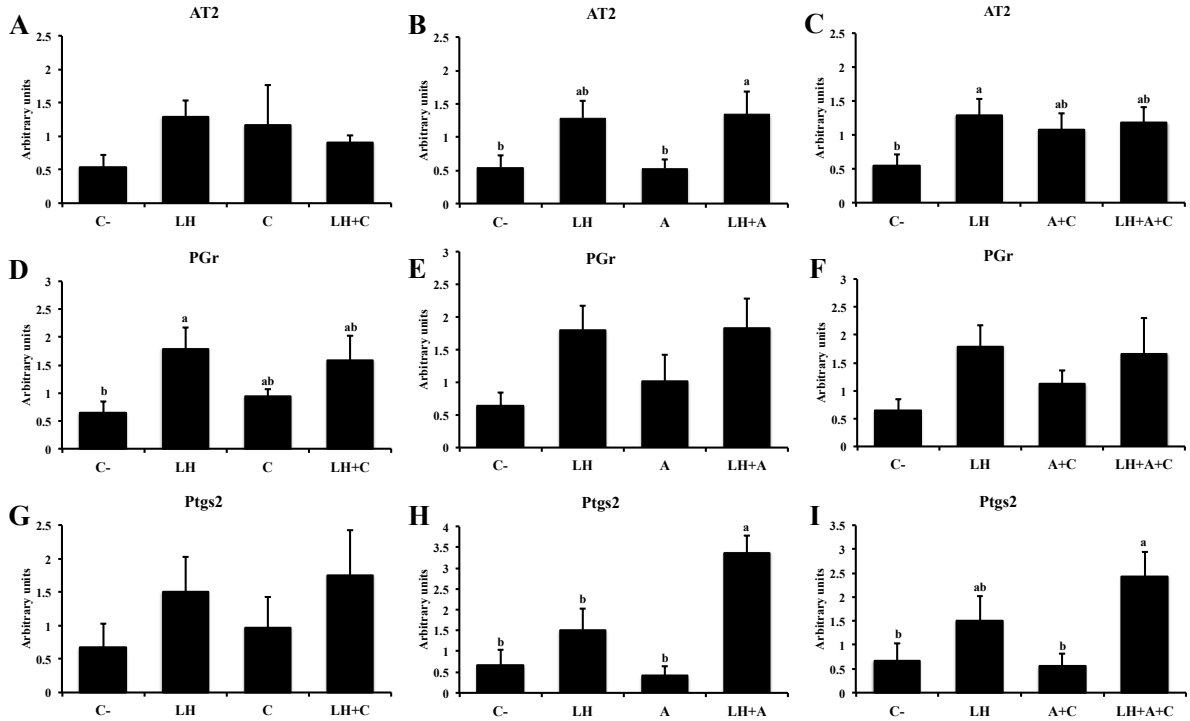

NPR-1
A
Arbitrary units
2.5
2
1.5
1
0.5
0
Cont.
AG
NPR-2
B
Arbitrary units
8
6
4
2
0
Cont.
AG
NPR-3
C
Arbitrary units
1
0.75
0.5
0.25
0
b
a
Cont.
AG
NPPC
D
Arbitrary units
2
1.5
1
0.5
0
a
b
Cont.
AG

Figure 5


A
AT2
Arbitrary units
C-
LH
C
LH+C
B
AT2
Arbitrary units
C-
LH
A
LH+A
C
AT2
Arbitrary units
C-
LH
A+C
LH+A+C
D
PGr
Arbitrary units
C-
LH
C
LH+C
E
PGr
Arbitrary units
C-
LH
A
LH+A
F
PGr
Arbitrary units
C-
LH
A+C
LH+A+C
G
Ptgs2
Arbitrary units
C-
LH
C
LH+C
H
Ptgs2
Arbitrary units
C-
LH
A
LH+A
I
Ptgs2
Arbitrary units
C-
LH
A+C
LH+A+C

6. ARTIGO 4

TRABALHO A SER SUBMETIDO PARA PUBLICAÇÃO:

REGULATION AND EFFECT OF NATRIURETIC PEPTIDE RECEPTOR-3 ON CUMULUS-OOCYTE COMPLEX IN CATTLE

Matheus Pedrotti De Cesaro[1,2], Mariana Priotto de Macedo[2], Paulo Roberto Antunes da Rosa[4]; Joabel Tonellotto dos Santos[3], Ricardo Della Mea[1], Janduí Escarião da Nóbrega Jr[1], Rajesha Duggavathi[2], Paulo Bayard Dias Gonçalves[1], Vilceu Bordignon[2*]

REPRODUCTION, 2017

REGULATION AND EFFECT OF NATRIURETIC PEPTIDE RECEPTOR-3 ON CUMULUS-OOCYTE COMPLEX IN CATTLE

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Short title: **NPR3 activation affect cumulus cells expansion**

Abstract

The natriuretic peptide (NP) receptors mRNA regulation and function of NP receptor 3 (NPR3) in cumulus cells during the oocyte maturation and cumulus cells expansion is unknowing in monovular species. We conducted experiments using *in vitro* models of well-characterized cumulus-oocyte complex (COC) culture to better understand these questions in cattle. The results demonstrated that in bovine cumulus cells the *NPR1* mRNA has low expression. Moreover, the *NPR2* mRNA was not up or downregulated by gonadotrophins, whereas the *NPR3* mRNA was downregulated by FSH and FSH+LH through epidermal growth factor receptor (EGFr). Similarly, to mimic post-gonadotropins signals the COCs were treated with forskolin and the *NPR3* mRNA was downregulated, but did not influence *NPR2*. Therefore, the regulation for *NPR2* and *NPR3* mRNA in bovine cumulus cells was different and opposite, respectively, compared to the multiovular species. Our functional results for NPR3 demonstrated that its activation, by a specific agonist ($cANP^{4-23}$), does not interfere in the nuclear maturation of oocytes, but, it inhibits the complete expansion of FSH+LH-stimulated cumulus cells, in cattle. In addition, when the NPR3 agonist was associated with CNP there was no increase in the rate or prolongation of meiosis inhibition compared to CNP alone, however, the inhibition of gonatrophin-stimulated cumulus cell expansion was potentiated. All these findings suggest that the decrease and non-activation of NPR3 appears to be important for cumulus expansion to occur, and the CNP also has functions on cumulus cells expansion beyond the delay of meiosis resumption in bovine oocyte.

Keywords: Natriuretic peptides receptors, cumulus expansion, gonadotropins, epidermal growth factor receptor, bovine.

1. Introduction

The pre-ovulatory peak of LH alter the cellular machinery of the follicle constituents and trigger the resumption of oocyte meiosis, expansion of cumulus cells and ovulation. The LH is G-protein-coupled receptor, predominantly expressed in the mural granulosa cells (Peng *et al.* 1991, Cotterill *et al.* 2012). Its activation induces the signaling cascade by the stimulation of adenylate cyclase and elevation of intracellular cyclic adenosine 3,5-monophosphate (cAMP) levels, activating the cAMP-dependent protein kinase (PKA) pathway (McFarland *et al.* 1989, Richards 2001, Conti *et al.* 2002), transcription and translation of genes designated as intrafollicular mediators of this hormone, as growth factors similar to epidermal growth factor (EGF) (Park *et al.* 2004, Shimada *et al.* 2006, Panigone *et al.* 2008, Hsieh *et al.* 2011, Li & Albertini 2013). In this context, understanding the regulation of genes present in cumulus cells during oocyte maturation and expansion of cumulus cells may be essential for understanding the biological events triggered by gonadotrophins.

The antral follicles have two somatic cell lines: granulosa and cumulus oophorus cells, which present hormone responsiveness and gene expression profile different (Russell & Robker 2007). The group of adjacent cells that surround the female gamete form the cumulus-oocyte complex (COC), which are extremely influenced by paracrine and autocrine factors (Eppig 2001, Russell & Robker 2007). The cumulus cells communicate with each other and with the oocyte intimately through the gap junctions, besides continuous filaments going through the zona pellucida that connect the cumulus cells with the plasma membrane of the oocyte by transzonal projections (TZPs) (Albertini *et al.* 2001, Li & Albertini 2013, El-Hayek & Clarke 2015). Failures in the regulation and expansion of cumulus cells compromise the nuclear and cytoplasmic maturation of the oocyte (Chian *et al.* 1994, Tanghe *et al.* 2002, Ferreira *et al.* 2009), the release of COC after rupture of follicle wall and subsequent capture by the cells of the infundibulum (fimbriae) (Chen *et al.* 1993, Zhuo & Kimata 2001) and the

microenvironment that facilitates the capacitation of spermatozoa for fertilization (Tanghe *et al.* 2002). However, the effect of natriuretic peptides (NP) on the expansion of cumulus cells is still unknown.

The NP family comprise three polypeptides: atrial NP (ANP), B-type NP (BNP), and C-type NP (CNP), as well as three receptors (NPR1, NPR2 and NPR3) located on the target of cell surface (Levin *et al.* 1998, Gardner *et al.* 2007, Misono *et al.* 2011, Potter 2011). The three NP receptors have an extracellular binding domain (approximately 450 amino acids) and a simple hydrophobic trans-membrane region (Porter *et al.* 1990, Gardner *et al.* 2007). The NPR1 and NPR2 signal is through the synthesis of cyclic guanosine 3,5-monophosphate (cGMP) (Potter *et al.* 2006, Potter *et al.* 2009). The NPR3 contains an intracellular domain with 37 amino acids and has no guanylate cyclase activity, thus, as main function controls NP concentrations by internalize and degrade the ligands (Levin *et al.* 1998, Matsukawa *et al.* 1999, Potter 2011). The CNP has been proposed as a major oocyte meiotic inhibitory factor. Produced by granulosa cells, the CNP, binds to NPR2 of cumulus cells and triggering the production of cGMP that diffuses into the oocyte through gap junctions and inhibits phosphodiesterase 3A, maintaining high levels of cAMP in mice (Vaccari *et al.* 2009, Norris *et al.* 2010, Zhang *et al.* 2010, Conti *et al.* 2012). Similar function was observed in goat (Peng *et al.* 2013, Zhang *et al.* 2015a), bovine (Franciosi *et al.* 2014) and cat (Zhong *et al.* 2015) for CNP *in vitro*. In pigs, both CNP and BNP bind to NPR2 and holds the oocyte meiosis resumption blocked (Hiradate *et al.* 2013, Zhang *et al.* 2014, Zhang *et al.* 2015b). With the collaboration of NPR3-specific agonist ($cANP^{4-23}$), recent study demonstrated for the first time function for the NPR3 in female reproductive process, in which this receptor participates in the inhibition of porcine oocyte meiotic resumption (Santiquet *et al.* 2014).

The regulation of NP system in follicular cells is intimately related to gonadotropins. The eCG stimulates the expression of NP precursor type C (*NPPC*) and *NPR2* in granulosa

and cumulus cells of mice, respectively, and the pre-ovulatory LH surge, by the epidermal growth factor receptor (EGFr) activation, induces the decrease of this expression (Kawamura *et al.* 2011, Zhang *et al.* 2011, Robinson *et al.* 2012, Tsuji *et al.* 2012, Lee *et al.* 2013, Wang *et al.* 2013). In pigs, in both granulosa and cumulus cells the NP precursor type B (*NPPB*), *NPPC* and *NPR2* mRNA expression is suppressed by EGFr-mediated signaling in pre-ovulatory follicles (Zhang *et al.* 2014, Zhang *et al.* 2015b). The *NPR3* mRNA expression increase quickly after LH surge in granulosa and cumulus cells, in mice (Lee *et al.* 2013). In cattle it was demonstrated the expression of three NP receptors and NP precursor type A (*NPPA*) and *NPPC* in granulosa and cumulus cells (De Cesaro *et al.* 2015, De Cesaro *et al.* submitted for publication). Furthermore, we demonstrated in bovine granulosa cells *in vivo* the up and downregulation of *NPPC* and *NPR3* after LH, respectively (De Cesaro *et al.* submitted for publication), and that this regulation is through EGFr (Santos *et al.* submitted for publication). However, the regulation of NP receptors in cumulus cells has not been demonstrated in monovular species.

The information summarized above allowed us to investigate the NP receptors mRNA regulation in bovine cumulus cells during the oocyte maturation and cumulus cells expansion. Furthermore, the effect of NPR3 activation in cumulus cells in these physiological events was investigated. In the present study, experiments were performed to test the hypothesis that *NPR3* mRNA were regulated by gonadotropins, through EGFr, and the activation of this receptor inhibit the bovine cumulus cells expansion, stimulated by gonadotropins, in synergism with CNP.

2. Material and methods

All chemicals used were purchased from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise indicated. The doses of 0.5 µg/mL of FSH (Folltropin®-V, Bioniche,

ON, CA) and 5.0 µg/mL LH (Lutropin®-V, Bioniche, ON, CA) were maintained in all experiments

2.1 Oocyte recovery and in vitro maturation

Bovine ovaries at different stages of the estrous cycle were obtained from an abattoir and transported to the laboratory in saline solution (0.9% NaCl) at 30°C containing 100 IU/mL penicillin and 50 µg/mL streptomycin sulfate. Cumulus–oocyte complexes were aspirated from follicles 3-8 mm in diameter and selected grades 1 and 2 according to the criteria outlined elsewhere (Leibfried & First 1979). Before COCs were randomly distributed to different treatments, they were washed three times in TCM-199 containing Earle's salts and L-glutamine (Gibco Labs, Grand Island, NY, USA) supplemented with 25 mM Hepes, 0.2 mM pyruvic acid, 0.4% fatty acid-free bovine serum albumin (BSA), 100 IU/mL penicillin, and 50 µg/mL streptomycin (TCM wash). The COCs were cultured in a four-well culture dishes (Nunc®, Roskilde, Denmark) containing 200 µL of maturation medium at 39°C in a saturated humidity atmosphere with 5% CO_2, for different times and with appropriate treatment. The basic culture medium was TCM-199 with Earle's salts and l-glutamine (Gibco Labs, Grand Island, NY, USA) supplemented with 25 mM Hepes, 2.2 mg/mL sodium bicarbonate, 0.2 mM pyruvic acid, 100 IU/mL penicillin, 50 µg/mL streptomycin, and 0.4% BSA.

2.2 Preparation of the follicular walls

Follicle with 2-5 mm in diameter and transparent with yellow follicular fluid were isolated from ovary and dissected from the stromal tissue (Richard & Sirard 1996). The follicle section and follicular hemisections culture procedures were conducted in according to

previous study of our laboratory (Giometti *et al.* 2005, Stefanello *et al.* 2006, Barreta *et al.* 2008, Siqueira *et al.* 2012, De Cesaro *et al.* 2013).

2.3 Analysis of nuclear maturation

At the end of the culture period, the cumulus cells were removed by repeated pipetting, and denuded oocytes were fixed in 4% paraformaldehyde for 15 min, followed by permeabilization of the nuclear membranes with 0.5% Triton X-100 for at least 2 hour. After this period, the oocytes were exposed to 10 μg/mL of bisbenzimide (Hoescht 33342) for 15 min. Stained oocytes were classified under UV light with a fluorescence microscope (Leica DMI4000 B, Wetzlar, Germany) and classified based on their nuclear chromatin configuration in germinal vesicle (GV), GV breakdown (GVBD), metaphase I (MI), anaphase I (AI), telophase I (TI) and metaphase II (MII).

2.4 Analysis of cumulus expansion

Using an inverted microscope, immediately before (0 hour) and at the end of the proposed culture period, images of the COCs were captured through the Leica Application Suite (LAS, Version 3.8) software. With the obtained images, the area line tool (available in LAS, Version 3.8) was used to measure the total COC area (Fig. 1A) at different times and treatments.

