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OS PROCESSOS DE OVULAÇÃO E REINÍCIO DA MEIOSE OOCITÁRIA SÃO MEDIADOS PELA INTERAÇÃO ENTRE ANGIOTENSINA II, PROGESTERONA E PROSTAGLANDINAS

TESE DE DOUTORADO

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Santa Maria, RS, Brasil

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OS PROCESSOS DE OVULAÇÃO E REINÍCIO DA MEIOSE OOCITÁRIA SÃO MEDIADOS PELA INTERAÇÃO ENTRE ANGIOTENSINA II, PROGESTERONA E PROSTAGLANDINAS

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Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Medicina Veterinária, Área de Concentração em Fisiopatologia da 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, PhD

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elaborada por
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como requisito parcial para obtenção do grau de
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RESUMO

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OS PROCESSOS DE OVULAÇÃO E REINÍCIO DA MEIOSE OOCITÁRIA SÃO MEDIADOS PELA INTERAÇÃO ENTRE ANGIOTENSINA II, PROGESTERONA E PROSTAGLANDINAS

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Data e Local da Defesa: Santa Maria, 28 de fevereiro de 2011.

A angiotensina II (AngII), progesterona (P_4) e prostaglandinas (PGs) são fatores essenciais para que ocorra a ovulação de um oócito fértil. Este trabalho buscou entender se esses fatores interagem durante o processo de ovulação e maturação oocitária. Para tanto, primeiramente caracterizamos a expressão de genes de interesse para o sistema renina angiotensina em células foliculares e a concentração de AngII no fluido folicular durante o período peri-ovulatório. Para tanto, folículos pré-ovulatórios de vacas foram coletados em diversos momentos após a administração intramuscular de GnRH. Após a coleta, o líquido folicular e as células da teca e granulosa foram separadas para realização da técnica de RT-PCR em tempo real. Neste estudo, foi observado que o pico de gonadotrofinas estimula a secreção folicular de AngII e a expressão de RNAm para receptores do tipo AGTR2 nas células da teca. Em seguida, avaliamos se a AngII é capaz de modular a secreção de esteróides e PGs pelas células foliculares. Células foliculares bovinas obtidas apartir de ovários de abatedouro foram cultivadas in vitro na presença de LH, AngII e/ou saralasina. Com este experimento, foi verificado que a AngII em sinergismo com LH estimula a síntese de P_4, PGE_2 e $PGF_2\alpha$ pelas células da granulosa. O papel da AngII e PGs como mediadoras da maturação nuclear de oócitos em ruminantes induzida por gonadotrofinas já havia sido demonstrado. No entanto, uma ação similar realizada pela P_4 ainda possuía caráter questionável em bovinos. No presente trabalho, utilizando modelos *in vitro* e *in vivo*, foi evidenciado que a P_4 não só participa desse processo, mas também é um fator intermediário entre AngII e PGs na cascata de eventos. Ao final deste trabalho foi possível concluir que AngII, P_4 e PGs são mediadores "conectados" da ação do pico pré-ovulatório de gonadotrofinas. Esses dados nos permitem propor um modelo unificado de eventos tanto para a ocorrência da ovulação quanto para a retomada da maturação nuclear do oócito. Neste modelo, o pico pré-ovulatório de gonadotrofinas aumenta a secreção folicular de AngII e a expressão de receptores AGTR2, os quais estimulam a secreção de P_4 e por consequência de PGE_2 e $PGF_2\alpha$, culminando com a ovulação de um gameta fértil.

Palavras-chave: Ovulação. Oócito. Bovinos. Angiotensina. Progesterona.

