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PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA VETERINÁRIA

CARACTERIZAÇÃO DO SISTEMA CALICREÍNA-CININA DURANTE O PROCESSO OVULATÓRIO DE BOVINOS

DISSERTAÇÃO DE MESTRADO

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Santa Maria, RS, Brasil

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CARACTERIZAÇÃO DO SISTEMA CALICREÍNA-CININA DURANTE O PROCESSO OVULATÓRIO DE BOVINOS

por

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Dissertação apresentada ao Curso de Mestrado do Programa de Pós-Graduação em Medicina Veterinária, Área de Concentração em Fisiopatologia da Reprodução, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de
Mestre em Medicina Veterinária.

Orientador: Prof. João Francisco Coelho de Oliveira

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como requisito parcial para obtenção do grau de
Mestre em Medicina Veterinária

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RESUMO

Dissertação de Mestrado
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CARACTERIZAÇÃO DO SISTEMA CALICREÍNA-CININA DURANTE O PROCESSO OVULATÓRIO DE BOVINOS

AUTOR: GUSTAVO FREITAS ILHA
ORIENTADOR: JOÃO FRANCISCO COELHO DE OLIVEIRA
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O sistema calicreína-cinina (KKS) tem sido descrito como um importante mediador de processos fisiológicos. Calicreínas utilizam o cininogênio (KNG) como substrato para formar a bradicinina, que é o principal peptídeo ativo do KKS o qual atua através de dois tipos de receptores, o B_1R e B_2R. O objetivo deste estudo foi caracterizar os principais componentes do KKS em diferentes compartimentos ovarianos durante o processo ovulatório de bovinos. A expressão de RNAm de KNG, B_1R e B_2R foi mensurada em células da teca e granulosa, e concentração de bradicinina e atividade de calicreína no fluido folicular de folículos peri-ovulatórios bovinos. Para obter um folículo peri-ovulatório (≥ 12mm), vinte e sete vacas foram submetidas a um protocolo de sincronização de cios e ovariectomizadas por colpotomia 0, 3, 6, 12 ou 24 horas após uma injeção de um análogo ao GnRH (gonadorelina; 100 μg, IM). O fluido folicular foi aspirado para os ensaios enzimáticos e as células da teca e granulosa dissecadas para análise do RNAm. A expressão do RNAm em células foliculares foi avaliada por PCR em tempo real e os dados representados em relação ao gene constitutivo ciclofilina. A concentração de bradicinina e atividade de calicreína foram mensuradas no fluido follicular por imunoensaio enzimático e clivagem de substrato seletivo, respectivamente, e sua absorbância mensurada por leitor de placas. A expressão de RNAm para o KNG não variou em ambos os tipos celulares nos diferentes tempos ($P>0,05$), enquanto que para B_2R a expressão em células da teca e expressão para B_1R nas células da teca e granulosa apresentaram diferentes padrões durante o período peri-ovulatório ($P<0,05$). A concentração de bradicinina e a atividade de calicreína no fluido follicular foram diferentes ($P<0,05$) de acordo com o tempo durante o processo ovulatório. Estes resultados demonstram que o KKS está presente e há indicativos de sua regulação durante a ovulação em bovinos.

Palavras-chave: ovulação; sistema calicreína-cinina; bradicinina; ovário; bovino.

ABSTRACT

Master Course Dissertation
Professional Graduation Program in Veterinary Medicine
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CHARACTERIZATION OF KALLIKREIN-KININ SYSTEM DURING THE OVULATION PROCESS IN BOVINE
AUTHOR: GUSTAVO FREITAS ILHA
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The kallikrein-kinin system (KKS) has been described as an important mediator of physiologic processes. Kallikreins use kininogen (KNG) as substrate to generate bradykinin, the principal active peptide of the KKS which acts through two types of receptors, the B_1R and B_2R. The objective of this study was to characterize some components of KKS in different compartments of ovary during the ovulation process in bovine. mRNA expression pattern of KNG, B_1R and B_2R was assessed in theca and granulosa cells and bradykinin concentration and kallikrein-like activity in follicular fluid of bovine peri-ovulatory follicles. In order to obtain a peri-ovulatory follicle (≥ 12mm), twenty-seven cows were submitted to estrus synchronization protocol and ovariectomized by colpotomy at 0, 3, 6, 12 or 24 hours after a GnRH-analog injection (gonadorelin; 100 μg, IM). Follicular fluid was aspirated for enzymatic assays and granulosa and theca cells were harvested for mRNA analysis. The mRNA expressions in follicular cells were evaluated by real-time RT-PCR and data represented as relative to housekeeping gene cyclophilin. Bradykinin concentration and Kallikrein-like activity was measured in follicular fluid by enzymatic immunoassay and selective substrate cleavage, respectively, and the absorbance measured using a plate reader. KNG mRNA expression was similar for both follicular cell types ($P>0.05$), while B_2R expression in theca cells and B_1R expression in theca and granulosa cells showed different profiles during peri-ovulatory period ($P<0.05$). Bradykinin concentration and kallikrein-like activity in follicular fluid were different ($P<0.05$) according the time during ovulation process. The results provide an important characterization of the presence and possible regulation of KKS during ovulation in bovine.

Key words: ovulation; kallikrein-kinin system; bradykinin; ovary; bovine.

LISTA DE FIGURAS

CAPÍTULO - 1

SUMÁRIO

1. INTRODUÇÃO

A liberação de uma célula germinal feminina, o óvulo, pelo ovário é um evento chave na reprodução dos mamíferos (RICHARDS et al., 2002). Esse processo, chamado de ovulação, é o somatório de processos seqüenciais que ocorrem simultaneamente em vários microambientes do ovário levando à ruptura do folículo culminando com a liberação do oócito maturo (RICHARDS et al., 2002). Esse evento tem sido comparado a uma resposta inflamatória devido a evidencias de que folículos ovulatórios possuem eventos similares a tecidos inflamados (ESPEY, 1980). A sequência de eventos que ocorrem durante esse processo inicia no folículo ovulatório após o pico do hormônio luteinizante (LH), o qual atua tanto nas células da teca quanto nas células da granulosa ativando diferentes cascatas e sinalização (RICHARDS et al., 2002). Tais cascatas induzem a transcrição de genes específicos, os quais são expressos antes da ruptura do folículo (ESPEY, 1980; RICHARDS et al., 2002). No entanto, fatores intrafoliculares que iniciam e controlam o processo ovulatório ainda não estão totalmente esclarecidos.