2.5 RNA extraction, reverse transcription and real-time PCR

Total RNA was extracted from cumulus and granulosa cells using Trizol (Invitrogen, São Paulo, SP, Brazil) according to the manufacturer's instructions. Quantity and RNA purity were measured using the NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA; absorbance ratio at 260/280 nm). Ratios above 1.8 were considered pure, and all

samples used in the present study were above this threshold. To generate the complementary DNA (cDNA), 500 ng RNA was first treated with 0.1U DNAse (Invitrogen; 37°C for 5 minutes). After DNAse inactivation at 65°C for 10 min, samples were incubated in a final volume of 20 µL with iScript cDNA Synthesis Kit (Bio-Rad) following the manufacturer's protocols.

Real time quantitative PCR (qPCR) reactions were run in the CFX384 real-time PCR detection system (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad), and bovine-specific primers (Table 1) taken from the literature or designed using Primer-Blast, and specificity was confirmed using BLAST (NCBI). Standard two-step qPCR was performed to amplify each transcript with an initial denaturation at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 58°C for 30 seconds and melting-curve analysis was used to verify the specificity of reaction products. Samples were run in duplicates, standard curve method was used to determine the abundance of mRNA for each gene, and expression was normalized to the abundance of the housekeeping genes *cyclophilin* and *β-actin*.

2.6 Experimental design

2.6.1 *Experiment 1. Kinetics of nuclear maturation and cumulus cells expansion of bovine COCs in the absence or presence of FSH and/or LH during 24 hours*

This experiment was designed to assess which gonadotropin alone or in association and which time is more suitable for study of oocyte maturation and cumulus cells expansion in bovine COCs. To conduct this experiment, 678 COCs were *in vitro* matured with basic culture medium (TCM) or in presence of FSH or with LH or in association of FSH+LH. After 6, 12, 18 or 24 hour of *in vitro* culture, besides 0 hour, images of each COCs were captured to

measure the total COC area, following the cumulus cells removal to asses the nuclear maturation of denuded oocyte. This experiment was performed in quadruplicate.

2.6.2 Experiment 2. Effect of gonadotropins on mRNA expression of natriuretic peptide receptors

Based on the previous experiment, we evaluated the regulation of NP receptors in the cumulus cells using an *in vitro* culture medium that delays nuclear maturation and does not stimulate cumulus expansion (TCM), and another culture medium that stimulates oocyte maturation and also cumulus cells expansion (FSH+LH). A total of 399 COCs were *in vitro* matured with basic culture medium (TCM) or with FSH+LH for 3, 6, 9 and 12 hours. At hour 0 and also at the end of the each time point images of COCs were captured to measure your total area, oocytes were denuded to evaluate the nuclear maturation and cumulus cells were properly stored to analyses mRNA expression of *NPR1*, *NPR2* and *NPR3*. This experiment was performed in quadruplicate and the expression of hyaluronan synthase 2 (*HAS2*), prostaglandin-endoperoxide synthase 2 (*PTGS2*) tumor necrosis factor alpha-induced protein 6 (*TNFAIP6*), and pentraxin-related protein 3 (*PTX3*) were also assessed to validate de model.

2.6.3 Experiment 3. Effect of epidermal growth factor receptor (EGFr) inhibition and follicular hemisection on mRNA expression of NP receptors in bovine cumulus cells

Firstly, we cultivate 356 COCs with gonadotropins (FSH+LH) or gonadotropins plus the selective tyrosine kinase inhibitor of the EGFr (AG1478; 5.0 μM AG1478) for 6, 12, 18 and 24 hours and we evaluated whether the dose of 5.0 μM AG1478 was effective in inhibiting nuclear maturation and expansion of cumulus cells, as described above. In a second moment, using a model closer to the follicular environment, COCs were co-cultured with follicular hemisection (Giometti *et al.* 2005, Stefanello *et al.* 2006, Barreta *et al.* 2008,

Siqueira *et al.* 2012, De Cesaro *et al.* 2013) to understand whether gonadotropins regulate NP receptors of cumulus cells via follicular cells or EGFr activation. For this, 168 COCs were *in vitro* matured for 12 hours with FSH in culture medium without follicular hemisections (positive control; C+) or with follicular hemisections (negative control; C-) or with follicular hemisections in presence of 5.0 μM AG1478 (AG), similar to the model used by Rosa *et al.* (submitted for publication). We also cultivated 162 COCs in the presence of FSH+LH for the same experimental groups and at the same time. At the end of 12 hours of *in vitro* culture, the COCs were denuded and the cumulus cells were collected for evaluation of mRNA expression of NP receptor. In the third part of this experiment, 382 COCs were cultured for 6 and 12 hours with FSH or FSH+LH without follicular hemisections (C+) or with follicular hemisections in presence of 5.0 μM AG1478 (AG). At hour 0 and also at the end of the each time point images of COCs were captured to measure your total area, oocytes were denuded to evaluate the nuclear maturation, cumulus and granulosa cells were properly stored to analyses mRNA expression of *NPR1*, *NPR2* and *NPR3*. To validate this model the expression of *HAS2*, *PTGS2*, *TNFAIP6* and *PTX3* in cumulus cells were also assessed. All parts of this experiment were performed in quadruplicate.

2.6.4 Experiment 4. Effect of cAMP on mRNA expression of NP receptors in bovine cumulus cells

To evaluate the effect of cAMP on the abundance of mRNA for NP receptors in cumulus cells, we *in vitro* cultured 557 COCs in presence of basic culture medium (TCM) or with FSH+LH or with forskolin (Forsk; adenylate cyclase stimulator) for 3, 6, 9 and 12 hours. At hour 0 and also at the end of each time point images of COCs were captured to measure your total area, oocytes were denuded to evaluate the nuclear maturation and cumulus cells were properly stored to analyses mRNA expression of *NPR1*, *NPR2* and *NPR3*. To validate

this model the expression of *HAS2*, *PTGS2*, *TNFAIP6* and *PTX3* in cumulus cells were also assessed. In addition, in each replicate, around 10 COCs (total = 110 COCs) per treatment remained in cultivation for 24 hours to evaluation of cumulus expansion. This experiment was performed in quadruplicate.

2.6.5 Experiment 5. Dose-response of cANP$^{4-23}$, with and without gonadotropins, on bovine oocyte maturation and cumulus expansion

Knowing that NPR3 is downregulated by gonadotropins, we evaluated the effect of the NPR3 agonist (cANP$^{4-23}$), in the absence (TCM) or presence of gonadotropins (FSH+LH), in different concentrations (0, 1, 10 and 100 μM) on oocyte maturation and cumulus cells expansion after 12 hours of *in vitro* culture. For this, 570 COCs were used. At hour 0 and at the end of the proposed period, images of COCs were captured to measure their total area and oocytes were denuded to evaluate the nuclear maturation. This experiment was performed in quadruplicate.

2.6.6 Experiment 6. Kinetics of oocyte maturation and cumulus cells expansion with 10μM cANP$^{4-23}$ in medium with gonadotropins

Based on the previous experiment, a total of 489 COCs were cultured for 6, 9 and 12 hours in presence of gonadotropins without or with 10 μM cANP$^{4-23}$ to evaluate the kinetics of oocyte maturation and cumulus cell expansion. At hour 0 and also at the end of each time point images of COCs were captured to measure your total area and oocytes were denuded to evaluate the nuclear maturation. This experiment was performed in quadruplicate.

2.6.7 *Experiment 7. Effect of the association of cANP$^{4-23}$ and CNP on the maturation and expansion of cumulus cells*

In order to evaluate whether the association of NPR3 agonist and CNP interferes with bovine oocyte maturation and/or cumulus expansion, 531 COCs were *in vitro* cultured for 6, 9 and 12 hours in the following groups: FSH+LH; 1 μM CNP; 10 μM $cANP^{4-23}$; 1 μM CNP+10 μM $cANP^{4-23}$. At hour 0 and also at the end of each time point images of COCs were captured to measure your total area and oocytes were denuded to evaluate the nuclear maturation. This experiment was performed in triplicate.

2.7 Statistical analysis

The analyses were performed with SAS and JMP software (SAS; SAS Institute, Inc., Cary, NC, USA). For gene expression experiment, the continuous data were tested for normal distribution using Shapiro–Wilk test, normalized when necessary and submitted to ANOVA. When a treatment effect occurred, the average among groups was compared using the LSM (*least squares means*). Results are presented as means ± S.E.M. and $P \leq 0.05$ was considered statistically significant. In oocyte maturation experiment, the analyses were performed using a statistical model for categorical data (PROC CATMOD) and P-values less than 0.05 were considered to represent significant differences. In the cumulus cells expansion experiments, completely randomized blocks were used, the different treatments performed simultaneously and each replication considered as a block. Results are presented as means ± S.E.M. and $P<0.01$ or $P<0.0001$ was considered statistically significant.

3. Results

3.1 *The association of gonadotropins is required to stimulate oocyte maturation and cumulus expansion in bovine COCs*

Initially, we observed that, despite the initiation of meiosis, the absence of gonadotropins showed a delay in the bovine oocyte maturation compared to the association of

FSH+LH during the 24 hours of *in vitro* maturation (Fig. 1B). Similarly, the expansion of cumulus cells was only complete with the association of gonadotropins (Fig. 1A and C). Thereby, allowing us to choose the interval from 0 to 12 hours to focus on the next experiments.

3.2 Gonadotropins downregulated the mRNA expression of NPR3 in bovine cumulus cells

Based on the previous experiment, we intend to understand the regulation of NP receptors in bovine cumulus cells. Using a culture medium without gonadotropins (TCM) and another with the association of FSH+LH, we first demonstrated that from the 6 hours of *in vitro* culture the GVBD in the oocytes (Fig. 2A) and the beginning of the cumulus cells expansion (Fig. 2B) can be observed in the FSH+LH group. This was well established by stimulation the expression of the key enzymes of cumulus expansion (*HAS2*, *PTGS2*, *TNFAIP6* and *PTX3*) only in the gonadotropin group (Fig. 2C). With the consolidated model (Fig. 2), we demonstrated that the FHS+LH group decreased *NPR3* mRNA expression in ~6.2 and ~4.7-fold compared to the TCM group at 9 and 12 hours of *in vitro* culture, respectively (Fig. 3). In addition, in the TCM group, there was an increase in the mRNA expression of *NPR3* at 12 hours in relation to hour 0, different from observed in the gonadotropin group (Fig. 3). Among the other NP receptors, we observed that *NPR2* mRNA expression was not regulated by FSH+LH compared to TCM at any time point observed (Fig. 3) and *NPR1* mRNA expression in bovine cumulus cells is extremely low (not shown).

3.3 *Gonadotropins downregulate mRNA expression of NPR3 in bovine cumulus cells through EGFr*

In order to evaluate whether gonadotropins decrease *NPR3* mRNA expression of cumulus via EGFr, we first demonstrated that the 5 μM of AG1478, in the presence of FSH+LH, did not inhibit oocyte meiosis resumption in cattle (Fig. 4A), however, prevented the cumulus cells expansion during 24 hours of *in vitro* culture (Fig. 4B). With the dose tested, based on the previous experiment and using a model with follicular hemesictions in co-culture with COCs (model closer to the follicular environment), we demonstrated that after 12 hours of *in vitro* maturation both the FSH or FSH+LH in absence (C+) or presence of follicular hemesictions (C-) presented lower abundance of *NPR3* mRNA compared to the group with follicular hemesictions and 5.0 μM of AG1478 (Fig. 5B). Likewise, we demonstrated that follicular hemesictions did not interfere in the cumulus expression of NP receptors when co-cultured with COCs (Fig. 5A and B). *NPR2* showed no difference in expression between the groups tested either with FSH or FSH+LH (Fig 5A). In order to understand the kinetics expression of NP receptors we used the same model with FSH (C+) or FSH+LH (C+) alone or in the presence of follicular hemisections plus 5.0μM AG1478 (AG) for 6 and 12 hours of *in vitro* culture. We first validated the model, in which more than 90% of oocytes in AG group remained in VG with both FSH (Fig. 6A) and FSH+LH (Fig. 6B). In addition, only the group with FSH+LH (C+) stimulated the cumulus cells expansion (Fig. 6C), evidenced by the expression of *HAS2*, *PTGS2*, *TNFAIP6* and *PTX3* (Fig. 6D). *NPR2* expression in cumulus cells was not altered by any treatment (Fig. 7A). Similarly, no regulation of *NPR2* and *NPR3* were observed in granulosa cells at 6 and 12 hours by FSH or FSH+LH (supplementary data). However, the AG group at both 6 and 12 hours prevented the decrease of *NPR3* expression in bovine cumulus cells stimulated FSH or FSH+LH (Fig. 7B). Compared with hour 0, the AG group with FSH+LH showed higher expression of *NPR3* mRNA at 6 and 12 hours of *in vitro* culture. But, for the AG group with FSH, this difference was only observed at 12 hours.

3.4 Effect of cAMP on mRNA expression of NP receptors in bovine cumulus cells

Knowing that both FSH and FSH+LH decrease the expression of NPR3 mRNA in bovine cumulus cells, we evaluated whether the second messenger of gonadotropins (cAMP) had the same effect. Using an adenylate cyclase stimulator (forskolin - Forsk), we first verified its functionality, demonstrating that with its use there was a delay in oocyte nuclear maturation compared with TCM and FSH+LH groups (Fig. 8A). The expansion of the bovine cumulus complex surrounding the oocyte on the Forsk group did not differ statistically from the TCM group until 12 hours, however, with 24 hours of *in vitro* culture the COCs from the Forsk and FSH+LH groups were similar and greater than the TCM group (Fig. 8B). When we evaluated the key enzymes of cumulus expansion (*HAS2*, *PTGS2*, *TNFAIP6* and *PTX3*) up to 12 hours, we observed that only *HAS2* differed between FSH+LH and Forsk, and the TCM group did not stimulate any of these enzymes (Fig. 8C). NPR2 expression did not differ between treatments at the same time (Fig. 9). However, mRNA expression for *NPR3* in the Forsk and FSH+LH groups was similar and lower than the TCM group at hours 6, 9 and 12 (Fig. 9). In addition, the FSH+LH and Forsk groups showed lower expression of *NPR3* mRNA at all times analyzed compared to hour 0 (Fig. 9).

3.5 $cANP^{4-23}$ inhibits cumulus cells expansion stimulated by gonadotropin

To understand the effect of NPR3 stimulation, we used an agonist of this receptor ($cANP^{4-23}$) and observed that in the absence (TCM) or presence of gonadotropins (FSH+LH) there was no change in nuclear maturation after 12 hours of *in vitro* maturation (Fig. 10A). However, the 3 doses of $cANP^{4-23}$ used (1, 10 and 100 μM) reduced the FSH+LH-stimulated bovine cumulus cells expansion (Fig. 10B).

3.6 Kinetics of oocyte maturation and cumulus cells expansion in the presence of 10μM cANP$^{4-23}$ in medium with gonadotropins

Using 10 μM of cANP$^{4-23}$ in medium with gonadotropins we observed that nuclear maturation was not altered at any time point evaluated (6, 9 and 12 hours; Fig. 11A). For cumulus cells expansion, only at 12 hours of *in vitro* culture were observed that COCs of cANP$^{4-23}$ group were lower than the FSH+LH group (Fig. 11B).