ABSTRACT

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THE OVULATORY AND MEIOTIC RESUMPTION PROCESSES ARE MEDIATED BY THE INTERACTION AMONG ANGIOTENSIN II, PROGESTERONE AND PROSTAGLANDINS

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Angiotensin II (AngII), progesterone (P_4) and prostaglandins (PGs) are essential for ovulation of a fertile oocyte. This study sought to understand how these factors are linked together during the ovulatory process. To this end, first we characterized the pattern of expression of genes of interest to the renin-angiotensin system in follicular cells and AngII concentration in the fluid during the periovulatory period. Cows were ovariectomized at various times after GnRH injection to obtain pre (at the time of GnRH treatment) and periovulatory follicles (3, 6, 12, and 24 h after GnRH treatment). Theca and granulosa cells were separated and processed to real time RT-PCR Herein we demonstrated that the gonadotropin surge stimulates the follicular secretion of AngII and theca cells expression of mRNA for AGTR2 receptors. Next, we assessed whether AngII can modulate the secretion of steroids and PGs by follicular cells using in vitro culture of theca and granulosa cells of bovine ovaries from abattoir. In this study we found that AngII in synergism with LH stimulates the synthesis of PGE_2 and $PGF_2\alpha$ and P_4 by granulosa cells. The role of AngII and PGs as mediators of nuclear maturation of oocytes in ruminants induced by gonadotropins had already been demonstrated. However, a similar action performed by P_4 is still controversial in cattle. In this work, using in vitro and in vivo models, we evidenced that P_4 not only participates in this process, but it is also an intermediate factor between AngII and PGs in the cascade of events. With the present work is possible to conclude that AngII, P_4 and PGs are "linked" mediators of the preovulatory gonadotropin surge. These data allowed us to propose a unified model for both, ovulation and the resumption of nuclear maturation of oocytes. In this model the preovulatory gonadotropin surge up regulates the production of AngII and follicular expression of AGTR2 receptors, stimulating the secretion of P_4 and the release of PGE_2 and $PGF_2\alpha$, culminating with the ovulation of a fertile gamete.

Key words: Ovulation. Oocyte. Cattle. Angiotensin. Progesterone.

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1. INTRODUÇÃO

O pico pré-ovulatório de gonadotrofinas desencadeia simultaneamente a retomada da maturação nuclear oocitária e a ovulação, através de uma complexa e dinâmica interação de fatores sistêmicos e locais. Apesar de décadas de estudos, utilizando-se diversos modelos experimentais, as rotas intracelulares que conectam as gonadotrofinas e a liberação de gametas femininos férteis e formação do corpos lúteos ainda estão incompletas (DUGGAVATHI & MURPHY, 2009). Sabe-se que a angiotensina II (AngII), a progesterona (P_4) e as prostaglandinas (PGs) são agentes intermediários das ações desencadeadas pelo pico pré-ovulatório de gonadotrofinas, pois sem suas ações, eventos como a ovulação e a liberação de um oócito maturo são afetados negativamente.

Sem a sinalização da AngII, tanto a ovulação quanto a maturação nuclear do oócitos induzidas pelo pico de gonadotrofinas são inibidas (FERREIRA et al., 2007; BARRETA et al., 2008). Apesar das evidências de que a AngII possui uma participação precoce no processo ovulatório (FERREIRA et al., 2007), estudos prévios detectaram apenas um aumento tardio nas concentrações foliculares desse peptídeo durante o processo ovulatório (ACOSTA et al., 2000). A AngII utiliza a rota das ciclooxigenases para induzir a retomada da meiose oocitária (BARRETA et al., 2008). No entanto, as rotas hormonais utilizadas por esse peptídeo para mediar o processo ovulatório ainda permanecem desconhecidas. Por outro lado, apesar de evidente o papel da P_4 para a ruptura do folículo, a participação desse esteróide na maturação oocitária de ruminantes ainda permanece controverso (SILVA & KNIGHT, 2000; WANG et al., 2006). É bem estabelecido que durante o processo ovulatório a P_4 torna-se o principal esteróide secretado pelas células foliculares, e é um dos hormônios a estimular a síntese de PGs pelas células da granulosa (BRIDGES et al., 2006; BRIDGES & FORTUNE, 2007).

As evidências sugerem que tanto a AngII quanto a P_4 utilizam a rota das PGs durante a cascata ovulatória. Entretanto, não se sabe se esses hormônios são apenas diferentes etapas de uma única sequência de eventos ou sinalizações paralelas que se convergem no uso desses eicosanoides. Sendo assim, o presente trabalho foi realizado com o objetivo de testar a hipótese de interação entre AngII, P_4 e PGs nos processos de retomada da maturação nuclear oocitária e da ovulação em bovinos.