O sistema calicreína-cinina (KKS) possui uma variedade de funções fisiológicas (BHOOLA et al., 1992), incluindo a participação no processo inflamatório em muitos tecidos (CLEMENTS et al., 1997). A bradicinina é a principal cinina mediadora da função do KKS nos diferentes tecidos. É gerada a partir da quebra do substrato primário, o cininogênio de alto peso molecular, pela enzima calicreína plasmática (BHOOLA et al., 1992). Os efeitos do KKS ocorrem via dois tipos de receptores que são o B_1R e o B_2R, embora a afinidade da bradicinina pelos receptores B_1R seja muito pequena, se comparada à afinidade pelos receptores B_2R (BHOOLA et al., 1992; MARCEAU et al., 1998).

A bradicinina é degradada rapidamente *in vivo*, com meia vida em torno de 16 segundos (BALLDIN et al., 1980), sua degradação é feita principalmente pela enzima conversora de angiotensina (ACE), também chamada de cininase II (MOREAU et al., 2005). Nosso grupo de pesquisa tem estudado o sistema renina-angiotensina (RAS), no qual a angiotensina II foi relacionada com uma série de eventos que controlam e desencadeiam as funções reprodutivas nas fêmeas, como desenvolvimento folicular, ovulação e maturação oocitária (FERREIRA et al., 2007; BARRETA et al., 2008; PORTELA et al., 2008). A ACE, responsável pela quebra da angiotensina I em angiotensina II é a principal responsável pela interação entre o RAS e KKS (CAMPBELL, 2003; SCHMAIER, 2003; SHEN & EL-DAHR, 2006). Assim, torna-se importante o estudo da participação do KKS na fisiologia reprodutiva, bem como a sua relação com o RAS nestes eventos fisiológicos. Melhores detalhes sobre a

participação do KKS estão sendo realizados *in vivo* por nosso grupo afim de melhor explicar a regulação desse sistema durante o processo ovulatório de bovinos, e posteriormente estudar sua relação com o RAS.

Baseado na idéia de que o processo ovulatório é relacionado a uma reação inflamatória, o envolvimento do KKS na ovulação tem sido estudado em espécies de ovulação múltipla (KIMURA et al., 2001). Estudos prévios nessa linha de pesquisa mostraram um aumento da atividade do KKS durante a ovulação em algumas espécies, tais como, roedores e suínos (SMITH & PERKS, 1983; ESPEY et al., 1986; GAO et al., 1992). Apesar das evidencias da participação do KKS em ovários de mamíferos, pouco é sabido sobre a distribuição celular e regulação dos componentes desse sistema em ovários monovulatórios. A partir disso, a proposta deste estudo foi determinar a distribuição temporal, nos diferentes compartimentos ovarianos, dos principais componentes do KKS durante o processo ovulatório de bovinos.

2. REVISÃO BIBLIOGRÁFICA

2.1 - Ovulação

O desenvolvimento do folículo e ovulação são controlados por dois tipos de hormônios pituitários, o hormônio folículo estimulante (FSH) e o LH (ESPEY, 1980). Em *bos taurus*, somente folículos com diâmetro ≥ 12mm atingem concentrações adequadas de receptores de LH (LHR) (SARTORI et al., 2001). Esses folículos são chamados de pré-ovulatórios, os quais, a partir do pico de LH sofrem uma série de eventos regulatórios (RICHARDS, 1994). Entende-se por período peri-ovulatório o tempo entre o pico de LH e a ovulação, caracterizando-se pelas mudanças morfológicas, bioquímicas e moleculares que culminam com a liberação do oócito maturo (RICHARDS & PANGAS, 2010). A ligação do LH aos LHR, os quais são ligados a proteína G, nas células da teca e granulosa, ativam a adenilil ciclase que catalisa a formação de AMPc (Adenosina monofosfato cíclica) levando a ativação transitória da proteína cinase A e assim dando início à cascata de ovulação. Juntamente com esse evento inicia-se o processo de luteinização, mas esses são funcionalmente dissociados (ESPEY, 1980; RICHARDS et al., 2002). A partir do pico de LH, o oócito inicia sua competência a reiniciar a meiose, e os andrógenos sintetizados nas células da teca são transportados para as células da granulosa onde a enzima aromatase converte-os para 17b-estradiol (ESPEY, 1980).

Com o pico de LH termina a expressão de genes associados com a foliculogênese e os padrões de sinalização rapidamente induzem a transcrição de genes ovulatórios específicos. Esses genes são expressos antes da ruptura do folículo (SIROTKIN, 2010) e iniciam ou alteram as cascatas de sinalização celulares causando ruptura folicular e promovendo o remodelamento folicular para formação do corpo lúteo (RICHARDS et al., 2002). Muitos eventos são espacialmente restritos a microambientes específicos dentro ou ao redor do folículo para permitir com sucesso a expulsão do complexo cumulus-oócito a partir do rompimento do folículo (DUGGAVATHI & MURPHY, 2009).

Os três principais grupos bioquímicos estudados durante o processo ovulatório são as rotas da progesterona, das prostaglandinas e das proteases (ESPEY, 1980). A progesterona foi sugerida também como mediadora da ovulação (FORTUNE et al., 2009). No ovário, o pico de LH rapidamente e seletivamente induz receptores de progesterona (PR) nas células da granulosa de folículos pré-ovulatórios (NATRAJ & RICHARDS, 1993). De outro modo, a deficiência desses receptores reduz a expressão de fatores de transcrição induzidos por

hipóxia (HIFs), os quais aumentam antes da ovulação e estão envolvidos tanto nesse processo quanto na formação do corpo lúteo (SIROTKIN, 2010). Da mesma forma, o bloqueio dos HIFs pode prevenir o efeito das gonadotrofinas na ovulação (KIM et al., 2009).

As proteases envolvidas no processo ovulatório mais estudadas são as enzimas ativadoras do plasminogênio (PAP), as metaloproteinases de matriz (MMPs) e ADAMTS -1 (A Disintegrin And Metalloproteinase with Thrombo Spondin motifs), esses são importantes para a ovulação, mas ainda estão sendo estudados (ROBKER et al., 2000). MMPs incluem uma variedade de subtipos que possuem expressão diferente de acordo com o tipo celular, teca ou granulosa (RICHARDS et al., 2002).

É sabido que o pico de LH pode estimular as proteínas cinases A B, e RAS e isso parece ser crucial para a ovulação (FAN et al., 2009) além de aumentar níveis de STAR e enzimas esteroidogênicas (CYP11A1, CYP17, HSD3B) e expressão de LHR (YOUNG & MCNEILLY, 2010). Em células da granulosa o LH também induz a expressão de fatores de crescimento associados ao fator de crescimento epidermal (EGF) como anfirregulina, epirregulina e β-celulina, que se ligam aos receptores EGF nessas células e induzem a expressão dos genes da cascata de produção de prostaglandinas (SHIMADA et al., 2006).