3.7 Association of cANP$^{4-23}$ and CNP act in synergism to inhibit the expansion of cumulus cells

Evaluating the association of cANP$^{4-23}$ and CNP (CNP+cANP$^{4-23}$) we observed that nuclear maturation was not altered. Thus, 1 μM of CNP inhibited the resumption of meiosis at 6 hours of maturation compared to the FSH+LH group (Fig. 12A). Similar to observed in association of cANP$^{4-23}$ and CNP. Since at 9 and 12 hours no difference was observed for the oocyte maturation between the groups. However, for the cumulus cells expansion we observed that at 9 hours of culture the CNP+cANP$^{4-23}$ group was lower than the FSH+LH group and similar to the CNP and cANP$^{4-23}$ groups alone (Fig. 12B). At 12 hours this difference and the association CNP+cANP$^{4-23}$ was lower than the CNP and cANP$^{4-23}$ groups alone, which in turn were lower than the FSH+LH group.

4. Discussion

The regulation of mRNA expression of NP receptors and the NPR3 activation in cumulus cells of monovular species is not known. In the present study, we used *in vitro* experimental models to understand the regulation of NP receptors expression in bovine cumulus cells during oocyte maturation and cumulus cells expansion stimulated by gonadotropins. We demonstrated that the CNP also has functions related to the expansion of

cumulus cells. Furthermore, we provide the first direct evidence in female reproductive events of the NPR3 function in monovular species. Our significant findings are: 1) gonadotropins downregulated the mRNA expression of *NPR3* in bovine cumulus cells, whereas, *NPR2* mRNA was not regulated and *NPR1* mRNA is extremely low in bovine cumulus cells; 2) FSH and FSH+LH downregulated mRNA expression of *NPR3* in bovine cumulus cells through EGFr; 3) forskolin had the same mRNA regulation of gonadotropins on NP receptors in bovine cumulus cells; 4) activation of NPR3 does not interfere in the nuclear maturation of bovine oocytes, however, it inhibits the complete expansion of FSH+LH-stimulated cumulus cells; 5) the NPR3 activation and CNP act in synergism to inhibit the expansion of bovine cumulus cells.

The approach used in this study allowed us to confirm that although the absence of gonadotropins is not essential to restart oocyte nuclear maturation in cattle, only the association of FSH plus LH showed adequate expansion of cumulus cells in this specie. This was confirmed by the increase in the total area of COC (Fig. 1A and C; Fig 2B; Fig 6C) and also by the stimulation of indispensable genes for cumulus expansion: *HAS2*, *PTGS2*, *TNFAIP6* and *PTX3* (Davis *et al.* 1999, Varani *et al.* 2002, Fulop *et al.* 2003, Ochsner *et al.* 2003, Salustri *et al.* 2004, Shimada *et al.* 2006, Sugiura *et al.* 2009) only in the treatment with the association of gonadotropins (Fig. 2C; Fig. 6D). To mimic post-gonadotropins signals the COCs were treated with forskolin (adenylate cyclase stimulator), however bovine cumulus cells did not expanded until 12 hours of *in vitro* culture, but presented similar expansion to FSH+LH after 24 hours (Fig. 8B). Although gonadotropins induce cumulus expansion via an increase in the cAMP levels (Buccione *et al.* 1990, Zhang *et al.* 2005, Reizel *et al.* 2010, Nagyova 2012), possibly forskolin delays in stimulating *PTGS2* in bovine cumulus cells (Fig. 8C), similar to observed in cumulus cells of mice by forskolin in comparison to FSH (Shimada *et al.* 2006). Thus, in addition to transient inhibition of nuclear maturation in bovine

oocytes (Sasseville *et al.* 2009, De Cesaro *et al.* 2015), the use of the forskolin may also be considered when studying factors that stimulate cumulus expansion via prostaglandins.

Although *NPR1* has low expression in bovine cumulus cells, it has been shown that its main ligand (ANP) increases cGMP levels in COC (Bilodeau-Goeseels 2007), cumulus and oocyte (De Cesaro *et al.* 2015) after 3 hours of *in vitro* culture, in cattle. In addition, in the study submitted for publication (Santos *et al.,* submitted for publication), we demonstrated that in the initial processes of ovulatory signaling in bovine granulosa cells, NPR1 is important to ANP binding and act like a cofactor of LH to stimulate *PTGS2* mRNA.

The NPR2 in the cumulus cells is indispensable to CNP binding and maintenance of the oocyte meiotic block (Tamura *et al.* 2004, Zhang *et al.* 2010, Tsuji *et al.* 2012), being broadly regulated in multiovular species. The eCG, estradiol and oocyte-derived factors uregulated the expression of *NPR2* in cumulus cells of mice (Zhang *et al.* 2010, Zhang *et al.* 2011) and pigs (Hiradate *et al.* 2013, Zhang *et al.* 2014, Zhang *et al.* 2015b). However, after the pre-ovulatory LH surge, through the EGFr activation, decrease the expression of this receptor in cumulus cells in both mice (Tsuji *et al.* 2012, Wang *et al.* 2013) and pigs (Zhang *et al.* 2014, Zhang *et al.* 2015b). Herein, we demonstrated that *NPR2* mRNA was not regulated by gonadotropins, follicular hemisections, EGFr and forskolin in bovine cumulus cells (Fig. 3; Fig. 5A; Fig. 7A; Fig.9). However, the CNP binding on NPR2 of cumulus cells and block the meiotic resumption in mice (Zhang *et al.* 2010), cat (Zhong *et al.* 2015), goat (Peng *et al.* 2013) (Zhang *et al.* 2015a), pig (Hiradate *et al.* 2013) and bovine (Franciosi *et al.* 2014), similarly our result (Fig. 12A). Thus, due the great importance of NPR2, it is still necessary to understand its regulation in monovular species.

Despite the knowledge that CNP can also bind to NPR3 (Suga *et al.* 1992), still little importance in reproductive events has been given for this receptor. Recently, Lee *et al.* (2013) demonstrate that NPR3 is not involved in the degradation of CNP in the follicular

environment in mice, even though gonadotropins upregulated the expression of this NP receptors in cumulus cells, in the same species. Our results, in cattle, showed that *NPR3* mRNA has opposite regulation to mice, being downregulated by gonadotropins and forskolin in cumulus (Fig. 9) and granulosa cells *in vivo* (De Cesaro *et al.* submitted for publication). Moreover, the absence of gonadotropins and the non-increase of cAMP or the blockade of EGFr in the presence of FSH or FSH+LH stimulated mechanisms of synthesis of *NPR3* mRNA or inhibition of its degradation (Fig. 3; Fig. 7b; Fig. 9). Thus, these results suggest that this receptor is participating in reproductive functions prior to the pre-ovulatory peak of LH in cattle.

Based on recent knowledge that NPR3 participates in the blockade of oocyte meiosis resumption in pigs (Santiquet *et al.* 2014), and that in the circulatory system of mice with hypertension, NPR3-specific agonist ($cANP^{4-23}$) attenuates the phosphorylation of extracellular-regulated protein kinases 1 and 2 (ERK1/2) and v-akt murine thymoma viral oncogene homolog (AKT) in smooth muscle cells, and controls blood pressure (El Andalousi *et al.* 2013), we attempt to understand whether NPR3 acts in the maturation and/or cumulus expansion in cattle. Thus, we observed that the activation of NPR3 inhibits the complete expansion of FSH+LH-stimulated cumulus cells in cattle, but without alter the meiosis resumption of oocyte (Fig. 10-12). Knowing that ERK1/2 and AKT are downstream effectors of LH and indispensable for oocyte maturation and cumulus expansion (Fan *et al.* 2009, Nagyova 2012, Prochazka *et al.* 2012), we may speculate that NPR3 activation has the ability to interfere in ERK1/2 and AKT phosphorylation sufficient to attenuate cumulus cells expansion without interfering with meiosis, in cattle. Furthermore, when the NPR3 agonist was associated with CNP there was no increase in the rate or prolongation of meiosis inhibition compared to CNP alone (Fig. 12A). However, the inhibition of gonatrophin-stimulated cumulus cell expansion was potentiated with the association of CNP+$cANP^{4-23}$

(Fig. 12B). It is important to highlight that the use of CNP sustains gap junction-mediated communication in bovine COCs (Franciosi *et al.* 2014), and the density of TZP were higher in COCs treated for 48 hours with CNP than PDE3 inhibitor (cilostamide) in mice (Romero *et al.* 2016). Thereby, these results offer promising insights that the decrease in cumulus expansion resulting of CNP+cANP$^{4-23}$ maintains the gap junction-mediated communication and TZP in bovine COCs functioning. Thus, it may be important for mechanisms of oocyte maturation inherent to the cell-to-cell communication, and extends the possibility of understanding possible fertility failures due to cumulus expansion.

In conclusion, our studies demonstrated that in bovine cumulus cells the *NPR2* is not regulated by gonadotropins. However, the *NPR3* mRNA is doweregulated by gonadotropins and the modulation of this expression occurs through the EGFr activation. Moreover, the decrease and non-activation of NPR3 appears to be important for cumulus expansion occur, and the CNP also has functions on cumulus cells expansion beyond the delay of meiosis resumption in bovine oocyte.

5. Declaration of interest

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

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Figure 1 – Kinetics of nuclear maturation and cumulus cells expansion of bovine COCs in the absence or presence of FSH and/or LH during 24 hours. A) Images of COCs and the total area at 0, 12, 18 and 24 hour with FSH+LH. Percentage of germinal vesicle (GV), GV breakdown (GVBD), metaphase I (MI), anaphase I (AI), telophase I (TI) and metaphase II (MII) in bovine ooyte (B), and total area of bovine COC (C) at 0, 6, 12 and 24 hours of *in vitro* culture with basic culture medium (TCM) or in presence of FSH or with LH or in association of FSH+LH. Different letters represent statistical difference (P<0.01).

Figure 2 – Effect of FSH+LH on oocyte meiotic resumption, cumulus cells expression and mRNA expression of key enzymes of cumulus expansion in cattle. A) percentage of GV-arrested oocytes during 12 hours of *in vitro* cultured COC without (TCM) or with FSH+LH. B) total area of COC. C) mRNA expression of hyaluronan synthase 2 (*HAS2*), prostaglandin-endoperoxide synthase 2 (*PTGS2*) tumor necrosis factor alpha-induced protein 6 (*TNFAIP6*), and pentraxin-related protein 3 (*PTX3*) in cumulus cells. In the total area of COC different letters (P<0.01) and asterisk (*; P<0.0001) represent statistical difference. In the expression of genes different letters represent statistical difference (P<0.0.5).

Figure 3 – Abundance of *NPR2* and *NPR3* mRNA in bovine cumulus cells of COCs *in vitro* cultured without (TCM) or with FSH+LH during 0, 3, 6, 9 and 12 hours. Different letters represent statistical difference (P<0.05).

Figure 4 – Effect of selective tyrosine kinase inhibitor (AG) of the epidermal growth factor receptor on oocyte meiotic resumption and cumulus cells expansion stimulated by FSH+LH during 24 hours of bovine COC *in vitro* cultured. Percentage of germinal vesicle (GV), GV breakdown (GVBD), metaphase I (MI), anaphase I (AI), telophase I (TI) and

metaphase II (MII) in bovine ooyte (A), and total area of bovine COC (B) at 0, 6, 12, 18 and 24 hours of *in vitro* culture with FSH+LH or FSH+LH+AG. Different letters represent statistical difference (P<0.01) between treatment and hour. Asterisk (*) represent statistical difference (P<0.0001) between treatments in the same hour.

Figure 5 – Effect of selective tyrosine kinase inhibitor (AG1478) of the epidermal growth factor receptor and follicular hemisections on *NPR2* and *NPR3* mRNA expression in bovine cumulus cells after 12 hours of *in vitro* culture. Abundance of NPR2 (A) and NPR3 (B) mRNA in bovine cumulus cells of COCs *in vitro* cultured for 12 hours with FSH or FSH+LH in absence (C+) or presence of follicular hemesictions (C-) or in association with AG1478 (AG). Different letters represent statistical difference (P<0.05).

Figure 6 – Effect of FSH or FSH+LH in absence (C+) or presence of follicular hemesections in association with AG1478 (AG) on oocyte meiotic resumption, cumulus cells expression and mRNA expression of key enzymes of cumulus expansion in cattle. Percentage of GV-arrested oocytes after 0, 6 and 12 hours of *in vitro* cultured COCs with FSH (A) or FSH+LH (B) in absence (C+) or presence of follicular hemesictions in association with AG1478 (AG). Total area of COC (C) and mRNA expression of hyaluronan synthase 2 (*HAS2*), prostaglandin-endoperoxide synthase 2 (*PTGS2*) tumor necrosis factor alpha-induced protein 6 (*TNFAIP6*), and pentraxin-related protein 3 (*PTX3*) in cumulus cells (D) after 0, 6 and 12 hours of *in vitro* cultured COCs with FSH or FSH+LH in absence (C+) or presence of follicular hemesictions in association with AG1478 (AG). In the total area of COC letters represent statistical difference (P<0.01) between treatment and hour, and asterisk (*) represent statistical difference (P<0.0001) between treatments in the same hour. In the expression of genes different letters represent statistical difference (P<0.0.5).

Figure 7 – Abundance of *NPR2* (A) and *NPR3* (B) mRNA in bovine cumulus cells of COCs *in vitro* cultured with FSH or FSH+LH in absence (C+) or presence of follicular hemesections in association with AG1478 (AG) during 0, 6 and 12 hours. Different letters represent statistical difference (P<0.05).

Figure 8 – Effect of forskolin on oocyte meiotic resumption, cumulus cells expression and mRNA expression of key enzymes of cumulus expansion in cattle. Percentage of GV-arrested oocytes (A) and total area of COC (B) during 12 hours of *in vitro* cultured COC without gonadotropins (TCM), with FSH+LH or with forskolin (FORSK). C) mRNA expression of hyaluronan synthase 2 (*HAS2*), prostaglandin-endoperoxide synthase 2 (*PTGS2*) tumor necrosis factor alpha-induced protein 6 (*TNFAIP6*), and pentraxin-related protein 3 (*PTX3*) in cumulus cells. In the total area of COC letters represent statistical difference (P<0.01) between treatment and hour, and asterisk (*) represent statistical difference (P<0.0001) between treatments in the same hour. In the expression of genes different letters represent statistical difference (P<0.0.5).

Figure 9 – Abundance of *NPR2* and *NPR3* mRNA in bovine cumulus cells of COCs *in vitro* cultured without gonadotropins (TCM), with FSH+LH or with forskolin (FORSK) during 0, 3, 6, 9 and 12 hours. Different letters represent statistical difference (P<0.05).

Figure 10 – Dose-effect of a specific NPR3 agonist ($cANP^{4-23}$) with and without gonadotropins on bovine oocyte maturation and cumulus cells expansion. A) Percentage of germinal vesicle (GV), GV breakdown (GVBD), metaphase I (MI), anaphase I (AI), telophase I (TI) and metaphase II (MII) in bovine ooyte (A), and total area of bovine COC (B) at 12

hours of *in vitro* culture without (TCM) and with gonadotropins (FSH+LH) in presence of 1, 10 and 100μM cANP$^{4-23}$ (cANP). Different letters represent statistical difference (P<0.01) between treatment and hour. Asterisk (*) represent statistical difference (P<0.0001) between treatments in the same hour.

Figure 11 – Kinetics of oocyte maturation and cumulus cells expansion in the presence of 10μM cANP$^{4-23}$ in medium with gonadotropins. A) percentage of germinal vesicle (GV), GV breakdown (GVBD), metaphase I (MI), anaphase I (AI), telophase I (TI) and metaphase II (MII) in bovine ooyte (A), and total area of bovine COC (B) at 0, 6, 9 and 12 hours of *in vitro* culture with FSH+LH or FSH+LH+cANP$^{4-23}$. Different letters represent statistical difference (P<0.01) between treatment and hour. Asterisk (*) represent statistical difference (P<0.0001) between treatments in the same hour.