2. REVISÃO BIBLIOGRÁFICA

A ovulação é um evento iniciado por um aumento abrupto na liberação das gonadotrofinas hipofisárias que desencadeia múltiplas rotas intracelulares paralelas que modulam simultaneamente a expressão gênica nas células foliculares. O pico de gonadotrofinas desencadeia uma cascata coordenada por alterações bioquímicas, morfológicas e moleculares no folículo dominante, que culmina na liberação do oócito maturo e posterior formação do corpo lúteo. Dentre essas modificações, pode ser destacado a marcante alteração nas rotas esteroidogênicas e o envolvimento de PGs (KOMAR et al., 2001; BRIDGES et al., 2006; BRIDGES & FORTUNE, 2007). A secreção desses hormônios no líquido folicular e a modulação da expressão para seus receptores durante o período periovulatório (período entre o pico de LH e a liberação do oócito) são eventos cruciais para a fertilidade da fêmea.

2.1. Papel da progesterona no processo ovulatório

A P_4 possui um papel central em diversos eventos reprodutivos, atuando via receptores nucleares para P_4 (PR; NATRAJ & RICHARDS, 1993; LYDON et al., 1995; CONNEELY et al., 2001). Em mulheres e ratas, os antagonistas de PR inibem a ovulação (SNYDER et al., 1984; KAGABU et al., 1999). Além disso, camundongas que tiveram o gene PR deletado são inférteis por falha no processo de ruptura do folículo (LYDON et al., 1995; KIM et al., 2009). No entanto, nesses animais, o pico de LH induz a maturação nuclear dos oócitos e a luteinização das células foliculares. Esse grupo de receptores possui duas isoformas (PR-A e PR-B) produzidas a partir de um único gene, porém com propriedades distintas quando ligadas à P_4 (KASTNER et al., 1990; WANG, 2005; SLEITER et al., 2009). Ambas as proteínas são estimuladas durante o processo ovulatório em células da granulosa em camundongas, no entanto, apenas o subtipo PR-A parece estar diretamente envolvido na inibição da ovulação (MULAC-JERICEVIC et al., 2000; CONNEELY et al., 2001; MULAC-JERICEVIC & CONNEELY, 2004).

Em bovinos, durante a transição folículo-luteal, a P_4 gradualmente passa a ser o esteróide primário a ser secretado. Isso porque, as células da teca e granulosa, que no folículo dominante convertiam o colesterol até a forma de andrógeno e estradiol, respectivamente,

diminuem a expressão das enzimas responsáveis por essas rotas esteoidogênicas durante o processo ovulatório (KOMAR et al., 2001). Além disso, logo após o pico de gonadotrofinas, os níveis de expressão de RNAm para PR nas células da teca e granulosa aumentam (JO et al., 2002). Entretanto, nesse mesmo estudo, foi observado que ocorre uma diminuição nas concentrações de P_4 e PR, 12 horas após o pico de gonadotrofinas, seguida por um novo aumento nos momentos próximos à ovulação. Experimentos *in vitro*, com células foliculares coletadas em momentos conhecidos em relação ao pico de gonadotrofinas, confirmaram que o LH é capaz de induzir a síntese de P_4 e a expressão de PR (JO et al., 2002; JO & FORTUNE, 2003). Porém, a P_4 parece não ser capaz de regular a expressão do seu receptor (JO et al., 2002).

A P4 é um dos mediadores entre o pico de gonadotrofinas e o aumento na secreção de PGs no fluido folicular no período próximo a ovulação (BRIDGES et al., 2006; BRIDGES & FORTUNE, 2007). Utilizando-se cultivo *in vitro* de células da granulosa, demonstrou-se que é possível inibir o aumento da secreção de PGs induzido por LH adicionando-se um bloqueador de PR, e que essa inibição pode ser revertida com a utilização de progestágenos (BRIDGES et al., 2006). Ainda, a P_4 se mostrou capaz de estimular a secreção de ocitocina, e esta parece estimular a rota das PGs (BRIDGES & FORTUNE, 2007). Outro achado interessante desse trabalho é que as PGs e a ocitocina são capazes de estimular a secreção de P_4 em células foliculares. Com isso, nota-se que ainda existem outros mecanismos de auto-regulação, possivelmente redundantes, que ocorrem no momento periovulatório, os quais provavelmente sejam importantes para garantir a ovulação do oócito maturo.