Células da granulosa do folículo pré-ovulatorio respondem ao FSH, estradiol e IGF-1. São altamente proliferativas e expressam genes específicos que regulam o ciclo celular como, por exemplo, ciclina D2 (RICHARDS, 1994). Essas células da granulosa se diferenciam em resposta a esses hormônios em adquirirem a habilidade de sintetizar estradiol via enzima aromatase e ao responder ao LH. A sinalização ocorre através de receptores nucleares (DUGGAVATHI et al., 2008) que ativam AMPc e os membros da família EGF (PANIGONE et al., 2008). Apesar de haver uma grande densidade de receptores nas células da teca, o processo de ovulação só ocorre quando as células da granulosa adquirem receptores para LH (RICHARDS, 1994). Esse induz a transcrição de vários fatores nas células da granulosa, entre eles o fator I de regulação do crescimento (Egr -1), o CAAT regulador de ligação da proteína beta (C/EBPβ) e o PR (NATRAJ & RICHARDS, 1993; RICHARDS & PANGAS, 2010). Proteínas cinases ativadoras de mitose (MAPK) intracelulares são padrões de sinalização em células da granulosa relacionadas com a ovulação, principalmente ERK 1 e ERK 2 são induzidas por LH e consequentemente relacionadas a ruptura do folículo e formação do corpo lúteo (FAN et al., 2008). De outra forma, a interleucina 6 pode mediar alguns processos a partir da ligação dos receptores de progesterona nas células da granulosa e as citocinas também são produzidas por células ovarianas durante a ovulação, mas isto ainda precisa ser melhor elucidado (RICHARDS & PANGAS, 2010).

Apesar das células da teca apresentarem um padrão de expressão de genes importantes para a ovulação, uma significante função a elas ainda não está claramente elucidada. Se a ovulação é de fato relacionada com inflamação, talvez a camada celular da teca possua um papel adicional, como por exemplo, uma barreira impedindo a liberação inapropriada do oócito ou para sua proteção (RICHARDS et al., 2002). Antes da ruptura do folículo deve ocorrer a digestão da matriz extracelular (MEC) nas camadas da teca e túnica albugínea na superfície do ovário (ESPEY, 1980; RICHARDS et al., 2002), processo no qual a protease catepsina L pode estar envolvida (ESPEY, 1980).

A presença das prostaglandinas no processo ovulatório leva a idéia que este processo seja parecido com uma resposta inflamatória. As prostaglandinas são produzidas a partir do ácido aracdônico pela ação das cicloxigenases (COX) 1 e 2 (SILVA et al., 2000). A COX-1 expressa em muitos tecidos de forma constitutiva (DEWITT & SMITH, 1995) enquanto COX-2 é uma enzima induzível relacionada a processos patofisiológicos (HINZ & BRUNE, 2002). Gonadotrofinas aumentam a expressão de COX-2 nas células da granulosa, mas não há mudança alguma na expressão de COX-1 (RICHARDS, 1997). Em bovinos a expressão de RNAm e proteína para COX-2 tem sido demonstrada em células da granulosa de folículos pré-ovulatórios (LIU et al., 1997; LIU & SIROIS, 1998). Após o pico de LH, o aumento na expressão de COX-2 está associado com a elevação nas concentrações das prostaglandinas no fluído folicular (SIROIS, 1994; LIU et al., 1997), assim como uma maior expressão do receptor para PGF2α, nas células foliculares bovinas (BRIDGES & FORTUNE, 2007).

Devido ao fato de que o processo ovulatório seja semelhante a uma resposta inflamatória, alguns grupos de pesquisa estão estudando o papel do KKS nesse evento fisiológico (GAO et al., 1992; HOLLAND et al., 2001; KIMURA et al., 2001; BRANN et al., 2002). Este sistema esta relacionado com o RAS (CAMPBELL, 2003; SCHMAIER, 2003), aumentando sua suspeita de participar desse evento fisiológico, pois a angiotensina II, principal peptídeo do RAS, recentemente foi reconhecida como essencial para a ovulação (FERREIRA et al., 2007).

2.2 - Sistema calicreína-cinina no ovário

O KKS possui uma variedade de funções fisiológicas que incluem vasodilatação, ativação e inativação de proteases, estimulação da síntese de prostaglandinas e contratibilidade da musculatura lisa (BHOOLA et al., 1992). O cininogênio é a proteína precursora das cininas e é clivado pelas cininases para gerar calidina e bradicinina, a principal cinina mediadora das respostas do KKS (BHOOLA et al., 1992; CAMPBELL,

2003). As cininas atuam via dois tipos de receptores, o tipo 1 (B_1R) e o tipo 2 (B_2R) (MARCEAU et al., 1998; LEEB-LUNDBERG et al., 2005). O B_2R normalmente predomina nos tecidos, enquanto o B_1R é induzido pela injúria tecidual (CAMPBELL, 2003).

Existem dois tipos de cininogênio no plasma dos mamíferos, chamados de cininogênio de baixo peso molecular (LMWK) e cininogênio alto peso molecular (HMWK) os quais são estruturalmente parecidos (KITAMURA et al., 1987). Os cininogênios são os únicos precursores do KKS e são codificados por um único gene (CAMPBELL, 2003), localizado no cromossomo 3q26 o qual tem origem a partir de duas duplicações sucessivas do gene primordial do cininogênio (MOREAU et al., 2005). O gene precursor consiste em 11 exons, os primeiros nove codificam a cadeia pesada, enquanto o décimo é responsável pela codificação da bradicinina e a cadeia leve é codificada pelo exon 11. Ambos HMWK e LMWK possuem uma sequência idêntica de aminoácidos que inicia na cadeia pesada (N-terminal) e continua 12 animoácidos além da porção da bradicinina, mas diferencia da porção C-terminal, como um "splicing" alternativo, proporcionando assim as duas metades com cininogênios diferentes de cadeia leve (KITAMURA et al., 1987). Na verdade, as proteínas nativas são produzidas como polipeptídios de cadeia única, e as nomenclaturas da cadeia leve ou pesada se referem à sua ponte dissulfeto gerada a partir da ativação da calicreína (MOREAU et al., 2005). Devido a este fator tem sido indicado que LMWK e HMWK são estruturalmente relacionados (KITAMURA et al., 1987), pois ambos possuem uma sequencia idêntica, que é a cadeia pesada (KITAMURA et al., 1987; MOREAU et al., 2005).

As cininogenases, as quais são proteases serinas que usam os cininogênios como substrato para gerar bradicinina e calidina, são membros de uma família multigênica em muitas espécies (CLEMENTS et al., 1997). Esta família de enzimas está envolvida em diversas respostas biológicas (HOLLAND et al., 2001), sendo estudadas tanto como biomarcadores quanto para modelos de regulação hormonal (LAWRENCE et al., 2010). Em humanos, calicreína plasmática forma bradicinina a partir do HMWK enquanto a calicreína tecidual forma calidina do LMWK (BHOOLA et al., 1992). Em contraste, tanto a calicreína plasmática quanto a tedicual gera bradicinina em roedores, que pode também ser gerada pela clivagem da calidina mediada por aminopeptidase (CAMPBELL, 2003).