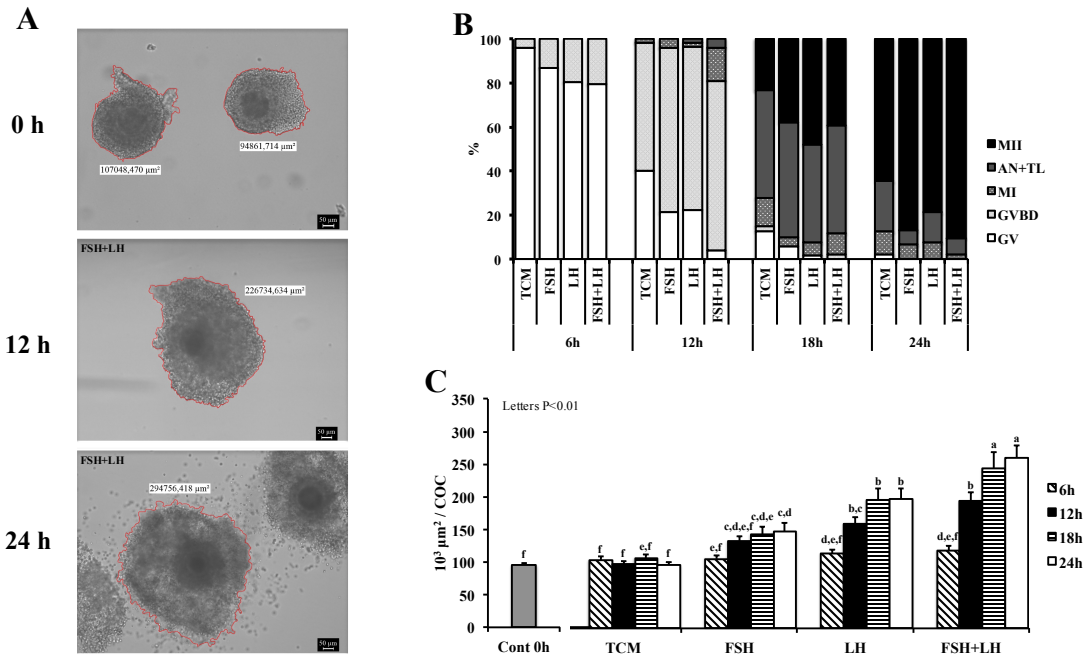
Figure 12 – Effect of the association of cANP$^{4-23}$ and CNP, in presence of gonadotropins, on the maturation and expansion of cumulus cells in cattle. A) percentage of germinal vesicle (GV), GV breakdown (GVBD), metaphase I (MI), anaphase I (AI), telophase I (TI) and metaphase II (MII) in bovine oocyte (A), and total area of bovine COC (B) at 0, 6, 9 and 12 hours of *in vitro* culture with FSH+LH in presence of 1μM CNP or 10μM cANP$^{4-23}$ or CNP+cANP$^{4-23}$. Different letters represent statistical difference (P<0.01) between treatment and hour. Asterisk (*) represent statistical difference (P<0.0001) between treatments in the same hour.

Table 1 - Primers used in the expression analysis of *Bos taurus taurus* genes.

Gene		Sequence	Reference or accession nº
Cyclophilin	F	GGTCATCGGTCTCTTTGGAA	(Ledoux *et al.* 2006)
	R	TCCTTGATCACACGATGGAA	
β-actin	F	TGTGGATCAGCAAGCAGGAGTA	NM_173979.3
	R	TGCGCAAGTTAGGTTTTGTCA	
HAS2	F	GCATGTCACCCAGTTGGTCT	NM_174079.2
	R	TGGGTCAAGCATGGTGTCTG	
COX2	F	CCCTTCTGCCTGACGTCTTT	(da Rosa *et al.* 2016)
	R	GGAAGATTCCTACCGCCAGC	
TNF	F	GCTCACGGATGGGGATTCAA	NM_001007813.2
	R	CGTGCTTCCCTGTGGTAGAC	
PTX3	F	CCGGCAGGTTGTGAAACAG	(Hung *et al.* 2015)
	R	CAGCGACCAGTCTGTTTTCC	
NPR1	F	AATTATGGCTCCCTACTAACCACAGA	(De Cesaro *et al.*2015)
	R	TCCGGTTCACACGTTTCACA	
NPR2	F	TCTGCTCCTAAGCTGGGTGAGT	(De Cesaro *et al.* 2015)
	R	CGGTCATCTGTGCGAGCAT	
NPR3	F	TTTGAAGCTAAGCAAGCGTACTCA	(De Cesaro *et al.* 2015)
	R	CAGAACTTTTCACCTCCATGGAA	

F, Forward primer; R, Reverse primer.

Figure 1


A
0 h
12 h
24 h
FSH+LH
FSH+LH
B
%
MII
AN+TL
MI
GVBD
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TCM
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6h
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Letters P<0.01
10³ µm² / COC
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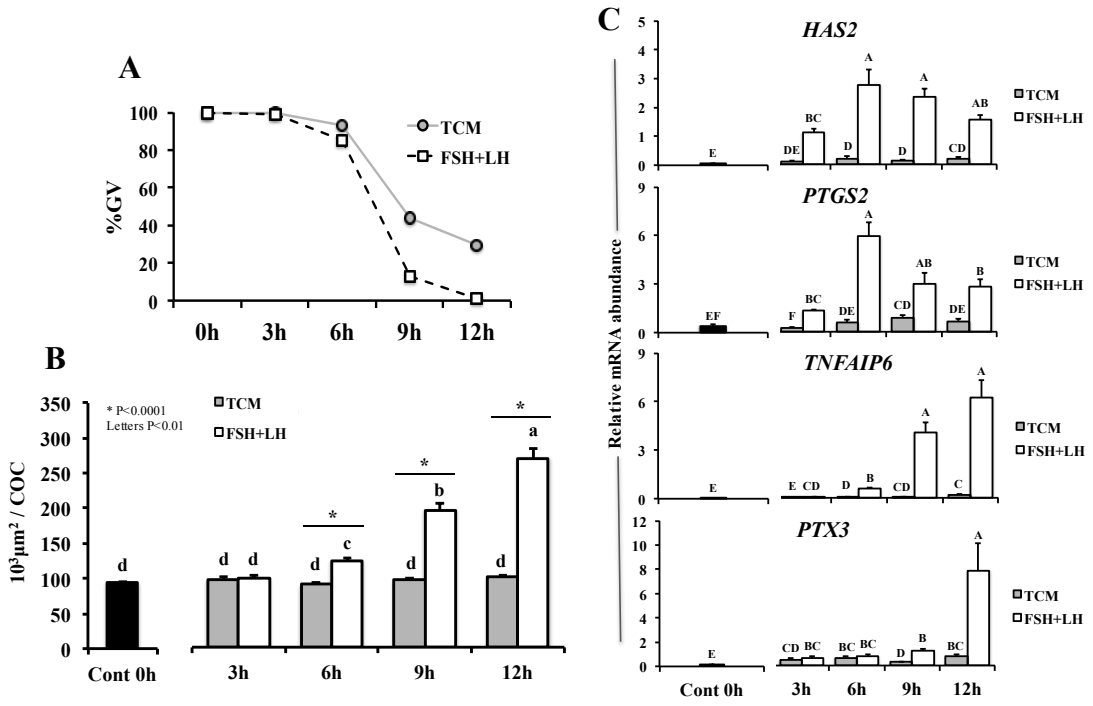
TCM
FSH
LH
FSH+LH
6h
12h
18h
24h

Figure 2


A
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TCM
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* P<0.0001
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6h
9h
12h
C
HAS2
PTGS2
TNFAIP6
PTX3
Relative mRNA abundance
TCM
FSH+LH
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6h
9h
12h

Figure 3

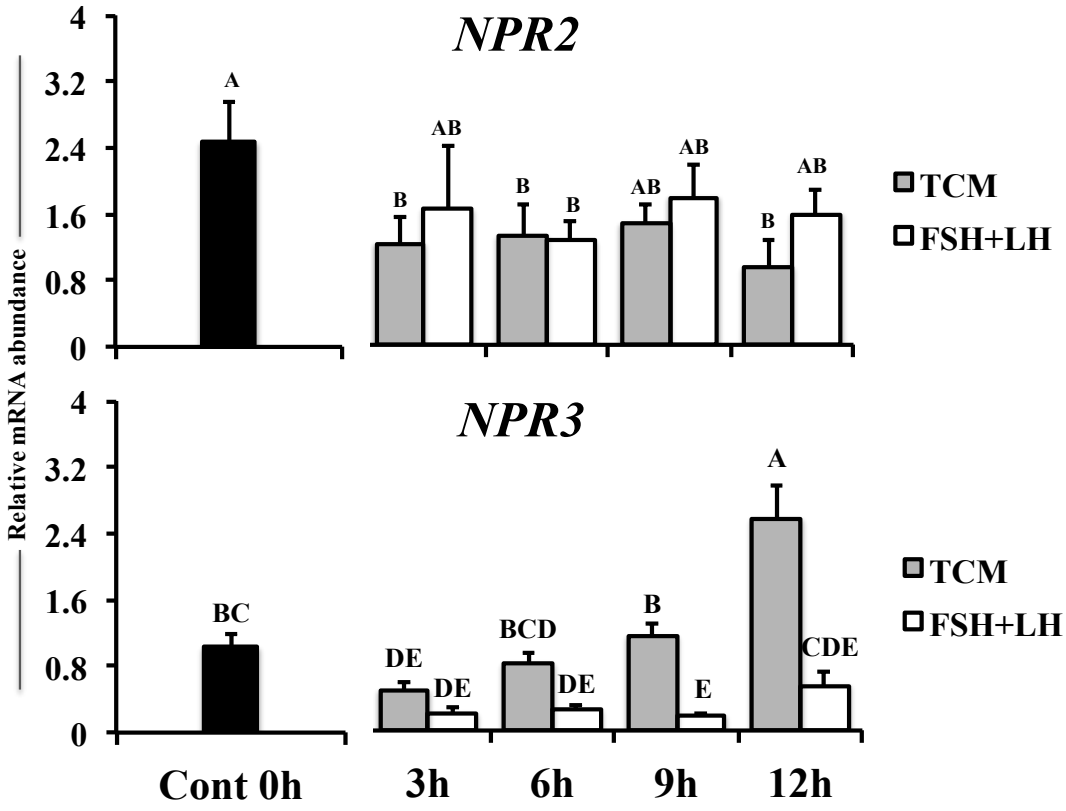
NPR2
NPR3
Relative mRNA abundance
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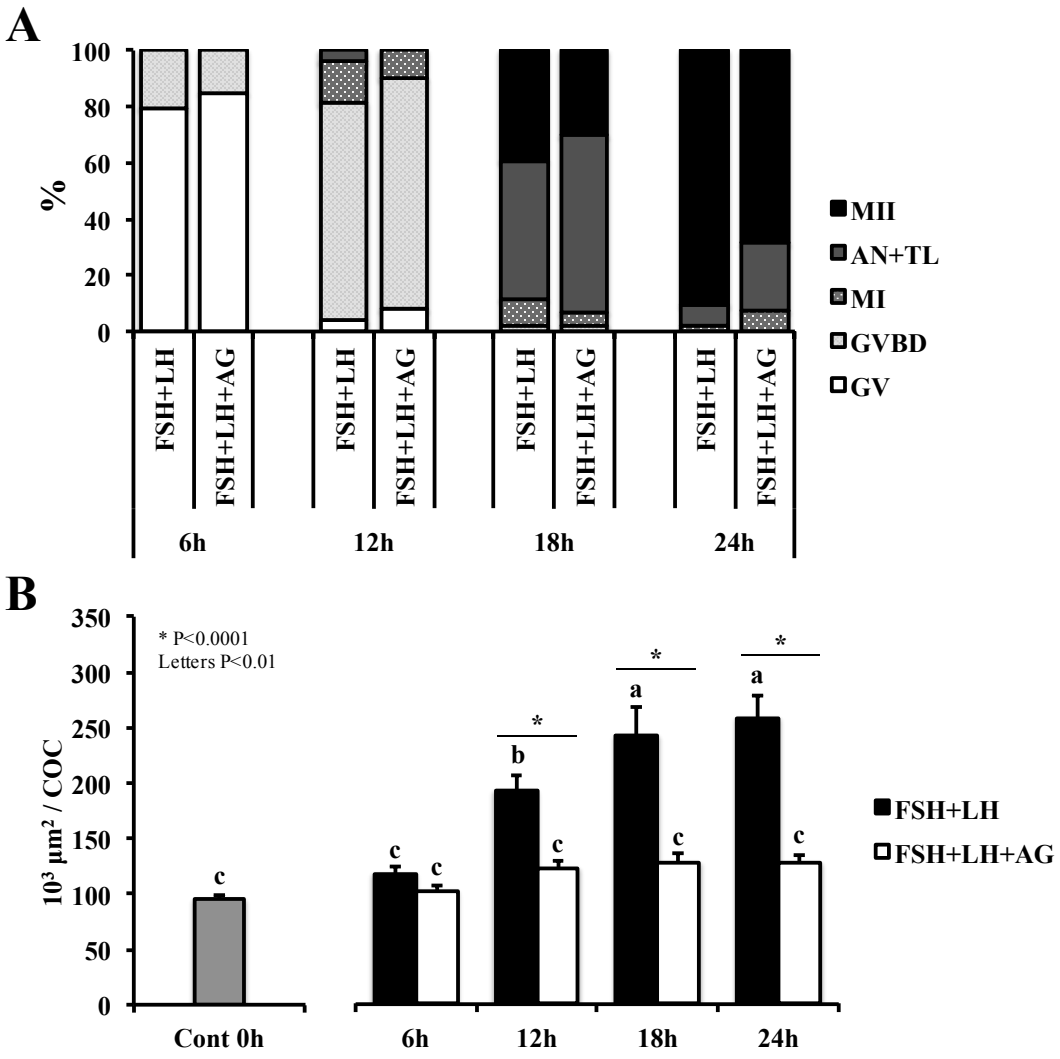
Figure 4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200
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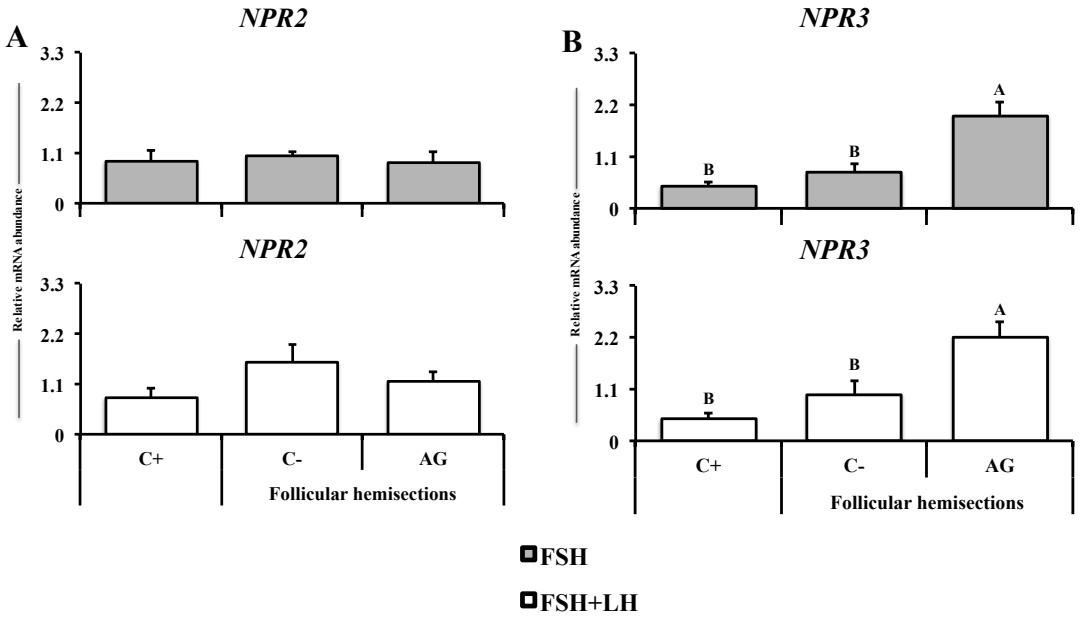
FSH+LH+AG