A capacidade de induzir a maturação nuclear de oócitos em ranídeos é uma das mais bem estudadas funções biológicas da P_4 (KIM et al., 2000; JESSUS & OZON, 2004). No entanto, em mamíferos o papel desse esteróide como agente indutor da maturação nuclear de oócitos permanece controverso. Enquanto em suínos a P_4 é capaz de antecipar a retomada da progressão meiótica na ausência de gonadotrofinas (SHIMADA & TERADA, 2002; SHIMADA et al., 2004), em roedores e humanos ela parece ser necessária apenas para ovulação, mas não para a maturação nuclear. Já em primatas e bovinos, diferentes estudos apresentam resultados contraditórios. De acordo com HIBBERT et al. (1996) a inibição da síntese de esteróide em folículos de macacas (Macaco Rhesus), pelo uso de um inibidor de 3-β hidroxi-esteróide-dehidrogenase, não afeta a maturação nuclear. No entanto, BORMAN et al. (2004) concluíram que, nessa mesma espécie, a administração de progestágenos induz o reinício da meiose de oócitos mesmo na ausência de um pico de gonadotrofinas. Em bovinos, a adição de P_4 ao meio de cultivo, foi considerada sem efeito na maturação nuclear ou até

mesmo prejudicial ao desenvolvimento embrionário (SILVA & KNIGHT, 2000; WANG et al., 2006). No entanto, salienta-se que nessa espécie a retomada da maturação nuclear ocorre espontaneamente após a remoção dos complexos cumulus-oócito do ambiente folicular, dificultando assim, o estudo de fatores estimulatórios sobre o reinicio da meiose.

2.2. Papel das PGs no processo ovulatório

As PGs são moléculas que possuem 20 carbonos na sua estrutura, sintetizadas a partir da clivagem do ácido aracdônico pela rota das ciclooxigenases (COX; revisado em: WEEMS et al., 2006). Essas enzimas catalisam a endoperoxidação desse ácido em intermediários instáveis, os quais por isomerização são convertidos a PGE_2 e $PGF_2\alpha$. As células da granulosa são responsáveis por grande parte da síntese dessas PGs nos folículos periovulatórios; no entanto, as células da teca e do cúmulos também são capazes de sintetizar pequenas quantidades desse hormônio (BRIDGES et al., 2006). Após o pico de gonadotrofinas pode ser observado um aumento inicial, porém modesto, na secreção de PGs pelo folículo ovulatório. Nos momentos próximos à ovulação, a expressão de COX-2 nas células da granulosa e a concentração de PGs no líquido folicular atingem seus níveis máximos. A PGE_2 e a $PGF_2\alpha$ exercem seus efeitos primariamente a partir de receptores específicos acoplados a proteína G, os quais possuem diversos subtipos (TSUBOI et al., 2002; HATA & BREYER, 2004). Esses receptores apresentam um complexo padrão de expressão durante todo o período pré-ovulatório em bovinos dificultando a identificação específica de suas funções (BRIDGES & FORTUNE, 2007).

O aumento das concentrações de PGs no líquido folicular após o pico pré-ovulatório de gonadotrofinas é componente chave no processo ovulatório. Utilizando-se inibidores da COX e, consequentemente, diminuindo a síntese de PGs, é possível inibir a maturação do oócito e a ovulação em mamíferos (ESPEY et al., 1986; DUFFY & STOUFFER, 2002). Camundongas que apresentam deleção para o gene da COX-2 apresentam taxas de ovulação reduzidas e ninhadas menores quando comparados a linhagens selvagens. As funções atribuídas às PGs incluem vasodilatação tecidual e alterações na região apical do folículo pré-ovulatório (MURDOCH et al., 1986; PRIDDY & KILLICK, 1993; MURDOCH, 1996). Ainda, possuem papel fundamental no processo de remodelamento da matriz extracelular (CURRY & OSTEEN, 2001). Recentemente, nosso laboratório demonstrou o envolvimento

das PGs como agentes intermediários entre o pico de LH e o reinício da meiose em oócitos bovinos (BARRETA et al., 2008).