A pré-calicreína e a calicreína estão presentes em células da granulosa de folículos imaturos e maturos em bovinos (PLENDL et al., 2002). O padrão da atividade de calicreína e de genes que a codificam é maior após o pico de LH e diminui até o momento que se aproxima da ovulação em ratos (GAO et al., 1992; HOLLAND et al., 2001). Esses diferentes padrões de expressão suportam uma diferente função para as calicreínas durante o processo

ovulatório (HOLLAND et al., 2001). Embora os genes que codificam calicreínas tem se mostrado como hormônio responsivos ainda não é claro que esses genes sejam metas específicas e diretas para a atuação desses hormônios (LAWRENCE et al., 2010). Portanto, mais estudos na regulação das calicreínas devem esclarecer os pontos entre a regulação hormonal e expressão de calicreínas (HOLLAND et al., 2001).

Bradicinina é um nonapeptídeo, da família das cininas, potente mediador da maioria das respostas do sistema calicreína-cininas (BHOOLA et al., 1992; CAMPBELL, 2003). Esta cinina foi relacionada com a indução da ovulação em coelhos (YOSHIMURA et al., 1988) e ratas (HELLBERG et al., 1991) além de potencializar a ação do LH nesta espécie (BRANNSTROM & HELLBERG, 1989). Existem evidências em que a bradicinina está envolvida na contração da parede folicular durante a ovulação em humanos (HELLBERG & NORSTROM, 1990). Além disso, estimula parcialmente a maturação nuclear do oócito em ratas, porém o mecanismo é desconhecido (HELLBERG et al., 1991). A bradicinina também pode ser um importante mediador de sinais esteróides no hipotálamo com função na liberação de LH pela hipófise (SHI et al., 1998).

Outros estudos indicaram que a bradicinina e um produto de sua degradação, a bradicinina 1-5, estão presentes nos folículos nos ovários de fêmeas suínas, sugerindo a importância da bradicinina no desenvolvimento folicular e ovulação (KIHARA et al., 2000). Essa cinina aumenta a expressão de MMPs em células da granulosa de suínos e esse pode ser o mecanismo utilizado pelo peptídeo na indução da ovulação nessa espécie (KIMURA et al., 2001).

A bradicinina é degradada rapidamente *in vivo*, com meia vida em torno de 16 segundos (BALLDIN et al., 1980). Várias cininases capazes de metabolizar as cininas tem sido estudadas (ERDOS & DEDDISH, 2002), entre elas a enzima conversora de angiotensina (ACE I), a aminopeptidase P, endopeptidase neutra (NEP) e as carboxipeptidases M e N (MOREAU et al., 2005). Estes estão presentes na forma solúvel em fluidos biológicos e dependem da espécie animal, abordagem, meio biológico e contexto fisiopatológico (ERDOS & DEDDISH, 2002).

Os efeitos do KKS ocorrem via dois tipos de receptores que são o B_1R e o B_2R, embora a afinidade da bradicinina pelos receptores B_1R seja muito pequena, se comparada à afinidade pelos receptores B_2R (BHOOLA et al., 1992; MARCEAU et al., 1998). O B_1R media a ação da des-Arg^9-BK e Lys-des-Arg^9-BK, que são os metabólitos bioativos formados através das ações das carboxipeptidases sobre a bradicinina e calidina, respectivamente (LEEB-LUNDBERG et al., 2005). Esses receptores estão ligados a proteína G e possuem

padrão distinto de expressão nos tecidos, pois, enquanto os receptores B_1R são induzidos por inflamação ou dano tecidual, os receptores B_2R são constitutivamente expressos em vários locais, como células da musculatura lisa e células endoteliais e são os responsáveis pelas principais ações do KKS (ABADIR et al., 2003).

Em eventos reprodutivos, o B_1R foi somente demonstrado em células de ovários de hamsters (AUSTIN et al., 1997). Por outro lado, muitos pesquisadores tem estudado os receptores B_2R (KIHARA et al., 2000; KIMURA et al., 2001; PLENDL et al., 2002; OHKURA et al., 2003; SINGH et al., 2010). A localização dos receptores B_2R em ovário suíno por hibridização e imunohistoquímica já foi descrita (KIMURA et al., 2001) e a sua expressão nas células da granulosa é diferente de acordo com a espécie (OHKURA et al., 2003). Esses pesquisadores indicaram que o nível de receptores B_2R no ovário de ratas é razoavelmente constante, e que o efeito biológico da bradicinina nesse órgão pode ser dependente de concentrações dos ligantes produzidos pelo KKS.

Devido às evidências do papel do KKS em ovaries de mamíferos, e ao fato de que pouco é sabido sobre a regulação dos seus componentes nos distintos compartimentos ovarianos e em ovários monovolatórios. Torna-se importante o conhecimento do papel deste sistema durante o processo ovulatório ajudando a um melhor entendimento das funções fisiológicas, aplicação em biotecnologias da reprodução e no tratamento de infertilidade. A proposta do presente estudo foi caracterizar a presenca e regulação dos principais componentes do KKS durante o processo ovulatório de bovinos.

3. CAPÍTULO 1

TRABALHO ENVIADO PARA PUBLICAÇÃO:

CHARACTERIZATION OF KALLIKREIN-KININ SYSTEM DURING THE OVULATION PROCESS IN BOVINE

Gustavo Freitas Ilha, Joabel Tonellotto dos Santos, Alisson Minozzo da Silveira, Karina Gutierrez, Camila de Campos Velho Gewehr, Sara Marchesan, Juliano Ferreira, Paulo Bayard Dias Gonçalves, João Francisco Coelho de Oliveira

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CHARACTERIZATION OF KALLIKREIN-KININ SYSTEM DURING THE OVULATION PROCESS IN BOVINE

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Abstract

The kallikrein-kinin system (KKS) has been described as an important mediator of physiologic processes. Kallikreins use kininogen (KNG) as substrate to generate bradykinin, the principal active peptide of the KKS which acts through two types of receptors, the B_1R and B_2R. The objective of this study was to characterize some components of KKS in different compartments of ovary during the ovulation process in bovine. mRNA expression pattern of KNG, B_1R and B_2R was assessed in theca and granulosa cells and bradykinin concentration and kallikrein-like activity in follicular fluid of bovine peri-ovulatory follicles. In order to obtain a peri-ovulatory follicle (≥ 12mm), twenty-seven cows were submitted to estrus synchronization protocol and ovariectomized by colpotomy at 0, 3, 6, 12 or 24 hours after a GnRH-analog injection (gonadorelin; 100 μg, IM). Follicular fluid was aspirated for enzymatic assays and granulosa and theca cells were harvested for mRNA analysis. The mRNA expressions in follicular cells were evaluated by real-time RT-PCR and data represented as relative to housekeeping gene cyclophilin. Bradykinin concentration and Kallikrein-like activity was measured in follicular fluid by enzymatic immunoassay and selective substrate cleavage, respectively, and the absorbance measured using a plate reader. KNG mRNA expression was similar for both follicular cell types ($P>0.05$), while B_2R expression in theca cells and B_1R expression in theca and granulosa cells showed different profiles during peri-ovulatory period ($P<0.05$). Bradykinin concentration and kallikrein-like activity in follicular fluid were different ($P<0.05$) according the time during ovulation process. The results provide an important characterization of the presence and possible regulation of KKS during ovulation in bovine.