Figure 5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Relative mRNA abundance
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C+
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AG
Follicular hemisections
B
NPR3
3.3
2.2
1.1
0
B
B
A
Relative mRNA abundance
NPR3
3.3
2.2
1.1
0
B
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A
C+
C-
AG
Follicular hemisections
FSH
FSH+LH

Figure 6

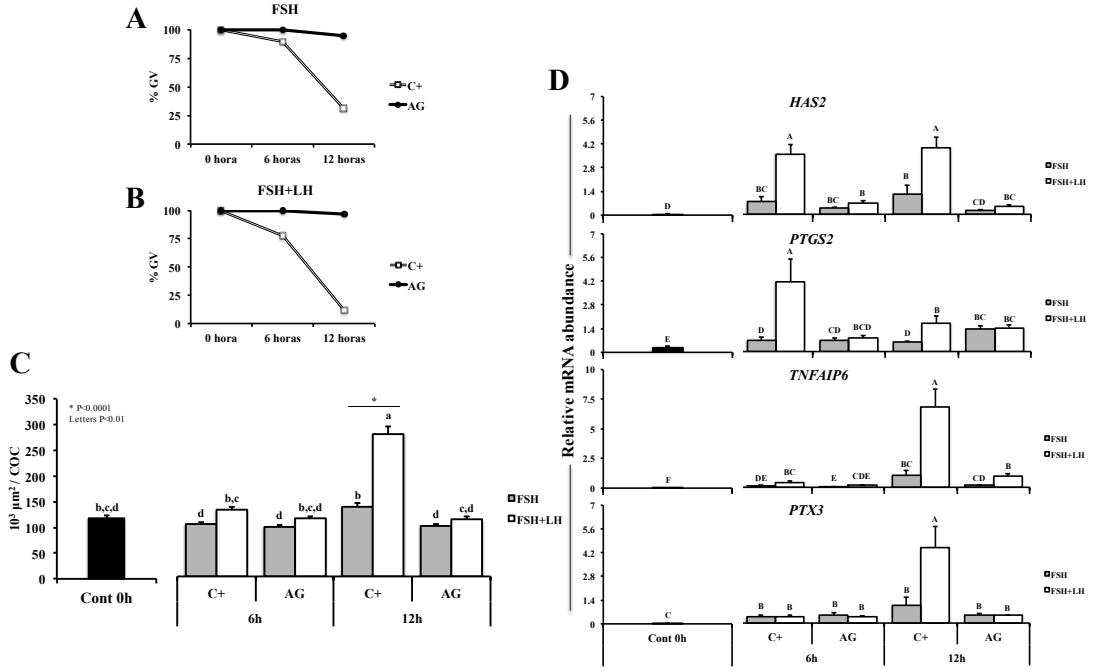

A
FSH
% GV
C+
AG
0 hora
6 horas
12 horas
B
FSH+LH
C
* P<0.0001
Letters P<0.01
10³ µm² / COC
Cont 0h
6h
12h
FSH
FSH+LH
D
HAS2
PTGS2
TNFAIP6
PTX3
Relative mRNA abundance

Figure 7

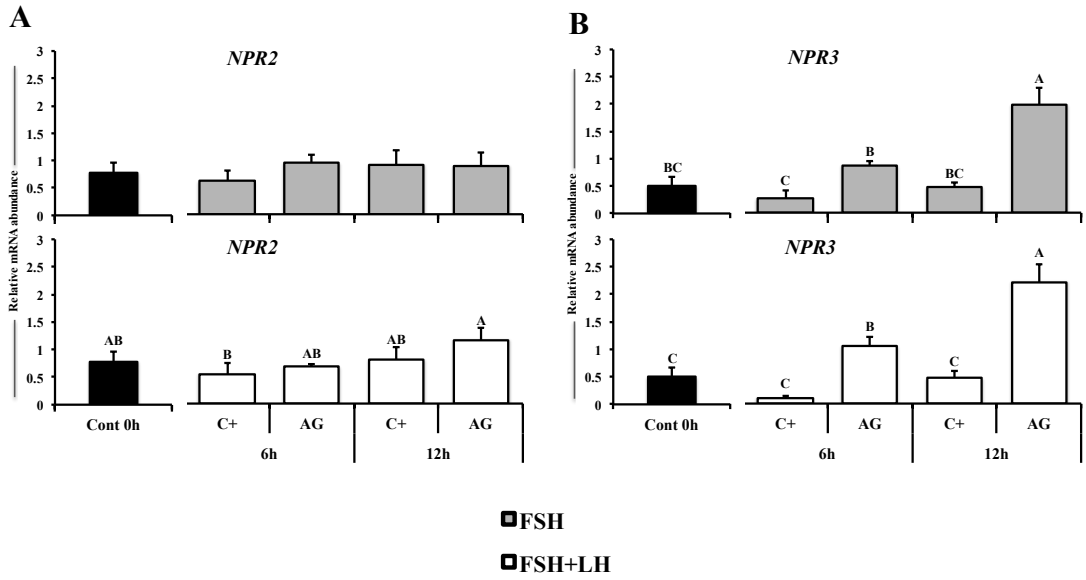

A
B
NPR2
NPR3
Relative mRNA abundance
Cont 0h
C+
AG
C+
AG
6h
12h
FSH
FSH+LH

Figure 8

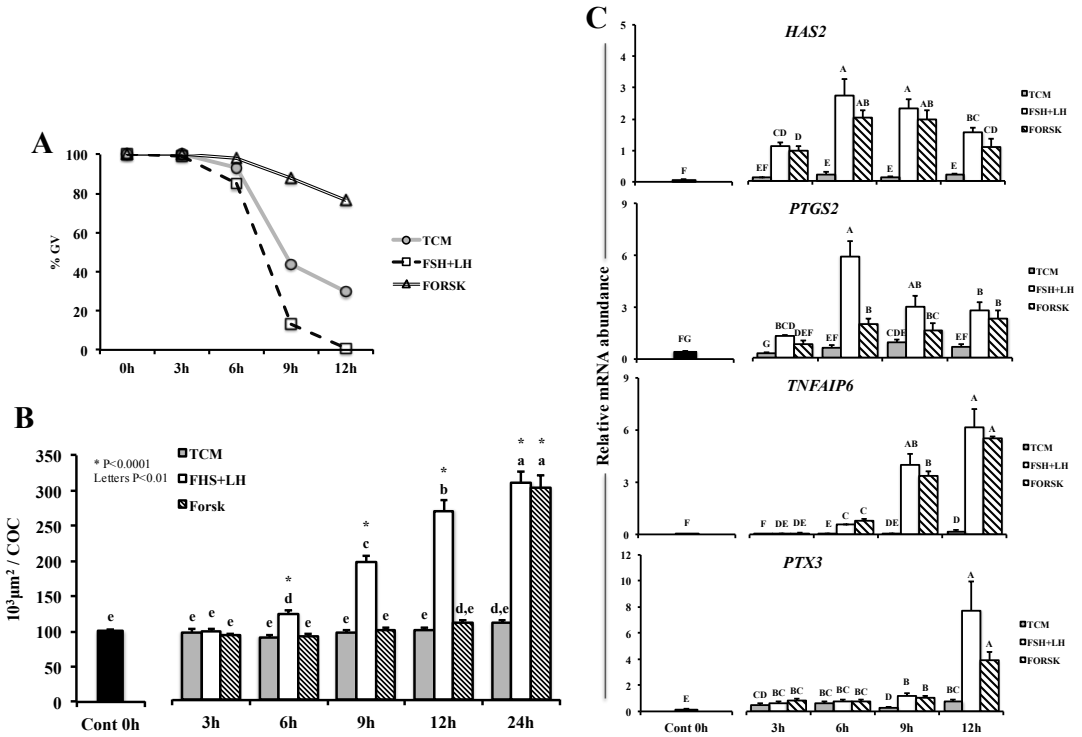

A
% GV
TCM
FSH+LH
FORSK
0h
3h
6h
9h
12h
B
* P<0.0001
Letters P<0.01
TCM
FHS+LH
Forsk
10³µm² / COC
Cont 0h
3h
6h
9h
12h
24h
C
HAS2
PTGS2
TNFAIP6
PTX3
Relative mRNA abundance
TCM
FSH+LH
FORSK
Cont 0h
3h
6h
9h
12h

Figure 9

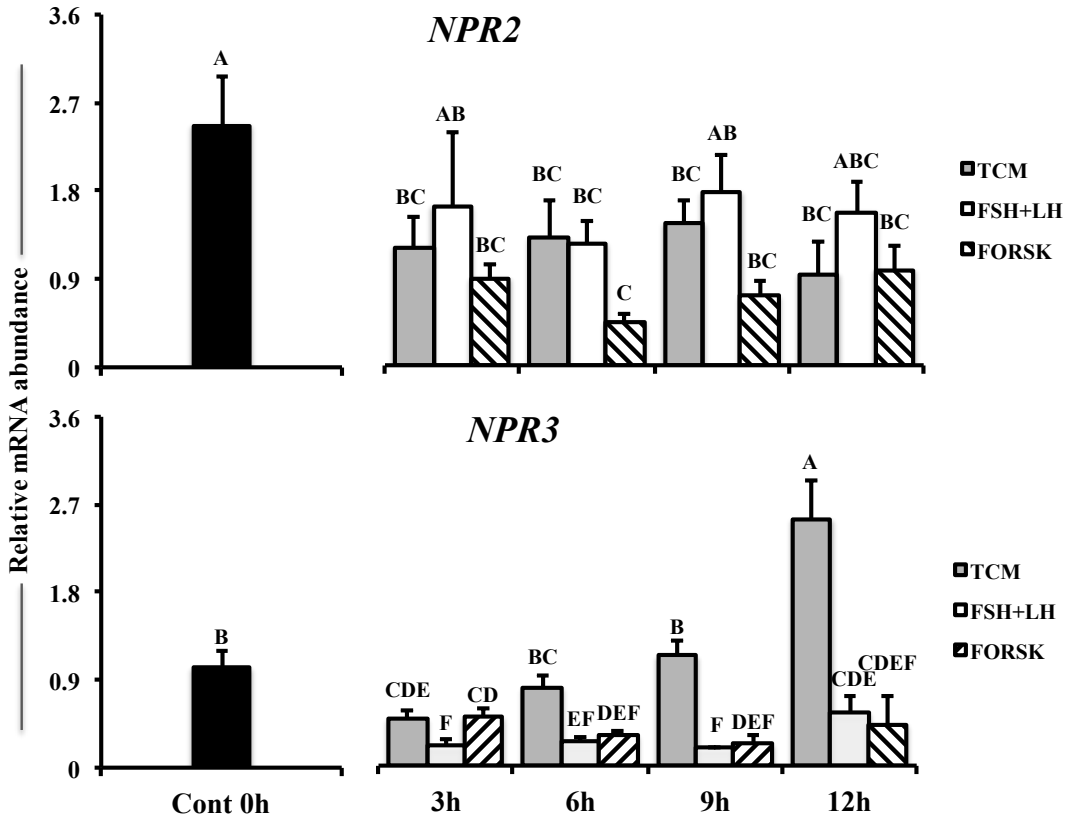

NPR2
NPR3
Relative mRNA abundance
3.6
2.7
1.8
0.9
0
A
AB
BC
BC
BC
BC
BC
C
AB
BC
BC
ABC
BC
BC
TCM
FSH+LH
FORSK
B
CDE
F
CD
BC
EF
DEF
B
F
DEF
A
CDE
CDEF
Cont 0h
3h
6h
9h
12h

Figure 10

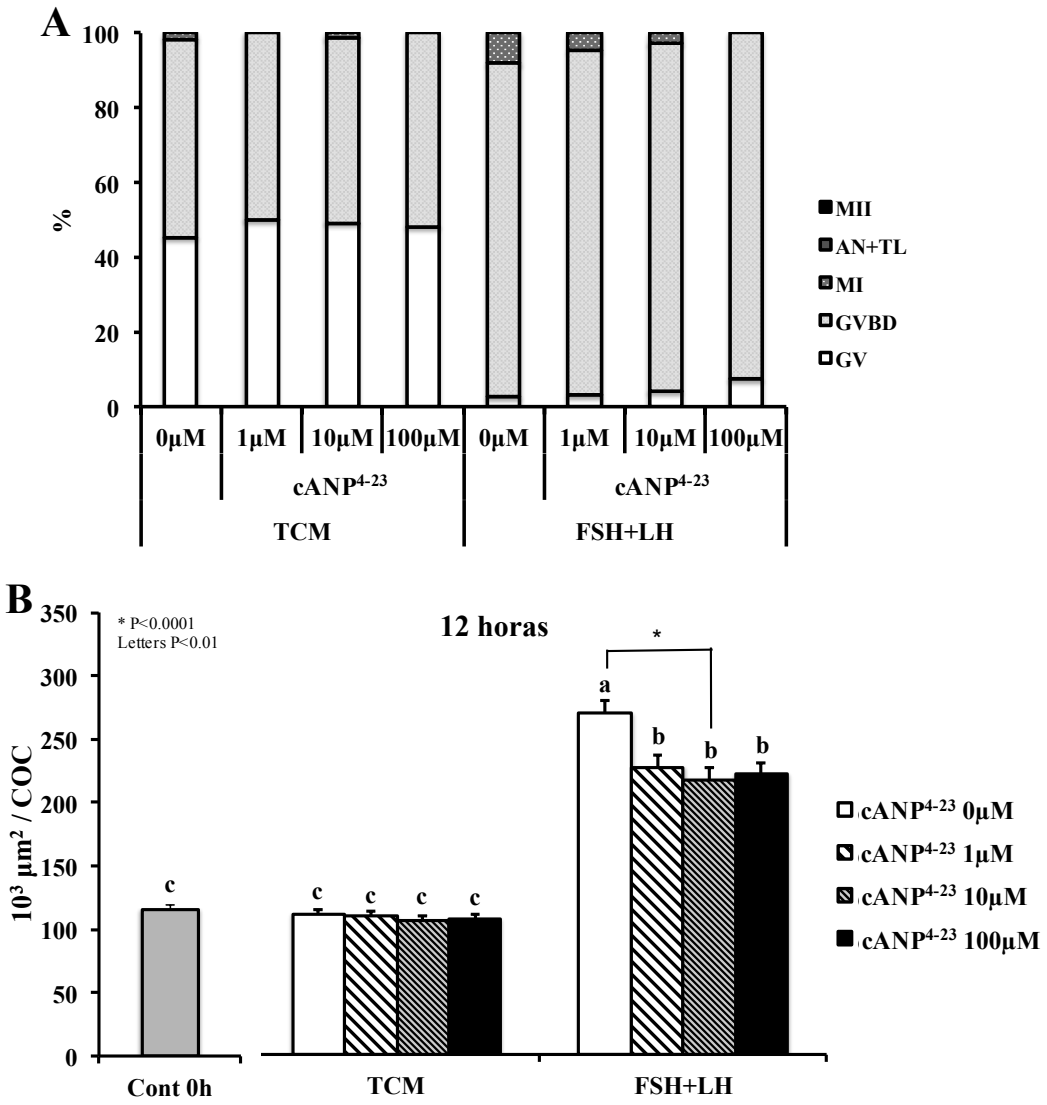

A
100
80
60
40
20
0
%
MII
AN+TL
MI
GVBD
GV
0µM
1µM
10µM
100µM
0µM
1µM
10µM
100µM
cANP4-23
cANP4-23
TCM
FSH+LH
B
* P<0.0001
Letters P<0.01
12 horas
10³ µm² / COC
350
300
250
200
150
100
50
0
a
b
b
b
c
c
c
c
c
*
cANP4-23 0µM
cANP4-23 1µM
cANP4-23 10µM
cANP4-23 100µM
Cont 0h
TCM
FSH+LH