As funções específicas da PGE_2 e $PGF_2\alpha$ durante o processo ovulatório e de luteinização não são totalmente entendidas. Em camundongas, enquanto a deleção dos receptores para $PGF_2\alpha$ não parece afetar a fertilidade, a ausência de receptores para PGE_2 diminui o tamanho da ninhada por diminuir a taxa de ovulação (SUGIMOTO et al., 1997; KENNEDY et al., 1999). No entanto, em ruminantes a $PGF_2\alpha$ *in vivo* parece possuir um papel central no processo ovulatório (MURDOCH et al., 1986). Além disso, BARRETA et al. (2008) demonstraram que a adição tanto de PGE_2 quanto a de $PGF_2\alpha$ ao meio de cultivo induz a maturação nuclear de oócitos bovinos. Esses resultados em conjunto apontam para um papel central e indispensável das PGs no processo de ovulação em mamíferos. Entretanto, os mecanismos intracelulares estimulados por PGs que levam à ovulação e a retomada da maturação nuclear do oócito ainda são pouco entendidos.

2.3. Angiotensina II na ovulação e maturação oocitária

A AngII é o principal peptídeo ativo do sistema renina-angiotensina (RAS). Nesse sistema, o precussor angiotensinogênio é clivado pela renina em Angiotensina I (CLAUSER et al., 1989). Esse decapeptídeo por sua vez sofre a ação da enzima conversora de angiotensina (ECA) que cliva-o em AngII. As ações desse octapeptídeo são mediadas por dois subtipos de receptores, AT1 e AGTR2, com estruturas glicoprotéicas com sete hélices transmembrânicas, mas com apenas 30% de homologia entre si. Os receptores AT1 são responsáveis pela maioria dos efeitos conhecidos da AngII na homeostase cardiovascular, enquanto os AGTR2 são mediadores de efeitos opostos ao produzidos pelo AT1. Ainda, o subtipo AGTR2 parece mediar as ações da AngII ligadas aos processos ovarianos (BOTTARI et al., 1993; FERREIRA et al., 2007; PORTELA et al., 2008a).

Apesar de estar bem determinado que folículos ovarianos secretam AngII, o tipo celular responsável pela sua síntese ainda não foi identificado. Todos os componentes do RAS podem ser encontrados no ovário. O angiotensinogênio parece ser produzido principalmente nas células da granulosa (THOMAS & SERNIA, 1990). A renina parece ser produzida exclusivamente no rins, pois animais nefrectomizados não apresentam níveis circulantes dessa enzima. No entanto, a concentração de pró-renina e renina ativa no fluido folicular bovino é regulada por gonadotrofinas (HAGEMANN et al., 1994). A ECA e os receptores AT1 e

AGTR2 são expressos nas células da teca e granulosa. No entanto, em bovinos, apenas a expressão de receptores AGTR2 e da ECA parecem ser moduladas durante a foliculogênese (PORTELA et al., 2008a).

Nosso laboratório tem estudado o papel da AngII no desenvolvimento folicular, ovulação e progressão meiótica de oócitos de bovinos. Usando modelos *in vivo* e *in vitro*, nossos resultados suportam uma importante função desse peptídeo na fisiologia ovariana, principalmente no desenvolvimento folicular, ovulação e maturação de oócito em bovinos (GIOMETTI et al., 2005; FERREIRA et al., 2007; BARRETA et al., 2008; PORTELA et al., 2008a). A AngII é fundamental na cascata ovulatória, tendo em vista que o bloqueio dos seus receptores inibe tanto a ovulação quanto o reinício da meiose em bovinos (FERREIRA et al., 2007; BARRETA et al., 2008).

Nossos resultados sugerem que a AngII possui um envolvimento precoce no período periovulatório, pois a injeção dos antagonistas da AngII 12 horas após o pico de gonadotrofinas não foi capaz de inibir a ovulação (FERREIRA et al., 2007). Segundo esse mesmo estudo, a AngII medeia o processo ovulatório atuando via seus receptores do subtipo AGTR2. A expressão desses receptores em células da granulosa de bovinos parece ser regulada por FSH e fatores de crescimento (PORTELA et al., 2008a). Sendo assim, os estudos *in vitro* suportam os dados coletados *in vivo* e apontam para possíveis rotas de ação da AngII no período periovulatório.