Key words: ovulation, kallikrein-kinin system, bradykinin, ovary, bovine.

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1. Introduction

The ovulation is characterized as a sequence of events that occur in a responsive preovulatory follicle after a surge of luteinizing hormone (LH) [12, 31]. This event is controlled by a complex interaction of factors, on that is including endocrines mechanisms, cellular messengers, proteases, cinases and activating enzymes [12]. The ovulation process, that culminates at the rupture of follicle releasing the oocyte on the surface of the ovary, has been compared to an inflammatory response and involves simultaneously several ovarian microenvironments [31].

The kallicrein-kinin system (KKS) is an important mediator of inflammatory response for acts on vasodilatation, activation and inactivation of proteases, stimulation of prostaglandin biosynthesis, as well as induction of smooth muscle contractility [4, 14, 25]. Kininogen (KNG) is a precursor protein of KKS, plasma kallikrein use KNG as substrate to generate bradykinin while tissue kallikrein liberates kallidin, that is cleaved to bradykinin [4, 11] . Bradykinin is a nonapeptide kinin main mediator of KKS responses [4, 8]. This system acts by two types of receptors, the type 1 (B_1R) and the type 2 receptor (B_2R),

Therefore the ovulation resembles an inflammatory process and the KKS is involved at inflammatory function, this system has been suggested as a possible important mediator of ovulatory process [5, 17-19]. Some groups have been studding this system in other reproductive processes in different species. The role of KKS was already described to participate of gonadotropin hormone secretion [35-36], follicular development [18], embryo implantation and uterine contraction at parturition [26, 39].

Despite the increasing evidences for the role of KKS in the ovaries of mammals, little is known about the regulation of these components at distinct ovarian compartments and in monovulatory ovaries. On the other hand, the intrafollicular factors that initiate and control the ovulatory process are not well understood [13]. Thus, the knowledge of the role of this system during the ovulatory process can allow a better control of physiological function to be applied to reproduction biotechnology and treatment of infertility. The purpose of the present study was characterizes the presence and regulation of some KKS components during the ovulation process in bovine.

2. Material and methods

The procedures performed in this experiment were approved to the Ethics and Animal Welfare, Universidade Federal de Santa Maria, 23081.007716/2010-61 protocol.

2.1. Animals and experimental design

Twenty-seven cyclic beef cows, predominantly hereford and angus, with an average body condition score of 3 (1–5, emaciated to obese) were pre-synchronized to obtain a GnRH (≥12mm; [32]) responsible follicle at beginning of experiment. The protocol utilized was fisrt discribed [13], on day zero was administered estradiol benzoate (EB, 2mg, IM) with progesterone intravaginal device (DIB®, 1g, Intervet/Schering-Plough, Brasil). At day 9, DIB was removed and the animals received PGF2α analog (Cloprostenol, 250 μg, IM, Sincrocio®, Ourofino, Brazil). In females that had ≥12mm pré-ovulatory follicles at Day 10, was administered GnRH analog (Gonadorelin, 100 μg IM, Profertil®, Tortuga, Brazil) and the ovaries were removed 0, 3, 6, 12 e 24 h after GnRH by colpotomy [10].

Immediately after ovariectomy, follicular fluid, granulosa and theca cells were recovery from the largest follicle and stored at -80ºC [7]. The granulosa cells were stored in buffer RLT^{e} (600Ul, Qiagen, Mississauga, Canada) and the theca cells in trizol (600uL, Invitrogen, Brasil), both in cryogenic tubs.

2.2. Nucleic Acid Extraction and Real-Time RT-PCR

Total RNA was extracted using Trizol (theca cells) or silica based protocol (granulosa cells; Qiagen, Mississauga, Canada) according to the manufacturer's instructions and was quantified by absorbance at 260 nm. Total RNA (1 μg) was first treated with 0.2U DNase (Invitrogen, Brazil) at 37°C for 5 minutes to digest any contaminating DNA, followed by heating to 65°C for 3 minutes. The RNA was reverse transcribed (RT) in the presence of 1 μM oligo(dT), primer, 4 U Omniscript RTase (Omniscript RT Kit; Qiagen, Mississauga, Canada), 0.5μM dideoxynucleotide triphosphate (dNTP) mix, and 10 U RNase Inhibitor (Invitrogen, Brazil) in a volume of 20 μL at 37°C for 1 hour. The reaction was terminated by incubation at 93°C for 5 minutes.

Real-time polymerase chain reaction (PCR) was conducted in a Step One Plus instrument (Applied Biosystems, Foster City, Canada) with Platinum SYBR Green qPCR SuperMix (Invitrogen, Brazil) and bovine-specific primers KNG (Initiator *sense*: TTGGCTGTGTGCATCCCATA and *anti-sense*: AGGTGGGAATGACTGGTGTTG); B_2R (Initiator *sense*: TCACCAACATCCTCCTGAACTCT and *anti-sense*:

CGTGGCCTTCCTCTCAGTCT); and B_1R (Initiator *sense*: CTCGACGGCGTCTGAACAC and *anti-sense*: CGGATGTTCTCTGCCCAGAA). Common thermal cycling parameters (3 minutes at 95°C, 40 cycles of 15 seconds at 95°C, 30 seconds at 60°C, and 30 seconds at 72°C) were used to amplify each transcript. Melting-curve analyses were performed to verify product identity. Samples were run in duplicate and were expressed relative to Cyclophilin as housekeeping gene. The relative quantification of gene expression across treatments was evaluated using the ddCT method [23]. Briefly, the dCT is calculated as the difference between the CT of the investigated gene and the CT of Cyclophilin in each sample. The ddCT of each investigated gene is calculated as the difference between the dCT in each treated sample and the dCT of the sample with lower gene expression (higher dCT). The fold change in relative mRNA concentrations was calculated using the formula 2^{-ddCT}. Bovine-specific primers were taken from literature or designed using Primer Express Software v3.0 (Applied Biosystems, USA) and synthesized by Invitrogen, Canada. The cross-contamination in granulosa and theca cells was tested by Real time PCR by deletion of mRNA that encodes P450 aromatase (CYP19A1) and 17α-hydroxylase (CYP17A1), respectively. Granulosa cells that expressed CYP17A1 and theca cells that expressed CYP19A1 were discarded [7].