Figure 11

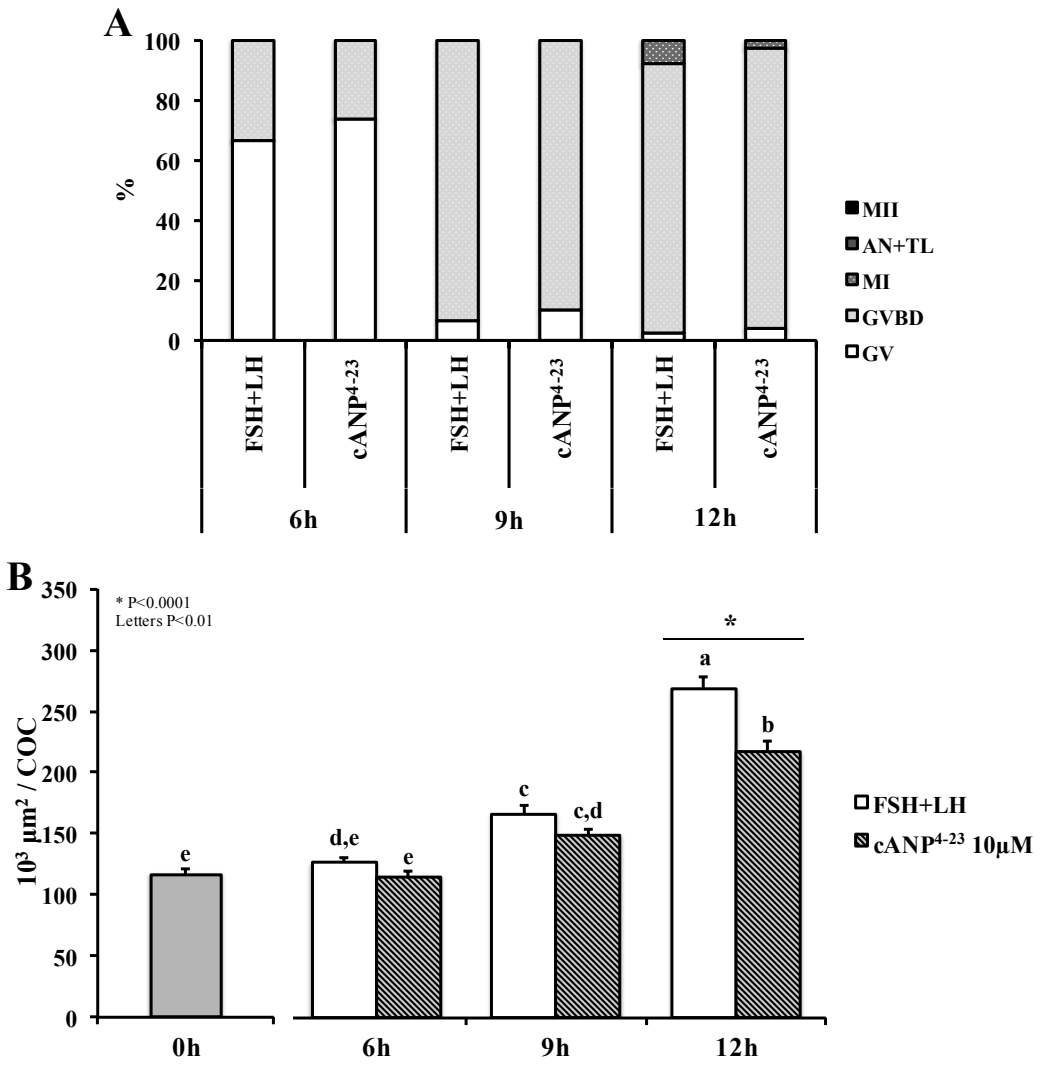
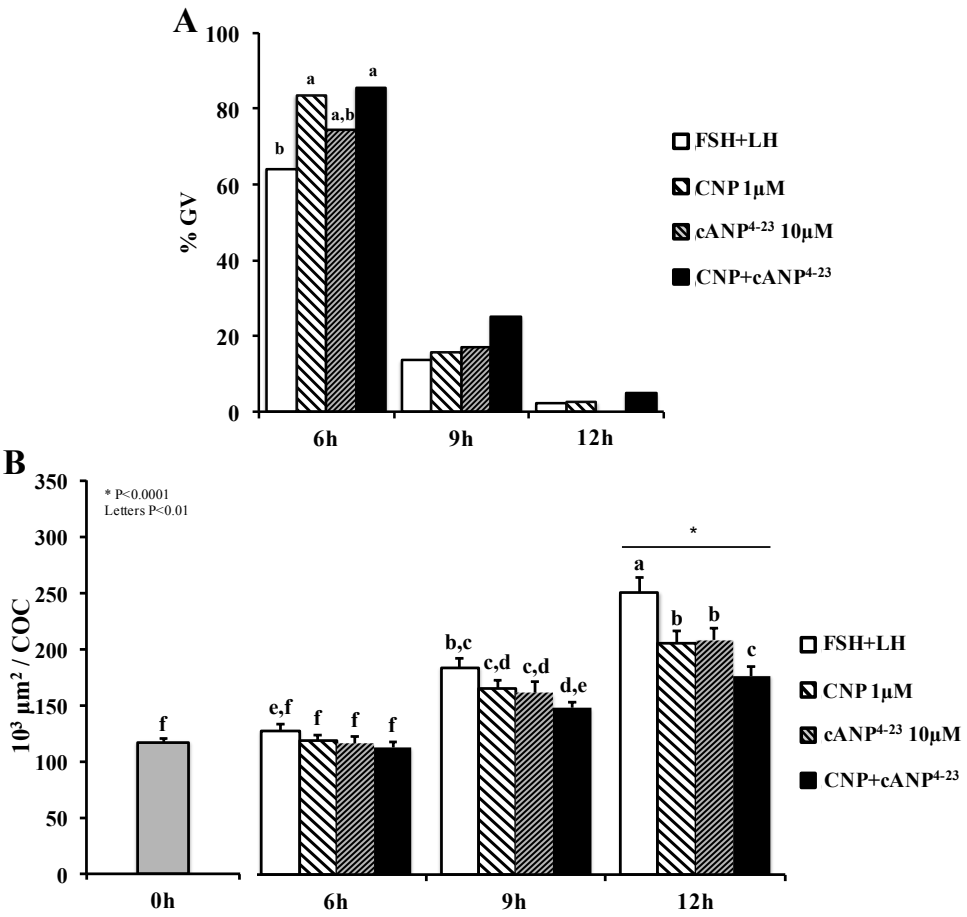
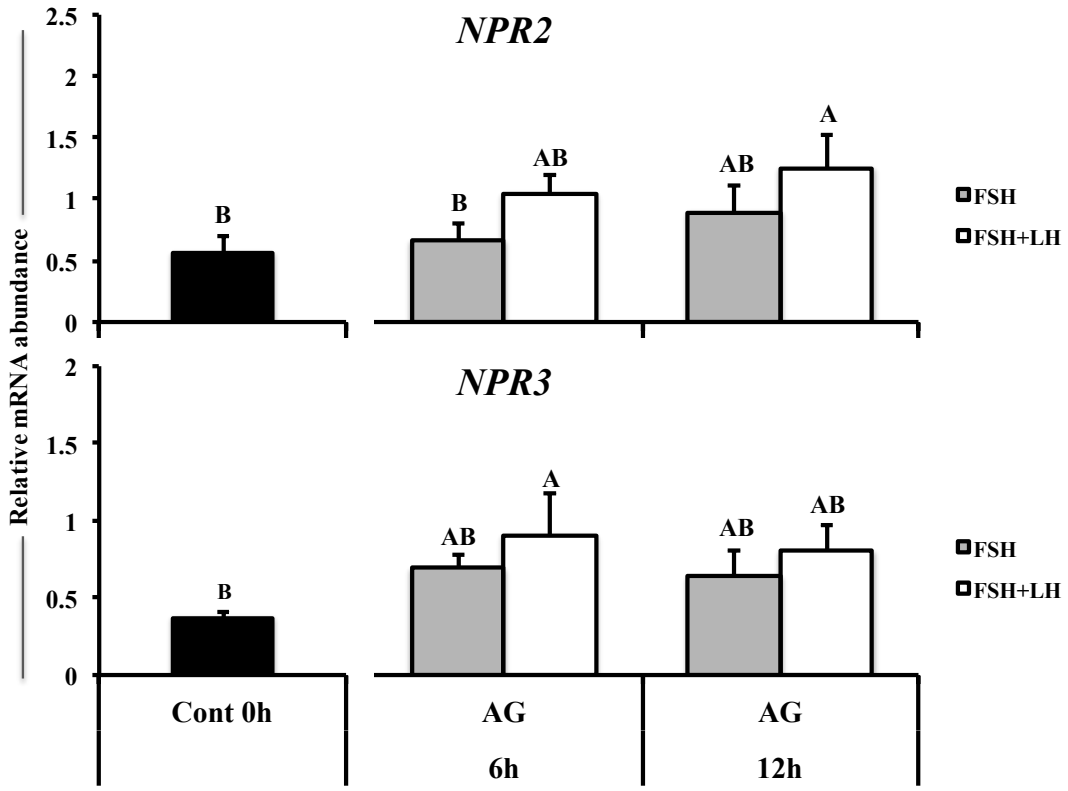

A
100
80
60
40
20
0
%
FSH+LH
cANP4-23
FSH+LH
cANP4-23
FSH+LH
cANP4-23
6h
9h
12h
MII
AN+TL
MI
GVBD
GV
B
* P<0.0001
Letters P<0.01
350
300
250
200
150
100
50
0
10³ µm² / COC
e
d,e
e
c
c,d
a
b
*
0h
6h
9h
12h
FSH+LH
cANP4-23 10µM

Figure 12


A
% GV
100
80
60
40
20
0
a
a
a,b
b
6h
9h
12h
FSH+LH
CNP 1µM
cANP4-23 10µM
CNP+cANP4-23
B
* P<0.0001
Letters P<0.01
10³ µm² / COC
350
300
250
200
150
100
50
0
*
a
b
b
c
b,c
c,d
c,d
d,e
e,f
f
f
f
f
0h
6h
9h
12h
FSH+LH
CNP 1µM
cANP4-23 10µM
CNP+cANP4-23

Supplementary data

Supplementary Figure 1 – Abundance of NPR2 and NPR3 mRNA in bovine granulosa cells (2-5mm in diameter) from follicular hemesection co-cultured with COCs *in vitro* in FSH or FSH+LH with AG1478 (AG) during 0, 6 and 12 hours. Different letters represent statistical difference (P<0.05).



7. RESULTADOS COMPLEMENTARES

7.1 Insulin-regulated aminopeptidase (IRAP)

Outro alvo do nosso estudo foi buscar evidências da participação da *insulin-regulated aminopeptidase* (IRAP) no processo de ovulação e retomada da meiose em resposta ao estímulo gonadotrófico em bovinos. Esta enzima é co-localizada em vesículas intracelulares e translocada à superfície da célula quando necessário (Demaegdt *et al.*, 2008), de maneira que, é classificada como ocitocinase pois metaboliza eficientemente ocitocina e vasopressina, sendo sugerida no controle da homeostase de tais hormônios durante a prenhez (Rogi *et al.*, 1996).

A IRAP também é conhecido como receptor IV da angiotensina (AT4), pelo fato de ter alta afinidade pela angiotensina IV (Ang IV), que é seu inibidor natural (Swanson *et al.*, 1992; Harding *et al.*, 1994). A Ang IV é formada a partir da clivagem do angiotensinogênio, sendo um hexapeptídeo que corresponde aos resíduos 3-8 da Ang II, e possui propriedades farmacológicas independente dos receptores AT1 e AT2. A Ang IV inibe a o sítio catalítico de IRAP e prolonga a meia vida da ocitocina (Lew *et al.*, 2003), como por exemplo nas amídalas cerebrais (Beyer *et al.*, 2010), na qual é essencial para o adequado funcionamento da memória e do aprendizado (Popik *et al.*, 1992; Arletti *et al.*, 1995; Popik *et al.*, 1996).

No trato reprodutivo de ovelhas (folículo, ovário, oviduto e útero) foi identificado a presença de IRAP, de maneira que o estradiol diminuiu os níveis deste receptor no miométrio (Mustafa *et al.*, 2004). Entretanto, até o momento não há trabalhos demonstrando a presença desta enzima nas células da granulosa e do cumulus e a sua possível interação com a ocitocina durante os processos iniciais de ovulação e retomada da meiose em bovinos.

Nosso grupo demonstrou que a Ang II é essencial para os processos iniciais de ovulação (Ferreira *et al.*, 2007), bem como para a retomada da meiose (Giometti *et al.*, 2005; Stefanello *et al.*, 2006; Barreta *et al.*, 2008). Além disso, demonstramos que após o pico pré-ovulatótio de LH a Ang II utiliza sequencialmente progesterona, ocitocina e prostaglandina para desencadear a retomada da meiose em bovinos (Barreta *et al.*, 2008; Siqueira *et al.*, 2012; De Cesaro *et al.*, 2013).

Um dos fatores indispensáveis para os processos finais de foliculogênese é a ocitocina. Este peptídeo é sintetizado concomitantemente com neurofisina I em bovinos (Schams *et al.*, 1985) e ovinos (Watkins *et al.*, 1984), de maneira que, a expressão de RNAm para

ocitocina/neurofisina I já foi observada nas células da granulosa de bovinos e resultou na síntese e secreção da ocitocina no fluido folicular (Einspanier *et al.*, 1986; Holtorf *et al.*, 1989; Luck *et al.*, 1990). A expressão do receptor de ocitocina também já foi demonstrado na granulosa e nas células do cumulus de bovinos (Furuya *et al.*, 1995; De Cesaro *et al.,* 2013). Após o pico de LH a expressão de ocitocina/neurofisina I nas células da granulosa tem um grande aumento (Jo e Fortune, 2003), sendo sua atuação demonstrada na ovulação (Viggiano *et al.*, 1989), maturação do oócito (Furuya *et al.*, 1995; De Cesaro *et al.*, 2013) e expansão do cumulus (Voss e Fortune, 1992).

Sabendo que a ocitocina e Ang II são fundamentais para a ovulação e retomada da meiose em bovinos, e que a IRAP pode metabolizar e ser inibida pela ocitocina e Ang IV (resíduo da Ang II), respectivamente, é de extrema importância a avaliação deste receptor nesses eventos fisiológicos.