O reinício da meiose de oócitos bovinos é um evento que ocorre durante o período periovulatório e que *in vivo* é desencadeado pelo pico de gonadotrofinas. Novamente, a AngII aparece como um fator mediador das ações das gonadotrofinas durante esse evento. BARRETA et al. (2008) demonstraram que a injeção intrafolicular *in vivo* de um antagonista da AngII previne o reinício da meiose induzido pelo GnRH. Visando estudar os mecanismos de ação da AngII na retomada da maturação nuclear, foi conduzida em nosso laboratório uma série de experimentos *in vitro*. Os resultados indicam que a AngII induz a retomada da maturação nuclear de oócitos bovinos (GIOMETTI et al., 2005) atuando via AGTR2 (BENETTI et al., 2008). Além disso, a AngII estimula a expressão de RNAm para COX-2 em cultivo de células da granulosa (PORTELA et al., 2008a). Nosso grupo também demonstrou que os inibidores da COX previnem o efeito estimulatório da AngII sobre a maturação oocitária (BARRETA et al., 2008). Esses resultados em conjunto apresentam evidências suficientes para sugerir que a AngII é um fator indispensável para a ovulação e também para a retomada da maturação nuclear do oócito, utilizando-se da rota das COX para exercer suas funções.

2.4. Fator de crescimento fibroblástico 10 nos processos ovarianos

O Fator de crescimento fibroblástico 10 (FGF10) pertence a uma grande família com mais de 20 ligantes, a qual possui uma ampla distribuição tecidual, participando em diversos eventos fisiopatológicos (GOSPODAROWICZ et al., 1987). Os primeiros efeitos atribuídos ao FGF10 estão ligados à organogênese, pois camundongos que não expressam esse gene, não desenvolvem o tecido pulmonar (SEKINE et al., 1999). Os ligantes dessa família atuam através de receptores tirosina-quinase, específicos para essa família (FGFR), onde 4 genes (FGFR1, FGFR2, FGFR3 e FGFR4) codificam diversas isoformas alternativas (ITOH & ORNITZ, 2004). Aparentemente, o FGF10 apresenta especificidade pelos receptores FGFR2b (IGARASHI et al., 1998).

O identificação do envolvimento dos FGFs nos eventos ovarianos são recentes e incluem a participação no crescimento inicial do oócito (CHO et al., 2008), na ativação de folículos primordiais (NILSSON et al., 2001) e no processo de divergência folicular (FGF2; BERISHA et al., 2000). Em bovinos, o FGF10 é expresso pelo oócito e células da teca. Sua expressão é regulada durante o crescimentos de folículos antrais, no qual folículos subordinados apresentam menor expressão de FGF10 nas células da teca (CASTILHO et al., 2008). Em cultivos de células da granulosa, o FGF10 inibe a secreção de estradiol, proliferação celular e expressão de receptores para AngII (AGTR2; PORTELA et al., 2008a). A proteína para o receptor FGFR2b foi inicialmente detectada nas células da granulosa e teca de folículos antrais (PARROTT & SKINNER, 1998; BERISHA et al., 2004; BURATINI et al., 2007). Recentemente, a expressão de RNAm para esse gene foi encontrada em células do cumulus e oócitos (ZHANG et al. 2011). No entanto, a ação específica do FGF10 no processo ovulatório não foi ainda descrita.