2.3. Kallikrein-like activity in follicular fluid

The activity of a tissue kallikrein-like enzyme was measured on the selective peptide-nitroanilide substrate D-Val-Leu-Arg-paranitroaniline (D-Val-Leu-Arg-pNA, dissolved in ultrapure water to a concentration of 1.5 mM and stored at 4°C). The method used for measurement of tissue kallikrein as previously described [30] with some modifications. To determine the kallikrein activity, 25µl of follicular fluid were added in 50µl of assay buffer (0.2M Tris-HCl, pH 8.2, with 0.01M EDTA) and 25µl of substrate (D-Val-Leu-Arg-pNA, 0.375mM) in 96 wells micro plates. The incubation mixtures were maintained at 37 ºC and the reaction was followed measuring the absorbance at 405 nm (measured in a Plate reader, Bioteck, USA), from zero until 100 minutes (0, 3, 10, 20, 30, 40, 50, 60, 70, 80, 90 e 100 minutes). For each experiment, we carried out a standard curve with the product generated by reaction (p-nitroaniline, 0.003-3 mM).

2.4. Estimation of kinin levels in follicular fluid

The bradykinin enzyme was measured by enzyme immunoassays used a high-sensitivity kit for BK (Bachem, USA) according to the manufacturer's protocol. Briefly, 50 µl of standard solutions or test samples were added to immunoplate multiwells, 25 µl of each primary antiserum and biotinylated peptide solution were then added, and the plates were

incubated for 2h at room temperature with mild agitation. The plates were then washed five times, and 100µl of diluted streptavidin-conjugated horseradish peroxidase solution was added to each well. After a 60min incubation at room temperature, the immunoplates were washed five times, and 100 µl of 3,3',5,5'-tetramethyl benzidine dihydrochloride (TMB) solution was added to each well. After a further 30min incubation at room temperature, the reaction was stopped with 100 µl of 2 N HCl. Absorbance was read at 450nm (measured in a Plate reader, Bioteck, USA), using 100 µl of TMB solution and 100 µl 2 N HCl as a blank control.

2.5. Statistical analysis

The differences on continuous data between hours during the ovulation process were accessed by paired Student's T test using cow as subject. Data were tested for normal distribution using Shapiro-Wilk test and normalized when necessary. All analyses were performed using JMP software (SAS Institute Inc., Cary, USA) and a $P<0.05$ was considered statistically significant. Data are presented as means ± sem.

3. Results

3.1. The ovulation process model

This experimental design aimed to recovery peri-ovulatory follicles in different periods of ovulation process. For this, the cows were ovaryectomized 0, 3, 6, 12 and 24 hours after LH peak induction and follicular cells, granulosa and theca, and follicular fluid were used for characterization of KKS during this physiologic event. There were no differences regarding follicular diameter in different time-points before ovariectomy (evaluated through ultrasound; data not shown). The concentration of oestradiol increased 3h after treatment with GnRH, that is expected time of endogenous LH peak, and gradually decreased thereafter, until 24 hours (data not shown).

3.2. Gene expression of KNG, B_1R and B_2R in follicular cells

The KKS precursor expression, or KNG, was similar for both follicular cell types, granulosa and theca, during the ovulation ($P>0.05$, Figure 1A and 1B). The mRNA expression of B_2R receptor was constant during the ovulation process in granulosa cells, with no difference ($P>0.05$) at different times after LH peak induction (Figure 1C). However, in theca cells the mRNA B_2R receptor expression showed an increase ($P<0.05$) after GnRH (hour zero) injection until 6h, and decrease gradually at 12h remaining constant until 24h (Figure

1D). The B_1R receptor mRNA expression was different in both follicular cells types during the assessed times. In granulosa cells (Figure 1E) the expression increased only at 6h and decrease after this. In theca cells (Figure 1F) B_1R expression increase at 3h and 6h decreasing at 12h and then remaining constant until 24h.

3.3. Follicular fluid concentration of kallikrein and bradykinin

The activity of kallikrein and the levels of kinins was measured for identify these components of KKS in follicular fluid and to test the hypothesis that after LH surge, during ovulation process, these components are regulated. Results for kallikrein-like activity in follicular fluid showed a decrease ($P<0.05$) between the LH ovulatory peak induction (hour zero) and 24h (Figure 2A), however there was no difference between zero and 12h. Bradykinin showed difference ($P<0.05$) during ovulation. BK increased after zero hour until 6h, decreasing until 12h and remaining constant until 24h (Figure 2B).

4. Discussion

The results confirm the presence of some components of KKS during the ovulation and show the differences in mRNA profiles in bovine ovaries. In this study we used an in vivo approach to study the ovulation event in bovine. This specie, in contrast with rodents, is a great monovulatory model and has a wide time between the LH surge and ovulation, around 24 to 30 hours. The presence of components of KKS system in bovine ovary was first shown in cultured granulosa cells [28]. According previous studies it was not clear if the KKS was synthesized at the ovary or are of hepatic origin, since an unknown mechanism would be responsible for moving such compounds into the follicles [18]. Our results support that KNG is synthesized at ovary. These are the first results, using a sensitive semi-quantitative RT-PCR and enzymatic assay for KKS components, where this system is assessed in different follicular cells types and in follicular fluid after LH surge induction until the moment preceding ovulation in a monovolatory specie.

KNG was expressed in both follicular cells and did not differ at the times during this event in bovine (Figure 1A and 1B). The kininogens are the sole precursors of the KKS and are coded by a single gene [8, 25]. There are at least two distinct kininogens in mammalian plasma, designated low molecular weight (LMWK) and high molecular weight (HMWK) kininogens and was indicated that LMWK and HMWK are structurally related [20]. Both HMWK and LMWK have an identical aminoacid sequence starting named heavy chain [20, 25]. On this study the primer used for KNG was designed based on the sequence of the heavy chain, so we assessed mRNA expression for total KNG, both LMWK and HMWK

kininogens, in different times and follicular cells at ovulation in bovine. KNG is highest in mature tertiary follicles of cultured bovine granulosa cells [28]. Different of our results, in equine chorionic gonadotropin (eCG) primed-immature rats the total ovarian KNG levels showed a progressive rise at time immediately preceding the beginning of ovulation [5, 14].