7.2 Resultados complementares

Primeiramente foi realizado um estudo *in vitro* com cultivo primário de células da granulosa bovina isoladas de folículos com diâmetro pré-ovulatório (≥12mm). As células foram cultivadas em uma densidade de $1x10^6$ células viáveis/poço em estufa com condição apropriada (37°C, 5%CO_2 e umidade saturada) por 6 h na ausência de gonadotrofinas (controle negativo) ou na presença de LH ou LH+5μM AG1478 (inibidor do receptor de EGF). Ao final do período de cultivo, as células foram adequadamente coletadas e armazenadas para posterior avaliação dos genes de interesse através de PCR em tempo real. Como resultado, observamos que apesar de estar presente nas células da granulosa, a IRAP não foi regulada neste horário independente do tratamento (Figura 1A), entretanto o LH estimulou a ocitocina (Figura 1B) e o seu receptor (Figura 1C). De modo que o grupo LH+AG1478 não diferiu estatisticamente do controle negativo e nem do LH para a expressão da ocitocina (Figura 1B).

Como forma de comprovar os resultados obtidos *in vitro*, foi realizado epxerimento para coleta de células da granulosa *in vivo*. Para isso, fêmeas bovinas cíclicas de raças europeias foram submetidas a um protocolo de indução da nova onda de crescimento folicular. Após a remoção do pessário intravaginal o crescimento folicular foi monitorado diariamente por ultrassonografia transvaginal. Quando os folículos atingiam o diâmetro de ≥12mm (pré-ovulatório) foi injetado intrafolicularmente solução fisiológica (Cont.) ou

AG1478 (AG), e administrado um análogo de GnRH. Após 6 h desse procedimento os ovários foram coletados por colpotomia e as células da granulosa adequadamente coletadas e armazenadas para posterior avaliação dos genes de interesse através de PCR em tempo real. Nossos resultados *in vivo* comprovaram que a IRAP não é regulada por LH e EGFr nas células da granulosa durante o início do processo ovulatótio em bovino (Figura 2A). Semelhante resultado foi observado para o receptor de ocitocina (Figura 2C). Entretanto, a expressão de ocitocina no grupo AG foi menor que no grupo que no grupo controle (Figura 2B).

Além de ser fundamental para o processo de ovulação, a ocitocina também é indispensável para a expansão das células do cumulus e retomada da meiose em oócitos bovinos. Dessa forma, avaliamos se a IRAP está presente nas células do cumulus e é regulado por gonadotrofinas. Num primeiro momento, realizamos cultivo *in vitro* de CCOs por 12 h, na qual coletamos células do cumulus a cada 3 h. Para isso, utilizamos 2 grupos experimentais: sem gonadotrofinas (TCM) e a associação das gonadotrofinas (FSH+LH). As células do cumulus foram processadas para a avalição de genes de interesse por PCR em tempo real. Como resultado, observamos que ocorre um aumento da expressão de IRAP a partir das 9 h, em comparação com a hora 0, independente de ausência ou presença de gonadotrofinas (Figura 3). Sugerindo que o IRAP poderia ser importante para a regulação da ocitocina nas células do cumulus.

Dessa forma, o próximo passo foi avaliar se o aumento da expressão de IRAP nas células do cumulus, estimulada por gonadotrofinas, era inibido pela presença de metades foliculares e AG1478. Ou seja, avaliar se as gonadotrofinas utilizam o EGFr para regular a expressão de IRAP. Para isso, utilizamos um sistema de maturação com metades foliculares oriundas de folículos de 3 a 5 mm associado a 5μM de AG1478 na presença de FSH e FSH+LH. Dessa forma, CCOs foram cultivados por 0, 6 e 12 h com FSH ou FSH+LH na ausência (C+) ou presença (C-) de metades foliculares ou metades foliculares+AG1478 (AG). Foi coletado as células da granulosa (das metades foliculares) e do cumulus para posterior processamento e avaliação da expressão de genes de interesse, através da técnica de PCR em tempo real. Como resultados, observamos que nas células da granulosa o FSH ou FSH+LH isoladamente ou na presença de AG1478 não alterou a expressão de IRAP dentro de cada horário avaliado (Figura 4). Porém, nas células do cumulus, a associação FSH+LH estimulou a expressão de IRAP com 12 h de cultivo *in vitro*, de modo que, a presença de AG1478 inibiu este aumento (Figura 5).

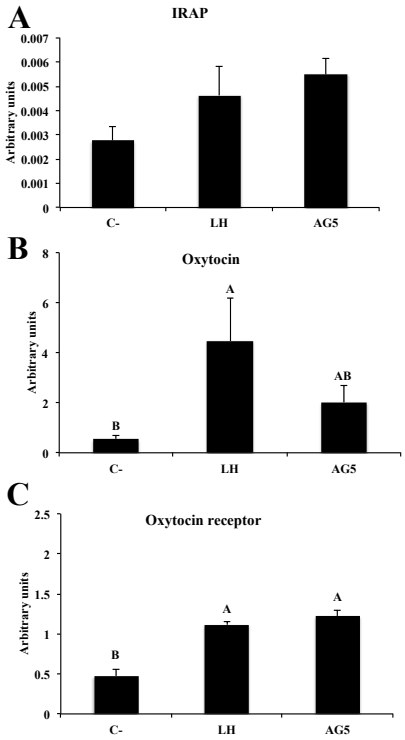


Figura 1. Abundância de mRNA nas células da granulosa bovina cultivadas *in vitro* por 6 h derivadas de folículos com diâmetro pré-ovulatório (≥12mm). (A) IRAP. (B) Ocitocina. (C) Receptor de ocitocina. C-: ausência de gonadotrofina; LH: hormônio luteinizante; AG5: hormônio luteinizante e 5μM de AG1478. Letras diferentes indicam diferença estatística ($P<0,05$).

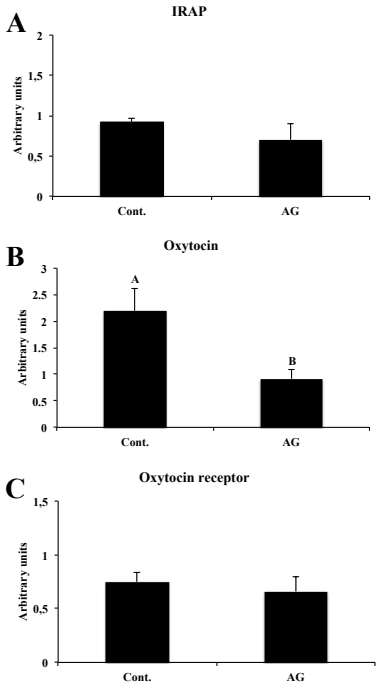


Figura 2. Abundância de mRNA nas células da granulosa bovina derivadas de folículos com diâmetro pré-ovulatório (≥12mm) após 6 h da administração de GnRH *in vivo*. (A) IRAP. (B) Ocitocina. (C) Receptor de ocitocina. Cont: GnRH e solução fisiológica intrafolicular; AG5: GnRH e 5μM de AG1478 intrafolicular. Letras diferentes indicam diferença estatística (P<0,05).

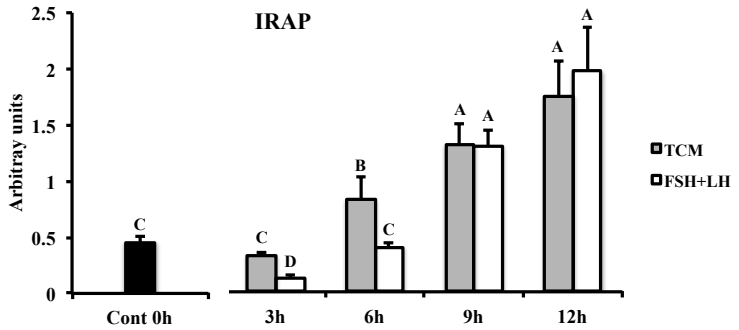


Figura 3. Abundância de mRNA para IRAP nas células do cumulus coletadas a cada 3 h durante 12 h de maturação *in vitro*. TCM: ausência de gonadotrofinas; FSH+LH: presença do hormônio folículo estimulante e hormônio luteinizante. Letras diferentes indicam diferença estatística (P<0,05).

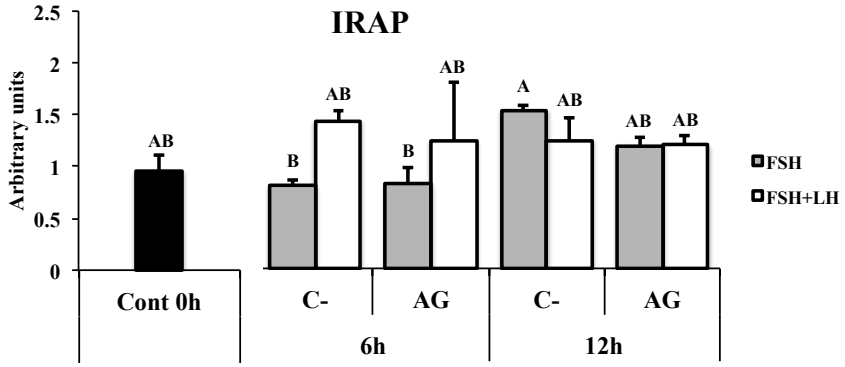


Figura 4. Níveis de mRNA para IRAP em células da granulosa derivadas das metades foliculares co-cultivadas *in vitro* com complexos cumulus-oócito (CCOs) bovino durante 0, 6 e 12 h. FSH+LH: hormônio folículo estimulante e hormônio luteinizante; C-: metades foliculares; AG: metades foliculares e 5μM de AG1478. Letras diferentes indicam diferença estatística (P<0,05).

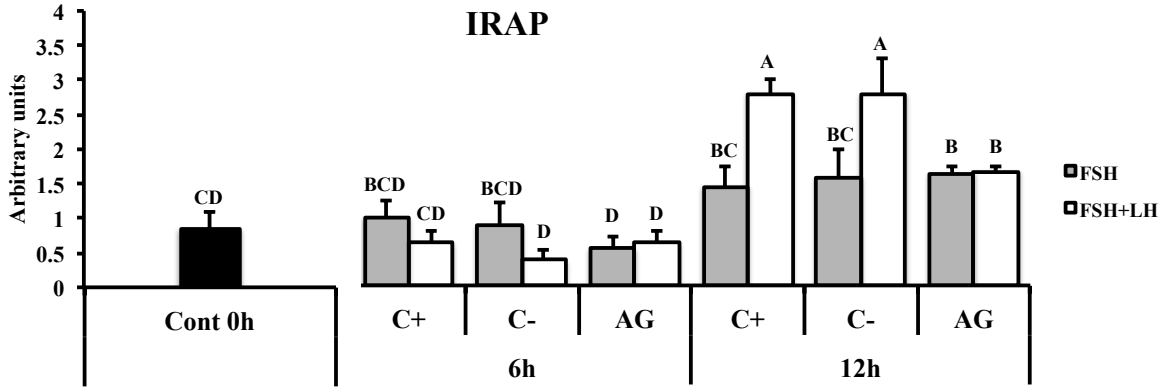


Figura 5. Níveis de mRNA para IRAP em células do cumulus derivadas de complexos cumulus-oócito bovino co-cultivados *in vitro* com metades foliculares durante 0, 6 e 12 h. FSH+ LH: hormônio folículo estimulante; LH: hormônio luteinizante; C+: somente CCOs; C-: metades foliculares; AG: metades foliculares e 5μM de AG1478. Letras diferentes indicam diferença estatística (P<0,05).

8. DISCUSSÃO

A grande importância de fatores locais em momentos críticos da foliculogênese instigam a investigação da sua regulação e atuação durante a fisiologia reprodutiva. Neste contexto, visto a grande importância do sistema peptídeos natriuréticos (NP), observado principalmente em animais com múltiplas ovulações, e a grande carência de conhecimento a este respeito em animais monovular, nos estimularam a avançar na busca pelo conhecimento nesta área. Além disso, mais do que o interesse para a pecuária bovina, o conhecimento acerca da reprodução gerado utilizando a fêmea bovina tem grande possibilidade de ser extrapolado para mulheres. Isso é possível, pois a regulação de eventos reprodutivos da fêmea bovina é considerada mais parecido da humana do que quando comparado a espécies multiovular (Shu *et al.,* 2008; Jaiswal *et al.,* 2009; Ginther, 2012). Dessa forma, utilizando modelos experimentais *in vivo* e *in vitro* bem estabelecidos, investigamos a regulação e função do sistema NP durante a dominância folicular, ovulação, retomada da meiose oocitária e expansão das células do cumulus em bovinos.

Inicialmente, caracterizamos o sistema NP no CCO de bovino e demonstramos que o NPPA e o NPPC aumentam os níveis de cGMP no cumulus e no oócito após 3 horas de cultivo, impedindo o aumento de cAMP no oócito na presença de forskolin. Além disso, através de sequenciamento do mRNA amplificado, confirmamos a homologia para o NPPA, NPPC, NPR-1, NPR-2 e NPR-3 em bovino. Os *primers* utilizados para amplificar esses genes em bovino foram descritos no primeiro artigo desta tese, já publicado (De Cesaro *et al.,* 2015), e usados em todos os experimentos. Dessa forma, permitiram grande confiabilidade na avaliação da expressão gênica.

Utilizando modelo *in vivo* para estudo da dominância folicular, validado pelo nosso laboratório e amplamente aceito pela comunidade científica (Ferreira *et al.,* 2011; Gasperin *et al.,* 2014; Rovani et al., 2014), demonstramos a presença de mRNA para NPR-1, NPR-2 e NPR-3 e ausência de expressão de NPPA, NPPB e NPPC em células da granulosa durante a divergência folicular. Além disso, observamos que a expressão do NPR-3 no folículo dominante, no momento esperado da divergência folicular, foi maior em comparação com o folículo iniciando atresia. No momento posterior, na qual a dominância folicular já estava consolidada, os três receptores NPs apresentavam alta expressão no folículo dominante. Sabendo que o FSH tem fundamental importância para este evento fisiológico, buscamos

compreender se esta gonadotrofina mantem a expressão dos receptores dos NPs nas células da granulosa do maior e segundo maior folículo, ambos folículos saudáveis. Para isso, outro modelo *in vivo* foi utilizado (Ilha *et al.,* 2015; Rovani *et al.,* 2014) e confirmamos que a expressão de NPR-1, NPR-2 e NPR-3 é mantida nas células da granulosa pelo FSH além do momento esperado da divergência folicular. Ou seja, os três receptores dos NPs apresentam alta expressão nas células da granulosa de folículos saudáveis. A próxima pergunta que buscamos responder foi a respeito da regulação da expressão dos receptores dos NPs pelo receptor de estradiol. Para isso, foi realizado injeção intrafolicular de um inibidor do receptor de estradiol (fulvestrant) no momento da divergência folicular e coletado células da granulosa 12 horas após (Ferreira *et al.,* 2011; Barreta *et al.,* 2013; Rovani *et al.,* 2014). Com esse modelo, observamos que somente o NPR-1 apresentou menor expressão de mRNA no grupo com inibição do receptor de estradiol do que no grupo controle.

Com estes resultados, demonstramos pela primeira vez a regulação dos membros do sistema dos NPs durante a divergência folicular em espécie monovular. Mais do que isso, nossos resultados demonstraram grandes diferenças quando comparados com espécies multiovular. Na qual, foi observado a expressão de NPPA e NPPC nas células da granulosa, durante o crescimento folicular, em roedores (Gutkowska *et al.,* 1999; Kawamura *et al.,* 2011; Zhang *et al.,* 2010), do NPPB e NPPC em suínos (Zhang *et al.,* 2015) e do NPPA, NPPB e NPPC em cabras (Peng *et al.,* 2013). Além de ser expresso nas células da granulosa de camundongos, o CNP também é capaz de estimular o crescimento de folículos pré antrais e antrais nesta espécie (Sato *et al.,* 2012). Dessa forma, podemos sugerir que durante a onda folicular, pelo menos em bovinos, o CNP não tem papel fundamental. No que diz respeito a regulação dos receptores dos NPs em espécies multiovular, durante o crescimento folicular, somente foi observado aumento da expressão do NPR-2 por FSH e estradiol (Kawamura *et al.,* 2011; Zhang *et al.,* 2011; Zhang *et al.,* 2015). Porém, nossos resultados sugerem que, nas células da granulosa, durante a divergência folicular, o estradiol não atua diretamente sobre NPR-2 e NPR-3 em bovinos. Além disso, entre os receptores dos NPs presentes nas células da granulosa de bovinos, podemos sugerir que o NPR-3 é o primeiro a ter alta expressão em folículos saudáveis e, de modo contrário, o NPR-1 parece ser mais sensitivo ao processo de atresia folicular, visto que, 12 horas após a indução da atresia (bloqueio dos receptores de estradiol) foi observado diminuição da sua expressão.