Pouco se conhece sobre o envolvimento dos membros da família FGF durante a maturação oocitária. Estudos em roedores (*Mus musculus*) sugerem que o os FGFR estão envolvidos no processo de prevenção da retomada da maturação nuclear durante a foliculogênese (PELUSO, 2006). Em contrariedade a esses achados, ZHANG et al. (2011) recentemente sugeriram que o FGF10 possa ser um fator estimulatório no processo de maturação de oócitos bovinos, aumentando a competência de embriões bovinos produzidos *in vitro*

3. CAPÍTULO 1

TRABALHO A SER ENVIADO PARA PUBLICAÇÃO:

Preovulatory changes in the angiotensin II system in bovine follicles

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Preovulatory changes in the angiotensin II system in bovine follicle

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Abstract

The present study evaluated whether the gonadotropin surge modulates components of the renin-angiotensin system and whether AngII play a role in the production of hormones by follicular cells during the ovulatory process. In experiment 1, cows were ovariectomized at various times after GnRH injection to obtain preovulatory (at the time of GnRH treatment) and periovulatory follicles (3, 6, 12, and 24 h after GnRH treatment). AngII was measured in follicular fluid and the levels of mRNA encoding AngII receptors and angiotensin-converting enzyme (ACE) were evaluated in theca and granulosa cells. The concentration of AngII in follicular fluid increased after GnRH and AGTR2 and ACE mRNA levels were transiently upregulated in theca cells. In experiment 2, using an in vitro culture, we determined whether AngII could modulate hormone production by healthy dominant follicles. In the absence of LH, AngII did not altered hormonal production by either theca or granulosa cells. The addition of AngII to medium containing LH increased progesterone, prostaglandin secretion by granulosa cells. In summary, the present work suggests that the renin-angiotensin system is intensely controlled during the preovulatory period and that AngII amplifies stimulatory effects of LH on secretion of progesterone and prostaglandins by granulosa cells.

1. Introduction

The periovulatory period is the time between gonadotropin surge and ovulation, characterized by a complex cascade of morphological, biochemical and molecular modifications that culminates with release of mature oocytes. Progesterone (P_4) and prostaglandins (PG) are essential in this cascade. Gonadotropin surge induces the shift in follicular steroidogenesis from androgen/estradiol to P_4, stimulating the secretion of PGs in granulosa cells (Komar *et al.* 2001; Bridges *et al.* 2006; Bridges and Fortune 2007). Whether the LH surge stimulates follicular P_4 production directly or if there are factors or hormones that mediate the effects of LH on steroid secretion remains to be elucidated.

Angiotensin II (AngII; the major bioactive peptide of the renin-angiotensin system) has recently been recognized as essential for ovulation. Although Yoshimura et al. (Yoshimura *et al.* 1992) have demonstrated that is possible to induce ovulation in rabbits with AngII in absence of gonadotropin, most of the knowledge points to AngII as only an intermediary factor between gonadotropin surge and ovulation. In vitro studies have suggested that AngII acts as a mediator in gonadotropin-induced ovulation in rabbits and rats (Peterson *et al.* 1993; Yoshimura *et al.* 1993). Using an in vivo model, we have demonstrated that AngII antagonists inhibit GnRH-induced ovulation in cattle (Ferreira *et al.* 2007). This inhibition occurs only if the receptor blocker is injected intrafollicularly at the same time or 6 hours after GnRH, but not if injected into follicles 12 h after GnRH. Together, these reports argue for an early pivotal role for AngII during ovulation. However, how the AngII participates in the ovulatory cascade and how the renin-angiotensin system is regulated within periovulatory follicles is currently unknown.

There is evidence that AngII can modulate follicular steroidogenesis and PG production. Our previous data suggest that AngII-receptor blocker inhibits follicular growth by inhibiting steroidogenesis (BENETTI 2008). Also, an in vitro study using microdialysis of the theca layer of bovine follicles had suggested that in absence of gonadotropin, AngII could stimulate P_4, estradiol and PGs secretion (Acosta *et al.* 1999). However, to our knowledge, no study was conducted to elucidate if AngII effects in follicular cells are gonadotropin-dependent or to identify in which follicular cell type AngII is modulating hormone secretion.

An active renin-angiotensin system is well described within the ovary. Although both AngII receptors (AGTR1 and AGTR2) are present in theca and granulosa cells, nothing is known about the pattern of expression of these receptors during the periovulatory period. Likewise, although it is known that antral follicles secrete AngII, virtually nothing is known about the temporal pattern of Ang II production during the periovulatory period. Early studies

using follicles microdialysed through the theca layer have suggested a 2-fold increase in AngII concentration during the late periovulatory period (24 h after LH surge; Acosta et al., 2000). Moreover, whether this increase represents systemic or intrafollicular changes in AngII concentrations is not known, since the theca layer is direct contact with peripheral blood. This late increase in AngII concentration after LH surge is not consistent with the early role for AngII during the ovulatory process suggested by our previous studies in vivo (Ferreira *et al.* 2007).