The enzyme that cleaves KNG, named kallikrein, was identified in follicular fluid confirming the hypothesis that this enzyme participates and is possible regulated during the ovulatory process in cattle (Figure 2A). There is a decrease after LH peak induction in relationship the moment before the ovulation moment, about 24 to 30h near the time of ovulation. Kallikreins, which are serine proteases that use KNG by substrate to generate the kallidin and bradykinin, are a member of a multigene family in several species [9]. This family of enzymes are involved in a diverse range of biological responses [17], and holds great promise not just as a panel of biomarkers and potential therapeutic targets, but also as an important model of hormonal regulation [21]. The expression and hormonal regulation of the tissue kallikrein gene family in the rat was previously extensively characterized [9]. While, pre-kallikrein and kallikrein has been described in cultured bovine granulosa cells of immature follicles cells and mature follicles cells, respectively [28].

The pattern of kallikrein activity and kallikrein genes in rat ovaries is high after LH peak induction and decrease as it approaches the time of ovulation in the well-established ovulatory model eCG primed, hCG stimulated immature rat [14, 17]. Our results suggest a similarity in kallikrein activity during ovulation in rats and cattle. Tissue kallikrein does play a role in ovulation, however, further studies are required to determine the sites of action. Although all kallikrein genes have been shown to be hormone-responsive, it is not yet clear which genes are specific and direct targets of each hormone receptor [21]. Future studies on the hormonal regulation of kallikreins should clarify this point and distinguish between hormone-related and hormone-regulated kallikrein expression.

Bradykinin, the mainly peptide of KKS, is present in follicular fluid and has an up regulation after GnRH treatment, reaching peak at 6h and decrease after this during the ovulation process in bovine (Figure 2B). Bradykinin is a nonapeptide kinin produced potent mediator of a wide variety of KKS responses [4, 8]. This peptide induces ovulation in perfused rabbit [40] and rat ovaries [16] and potentiates the action of LH [6]. There are evidences that this peptide is involved in follicular-wall contraction during ovulation [15]. These researches indicate a modulator role of bradykinin, possibly involving prostacyclin late in the ovulatory process, in the rat. Other studies demonstrated that when using porcine ovaries, not only all components of the KKS but also its product, bradykinin, are present

within the follicles [18]. On the other hand, bradykinin in neurons appear to be important mediators of steroid signals in the hypothalamus to produce the LH surge, through B_2R in female rat [36]. This study showed the presence of bradykinin in follicular fluid, but more studies have to do for clarify the importance of this kinin in bovine reproduction.

Bradykinin is known to be degraded rapidly in vivo, with a half-life of about 16 seconds [2]. Various peptidases are capable of metabolizing kinins have been extensively reviewed [11]. These are angiotensin I-converting enzyme (ACE), aminopeptidase P, neutral endopeptidase 24.11 (NEP, neprilysin), and carboxypeptidases M and N [25]. They are all present in a soluble form in biologic fluids and depend on the animal species, the analytical approach, the biological milieu, and the pathophysiological context [25]. In similar ovulation experiment model, our group showed that ACE mRNA expressions were transiently up regulated reaching greater expression 6h post-GnRH treatment in theca but not in granulosa cells (SIQUEIRA et al. data not published). On the other hand, mRNA expression of NEP increased 12 and 24h after GnRH treatment in granulosa cells but not in theca cells (SANTOS et al. data not published). These results show a possible participation of peptidases at bradykinin at down regulation in bovine follicles but more studies are necessary to explain this hypothesis.

The expression of KKS receptors in different follicular cell types showed that B_1R was induced in both follicular cells types while B_2R was constitutively expressed in granulosa cells (Figure 1E and 1F) and possible induced in theca cells (Figure 1D and 1E). These two types of G-protein-coupled receptors mediate the cellular effects of kinins [22, 24]. The effects of bradykinin and kallidin are believed to be mediated particularly the B_2R [4, 24]. Whereas the B_1R mediates the action of des-Arg9-BK and Lys-des-Arg9-BK, the second set of bioactive kinins formed through the actions of carboxypeptidases on bradykinin and kallidin, respectively [22]. These receptors are expressed under biologically different circumstances [24]. B_2R are constitutively expressed on many cell types and are responsible for the majority of the observed effects of kinins, however B_1R is induced only in inflammation [1, 28].

At reproductive events, B_1R was only showed in chinese hamster ovary cells [1], while some researchers has been studding B_2R [18-19, 27-28, 37]. The presence of B_2R is different into de species [18], and the expression is constant in theca and granulosa porcine cells and in mouse ovaries [19, 27]. The results of our study, besides highlight the difference of B_2R expression into species, shows that there are B_1R and B_2R expression in theca and granulosa cells in bovine ovary and demonstrates that the pattern of expression is different

between the two follicular cells types. Moreover, for the first time demonstrated that the expression of B_1R in both follicular cells and B_2R in theca cells can be regulated during ovulation in cattle. With these results we can postulate a possible regulation of these KKS receptors at ovulation in cattle.

Our research group has been studding the renin-angiotensin system (RAS), in which angiotensin II was related to a series of events that control and trigger the reproductive functions in females, such as follicle development, ovulation and oocyte maturation [3, 13, 29, 38]. Some researches has been working which the interaction in some physiological events between the RAS and KKS [8, 33-34]. Better information about reproductive functions of have to be understood. Thus, it becomes important to study the involvement of KKS in reproductive physiology, as well as their relationship with KKS.

This study, using an in vivo approach, confirms the presence of some components of KKS during the ovulation in bovine. According our results, KNG is synthesized in ovary, kallikrein has a possible down regulation while bradykinin has an up regulation and decrease after this. We show that there are B_1R and B_2R expression in theca and granulosa cells and demonstrates that the pattern of expression is different between the two follicular cells types and in different times. In summary, the KKS is present and there are evidences of its regulation at the ovulatory process in bovine. More research has to do for clarify the regulation and importance of KKS during this physiological event.