Buscando ampliar o conhecimento a respeito do sistema NP nas células da granulosa de bovinos, estudamos a regulação deste sistema durante o processo ovulatório. Utilizando um modelo *in vivo* (Santos *et al.,* 2012; Siqueira *et al.,* 2013) buscamos caracterizar a

expressão gênica dos membros do sistema NP nas células da granulosa de folículos pré-ovulatórios (≥12mm) em diferentes tempos (0, 3, 6, 12 e 24 horas) após a administração de um análogo de GnRH. Primeiramente observamos que a expressão de NPPC aumentou rapidamente (3 horas) após o GnRH. Estando este resultado em divergência ao observado em camundongos (Kawamura *et al.,* 2011; Tsuji *et al.,* 2012; Lee *et al.,* 2013), cabras (Peng *et al.,* 2013) e suínos (Zhang *et al.,* 2014; Zhang *et al.,* 2015), na qual, após o LH ocorre diminuição da expressão do NPPC. Além disso, Yang *et al.* (2016) demonstraram que em cultivo de células da granulosa de bovinos isoladas de folículos entre 2-6mm de diâmetro o LH diminui a expressão de NPPC. Entretanto, além das condições de cultivo *in vitro* apresentarem ausência de interações com outros tipos de células e fatores que ocorrem *in vivo* (Dufour *et al.,* 1999), foi demonstrado que em condições fisiológicas *in vivo*, folículos bovinos com diâmetro ≤8,5mm não são capazes de ovular mesmo quando utilizado doses de LH 10 vezes maiores que a requerida para ovulação de folículos com diâmetro ≥12mm (Sartori *et al.,* 2001). Assim, demonstrando a importância e a segurança dos nossos resultados *in vivo*, e indicando significativas disparidades nos mecanismos de ovulação entre mono e multiovular espécies no que diz respeito ao sistema NP. Para os receptores NP, observamos que após o GnRH ocorreu uma diminuição significativa do NPR-3 com 6 horas, e aumento do NPR-1 com 24 horas. Entretanto, não foi observado regulação para o NPR-2 após o GnRH nas células da granulosa de folículos pré ovulatório em bovinos. Esses resultados são diferentes dos observados em espécies multiovular, na qual, após o LH ocorre diminuição da expressão do NPR-2 nas células da granulosa (Kawamura *et al.,* 2011; Lee *et al.,* 2013; Tsuji *et al.,* 2012; Zhang *et al.,* 2014; Zhang *et al.,* 2015). Dessa forma, permitindo sugerir que a regulação do NPPC/NPR-2 durante o processo ovulatório pode apresenta regulação espécie específica.

Diferente do observado nas células da granulosa durante a divergência em bovinos, na qual observamos somente a presença dos receptores NPs, no período periovulatório foi demontrado a presença de mRNA para os 3 NPs. Dessa forma, as enzimas convertases corin e furin, responsáveis pelo processamento do NPPA (Yan *et al.,* 2000) e NPPC (Wu *et al.,* 2003), respectivamente, em ANP e CNP foram avaliadas. Utilizando o mesmo modelo experimental *in vivo* (0, 3, 6, 12 e 24 horas após GnRH), demonstramos a presença de mRNA para ambas as enzimas convertases nas células da granulosa de folículos pré-ovulatórios de bovinos, de modo que, a abundância de mRNA para furin aumentou após 24 horas do GnRH e a expressão de corin não se alterou durante os momentos avaliados.

Entre as vias de sinalização envolvidas na cascata de eventos celulares que ocorrem após o LH, e desencadeiam o processo ovulatório, o fator de crescimento epidermal (EGF) possui papel central, e é considerado, pelo menos em animais de laboratório, o principal mediador intrafolicular do LH (Park *et al.,* 2004; Conti *et al.,* 2006; Panigone *et al.,* 2008). Aliado a istso, a importância do sistema NP e o conhecimento dos resultados supracitados em bovinos, buscamos compreender a importância do receptor de EGF (EGFr) para a ovulação e sobre regulação do sistema NP induzido pelo GnRH nas células da granulosa de folículos pré ovulatórios em bovinos. Além disso, hipotetizamos que o ANP e o CNP em associação ou não com o LH alteram a expressão de importantes genes presentes na granulosa e sabidamente envolvidos no processo ovulatório. Utilizando somente um horário (6 horas) e o inibidor específico do EGFr (AG1478) em cultivo de células da granulosa (diâmetro ≥12mm) e por injeção intrafolicular em folículos pré ovulatórios, demonstramos que o LH aumenta a expressão de mRNA para o NPPC nas células da granulosa através do EGFr tanto *in vitro* como *in vivo*. Entretanto, a diminuição da expressão do NPR-3, via EGFr, somente foi observado no modelo *in vivo*. Além disso, como já esperado, devido ao conhecimento descrito anteriormente, a expressão de NPR-1 e NPR-2 não foi regulada por LH nem por AG1478 nas células da granulosa de bovinos tanto *in vitro* como *in vivo* após 6 horas. De forma contrária, o LH, através da ativação do EGFr, diminui a expressão de mRNA para NPPC na granulosa (Kawamura et al., 2011; Tsuji et al., 2012) e NPR-2 no cumulus (Robinson et al., 2012; Wang et al., 2013) em camundongos. De forma semelhante, a expressão de NPPB, NPPC e NPR-2 é diminuída pelo LH via EGFr em suínos (Zhang et al., 2014).

A cascata de eventos desencadeada pelo LH, nas células da granulosa, é extremamente complexa e envolve diversos genes. Os resultados descrito até aqui, nesta tese, permitiu-nos investigar a possibilidade do ANP e CNP estarem participando isoladamente ou com o LH na estimulação de genes envolvidos no processo ovulatório nas células da granulosa de bovinos. Para isso, cultivos *in vitro* de células da granulosa de folículos pré ovulatórios foram realizados. Entre os genes estudados (AREG, EREG, AT2, COX2, receptor nuclear de P4) observamos que a associação de LH+ANP aumentou a expressão de mRNA para COX2 quando comparado ao grupo sem gonadadotrofina somente com ANP e somente LH. O aumento da expressão desse gene, nas células da granulosa de bovino em cultivo está diretamente relacionados com o aumento da proteína desta enzima (Portela *et al.,* 2011). De maneira que, COX2 é responsável por produzir prostaglandinas (E2 e F2α) e indispensável para que ocorra a maturação oocitária (Barreta et al., 2008; Siqueira et al., 2012; De Cesaro et al., 2013), expansão das células do cumulus (Davis *et al.,* 1999) e a adequada ovulação (Davis

et al., 1999; Espey *et al.,* 1986; Peters *et al.,* 2004; Sena and Liu, 2008). Apesar de isoladamente o ANP não estimular mRNA para COX2, estes resultados sugerem que este NP é um cofator do LH para o estímulo desta enzima, semelhante os observado por Angiotensina II (Portela *et al.,* 2011).

A ativação do EGFr é indispensável para regular NPPC e NPPR-3 nas células da granulosa de folículos pré ovulatório de bovinos. Além disso, a ativação deste receptor (EGFr) induz a fosforilação de ERK1/2 que é essencial para mediar os efeitos do LH no ambiente folicular, como maturação do oócito, expansão das células do cumulus e ovulação. Porém, sabe-se que em camundongos também existe uma via alternativa e independente de EGFr para esta fosforilação (Duggavathi and Murphy, 2009). Na qual, apesar do *knockout* (Hsieh *et al.,* 2011) ou o inibição (Panigone *et al.,* 2008) do EGFr, o LH parcialmente fosforila ERK1/2. Para entender o que ocorre com a inibição do EGFr sobre a ovulação em bovinos, foi realizado injeção intrafolicular de AG1478 ou solução fisiológica em folículos pré ovulatórios, desafiado com análogo de GnRH e avaliado a ovulação do folículo desses animais. Nesse experimento, observamos que a inibição de EGFr não inibiu a ovulação em bovinos. Com o mesmo modelo, porém utilizando saralasina (inibidor do receptor de angiotensina II), foi demonstrado o bloqueio da ovulação em bovinos (Ferreira *et al.,* 2007). Assim, fortes evidências sugerem uma via secundária ao EGFr para a ocorrência da ovulação, possivelmente fosforilando ERK1/2.

Após a compreensão da regulação do sistema NP durante a divergência e ovulação, e saber que este sistema está presente no complexo-cumulus ooócito (CCO) de bovinos (De Cesaro *et al.,* 2015) passamos a nos questionar a respeito da regulação dos receptores deste sistema e a função do NPR-3 no CCO. Para responder estas questões utilizamos modelos *in vitro*. Semelhante ao observado em células da granulosa de folículos pré ovulatórios, as gonadotrofinas diminuíram a expressão de NPR-3 no cumulus. A abundância de mRNA para o NPR-2 não foi regulada nas células do cumulus de bovinos, e para o NPR-1 foi fracamente detectável. Além disso, demonstramos que tanto o FSH como o FSH+LH diminuem a expressão do NPR-3 via EGFr no cumulus. Nas espécies com múltiplas ovulações, o NPR-2 é amplamente regulado, de maneira que, eCG/FSH, crescimento folicular e fatores derivados do oócito (BMP15, GDF9 e FGF8) mantém ou aumentam a expressão deste receptor nas células do cumulus de camundongos (Zhang *et al.,* 2010; Zhang *et al.,* 2011) e suínos (Hiradate *et al.,* 2013; Zhang *et al.,* 2014; Zhang *et al.,* 2015). Mais do que isso, após o estímulo do LH, através do EGFr, a expressão deste receptor diminui tanto em camundongos (Tsuji *et al.,* 2012; Wang *et al.,* 2013) como em suínos (Zhang *et al.,* 2014; Zhang *et al.,* 2015). Além do

NPR-2 não ser regulado por ganadotrofinas nas células do cumulus de bovino, também demonstramos que este receptor não é regulado por EGFr, metades forlicular e forskolin (estimulador adenilato ciclase). Entretanto, é sabido que a ligação do CNP ao NPR-2 do cumulus inibe a retomada da meiose em camundongos (Zhang *et al.,* 2010), suínos (Hiradate *et al.,* 2013), cabras (Peng *et al.,* 2013), gatas (Zhong *et al.,* 2015) e bovinos (Franciosi *et al.,* 2014), semelhante ao observado nos resultados dessa tese. Assim, devido a grande importância deste receptor, ainda é necessário entender a sua regulação nos processos reprodutivos de espécies monovular.

Apesar do conhecimento que o CNP também se liga os NPR-3 (Suga *et al.,* 1992), ainda é dada pouca importância para este receptor nos eventos reprodutivos. Recentemente foi demonstrado que o NPR-3 aumenta a expressão nas células do cumulus após o LH e não está envolvido com a degradação do CNP em camundongos (Lee *et al.,* 2013). Sendo que, nas células do cumulus de bovinos observamos regulação oposta para este receptor. Sugerindo assim, que o NPR-3 possa estar participando em eventos reprodutivos anteriores ao pico pré ovulatório de LH em bovinos. Baseado nisso, em recentes resultados que mostraram a participação do NPR-3 em auxiliar o bloqueio da meiose em suínos (Santiquet *et al.,* 2014), e que em células da musculares lisas do sistema circulatório de camundongos com hipertensão quando o NPR-3 é ativado ocorre controle da pressão arterial através da atenuação da fosforilação de ERK1/2 e AKT (El Andalousi *et al.,* 2013), buscamos entender se o NPR-3 atua na maturação e/ou expansão das células do cumulus em bovinos. Dessa forma, demonstramos que a ativação do NPR-3, por um agonista específico ($cANP^{4-23}$), inibiu a completa expansão das células do cumulus de bovinos estimulada por FSH+LH, porém, sem alterar a retomada da meiose. Assim, demonstrando pela primeira vez uma evidência direta da função do NPR-3 em eventos reprodutivos de fêmeas monovular. Além disso, quando associamos $cANP^{4-23}$ e CNP observamos uma potencialização da inibição da expansão das células do cumulus estimulada por gonadotrofinas em bovinos. É importante destacar que também observamos inibição da retomada da meiose no grupo com CNP, semelhante ao descrito por Franciosi *et al.* (2014), porém, a associação deste NP e do agonista do NPR-3 não apresentou maior taxa ou aumento do tempo de inibição da retomada da meiose que o observado para o CNP isoladamente. No CCO de bovinos, foi demonstrado que o CNP, além de atrasar a retomada da meiose, também mantem funcional as comunicações tipo *gap* (Franciosi *et al.,* 2014), e que em camundongos as TZP apresentam maior densidade nos CCOs tratados com CNP quando comparados com cilostamide (inibidor PDE3) após 48 horas de cultivo *in vitro* (Romero *et al.,* 2016). Dessa forma, esses resultados oferecem perspectivas

promissoras que a diminuição da expansão das células do cumulus pelo CNP+cANP$^{4-23}$ mantem as comunicações tipo gap e as TZP funcionais nos CCOs de bovinos. Consequentemente, podendo ser um mecanismo importante da maturação oocitária inerente da comunicação célula-célula e possibilita o entendimento de possíveis problemas de fertilidade decorrentes da falha de expansão das células do cumulus.

9. CONCLUSÃO

Os resultados em conjunto, demonstram a caracterização e regulação do sistema peptídeos natriuréticos (NP) nas células da granulosa durante a divergência folicular e ovulação *in vivo*, no CCO e também nas células do cumulus durante a maturação oocitária e expansão das células do cumulus em bovinos. Além disso, foi demonstrado que o NPPA e o NPPC aumentam os níveis de cGMP no cumulus e no oócito após 3 horas de cultivo, impedindo o aumento de cAMP no oócito na presença de forskolin.

Apesar de não ser detectado mRNA para nenhum NP na granulosa de folículos durante a divergência folicular, há grande abundância de mRNA para os receptores NPs nos folículos dominates. Nas células da granulosa de folículos pré ovulatório *in vivo*, caracterizamos a presença de mRNA para NPPA, NPPB, NPR-1, NPR-2 e NPR-3 além de corin e furin. De maneira que, após o pico de LH, através do EGFr, ocorre rápido aumento da expressão de NPPC e diminuição de NPR-3. Contudo, a ovulação em bovinos pode ocorrer independente da ativação de EGFr. Além disso, o ANP parece ser um cofator do LH para o estímulo de mRNA para COX2 nas células da granulosa de folículos pré ovulatório de bovinos.

Nas células do cumulus de bovinos tanto o FSH como FSH+LH diminuem a expressão do NPR-3, via EGFr. Entretanto, o mRNA para NPR-2 não foi regulado. Além disso, a ativação do NPR-3 do cumulus por um agonista específico ($cANP^{4-23}$) não interfere na maturação nuclear oocitária em bovinos, porém, inibe a completa expansão das células do cumulus estimulada por FSH+LH, sendo que, a associação do $cANP^{4-23}$ com CNP potencializa este efeito.

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