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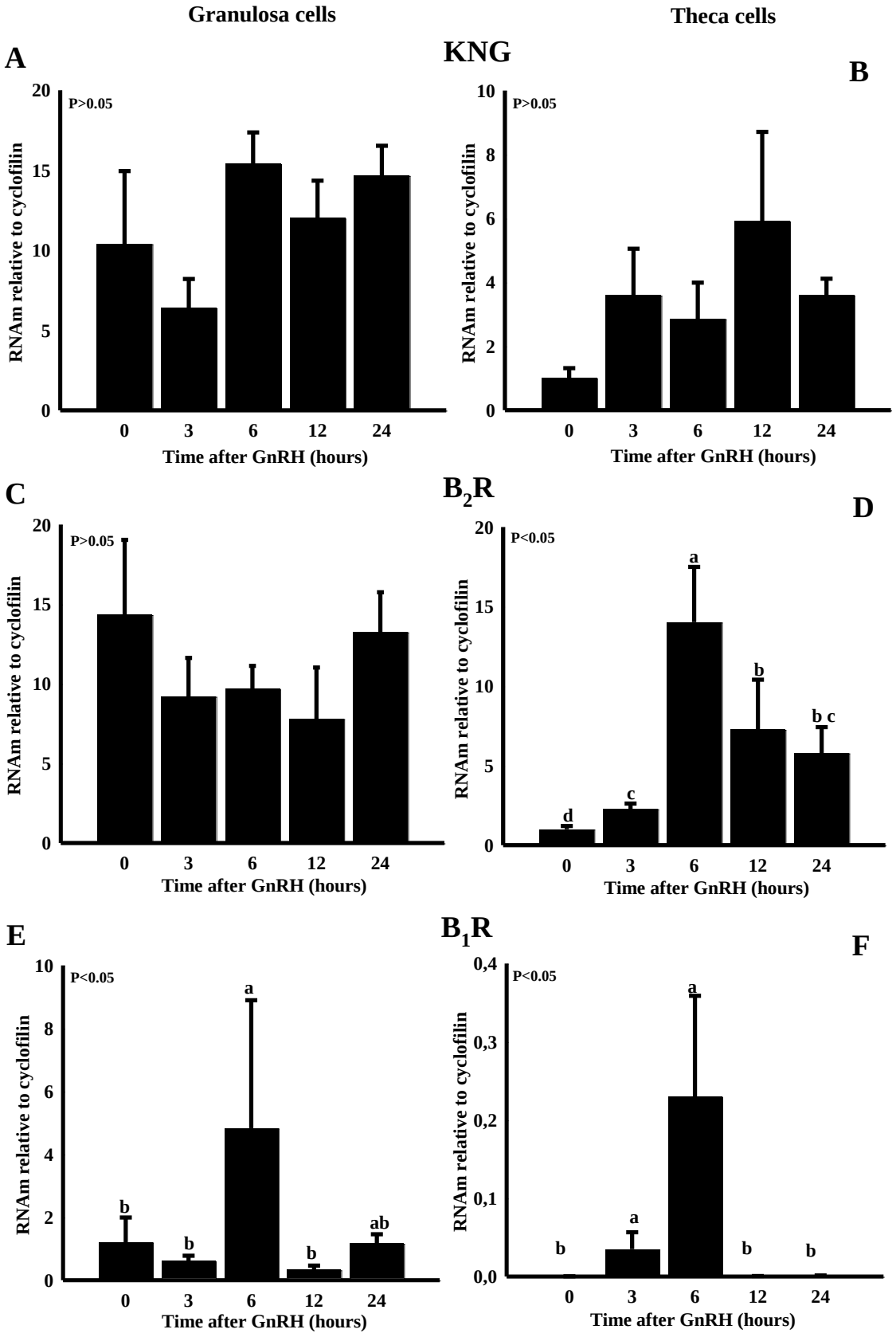
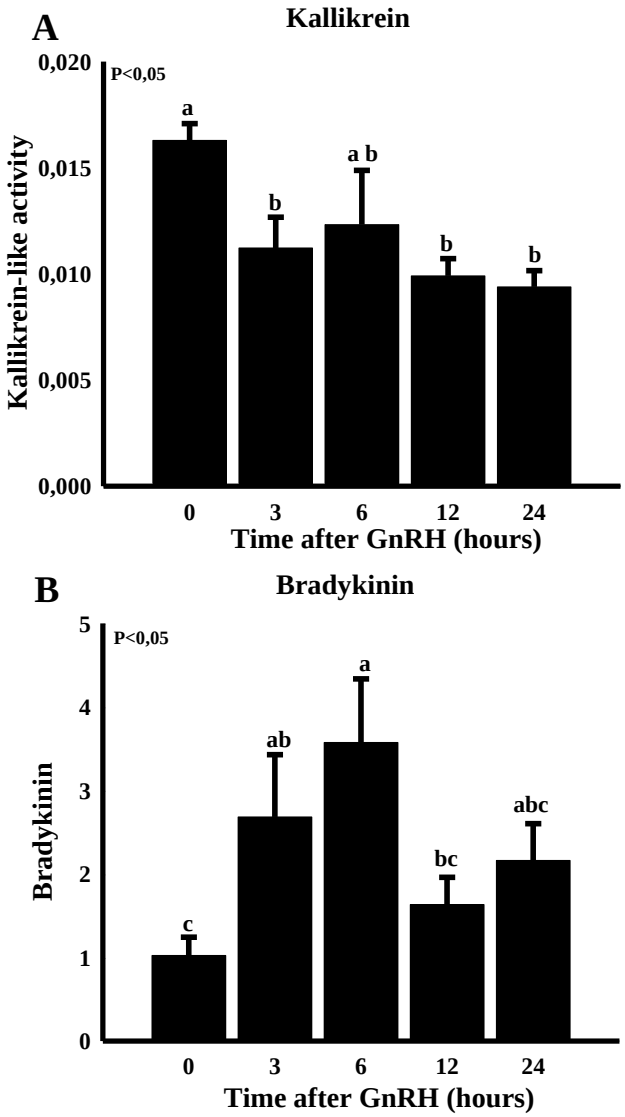

Granulosa cells
Theca cells
A
KNG
B
P>0.05
RNAm relative to cyclofilin
Time after GnRH (hours)
C
B2R
D
P<0.05
E
B1R
F
0
3
6
12
24

379

Figure 1. mRNA expression of KNG (A and B), B_2R (C and D) e B_1R (E and F) in follicular cells, granulosa and theca, relative to housekeeping cyclophilin. To obtain a pre-ovulatory bovine follicle (>12mm) the animals were pre-synchronized and the follicular cells were recovery in different times after GnRH analog injection. Different letters indicates statistical difference ($P<0.05$).



385 Figure 2. Kallikrein activity (nm/mg protein/min, A) and bradykinin (ng/ mg protein, B) in follicular fluid in pre-
386 ovulatory follicules. To obtain a pre-ovulatory bovine follicle (>12mm) the animals were pre-synchronized and
387 the follicular fluid was recovery in different times after GnRH analog injection. Different letters indicates
388 statistical difference (P<0.05).
389

4. CONCLUSÃO

O presente estudo confirmou, *in vivo*, a presença dos principais componentes do sistema calicreína-cinina durante o processo ovulatório de bovinos. Os resultados mostram que o KKS é produzido no ovário e que além da sua presença há indicativos de sua regulação durante a ovulação. Mais pesquisas devem ser realizadas nesta área a fim de esclarecer essa possível regulação e a importância do sistema calicreína-cinina durante este evento fisiológico.

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