The objectives of this study were: to characterize the follicular AngII concentration and levels of mRNA for AGTR1, AGTR2 and ACE during the periovulatory period in vivo and to determine the effect of AngII on follicular secretion of hormones involved in ovulation. The main hypotheses were: AngII concentration in follicular fluid increases before 6 h after GnRH treatment; AGTR2 mRNA expression is upregulated sometime between the LH surge and ovulation in follicular cells; and AngII mediates the LH-induced increase in follicular cell secretion of P4 and PGs.

2. Materials and methods

2.1. Animals and follicles isolation during the periovulatory period

Follicular fluid and cells were obtained from cows (Bos taurus taurus) with regular estrous cycles in accordance with procedures approved by the Ethics and Animal Welfare Committee of the Federal University of Santa Maria (CCR / UFSM). A new follicular wave was initiated by inserting an intravaginal P_4-releasing device and 2 mg im of estradiol benzoate on day -9. At day 0, luteolysis was induced by injecting 125 μg im of PG analog (Sodium cloprostenol, Schering-Plough Animal Health, Brazil), 12 h before and at the time of vaginal device removal to initiate a new follicular phase. Twenty-four hours after device removal, animals received im 100 μg GnRH analog (Gonadorelin, Tortuga, Brazil) to elicit a gonadotropin surge. The ovaries bearing the preovulatory follicles were removed by colpotomy at 0, 3, 6, 12 or 24 h post-GnRH injection (at least 5 ovaries / time point). Ovaries were examined at time of PG injection and 24 hours later (at the time of GnRH) by transrectal ultrasonography, to ensure follicular growth and a minimal diameter of 12 mm at GnRH injection (cows presenting follicles with a diameter smaller than 12 mm were excluded from the experiment). Ovaries were put in PBS and preovulatory follicles were quickly (~2 min) dissected from the ovarian stroma. Follicular fluid was aspirated and stored in the presence of

protease inhibitors (10^{-5} M phenylmethylsul-fonylfluoride, 10^{-5} M pepstatin A, 10^{-5} M EDTA, 10^{-5} M p-hydroxymercuribenzoate, and $9x10^{-4}$ M orthophenanthroline, all purchased from Sigma-Aldrich Corp) and follicular cells (theca and granulosa) were separated and processed as previously described (Bridges *et al.* 2006). Samples were frozen in liquid N_2 and stored at -80 ^{o}C. AngII was measured as described by (Costa *et al.* 2003).

2.3. Nucleic Acid Extraction and RT-PCR

Total RNA was extracted using Trizol (theca cells) or silica-based protocol (granulosa cells; Qiagen, Mississauga, ON, Canada) according to the manufacturer's instructions and was quantified by absorbance at 260 nm. Total RNA (1 µg) was first treated with 0.2 U DNase (Invitrogen) 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, ON, Canada), 0.5 µM dideoxynucleotide triphosphate (dNTP) mix, and 10 U RNase Inhibitor (Invitrogen) 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 (qRT-PCR) was conducted in a Step One Plus instrument (Applied Biosystems, Foster City, CA) with Platinum SYBR Green qPCR SuperMix (Invitrogen) and bovine-specific primers (Table 1). 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 D2 as housekeeping gene. The relative quantification of gene expression across treatments was evaluated using the ddCT method (Livak and Schmittgen 2001). Briefly, the dCT is calculated as the difference between the CT of the investigated gene and the CT of Cyclophilin D2 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 (Table 1) were taken from literature or designed using Primer Express Software v3.0 (Applied Biosystems) and synthesized by Invitrogen.

2.4. Follicular cell culture and hormone measurement

Pairs of ovaries were obtained from an abattoir and healthy dominant follicles were identified and dissected from the ovary. A follicle was considered healthy based on their

vascularization and translucent appearance under stereomicroscope and on a high estradiol:P_4 ratio in the follicular fluid. Granulosa cells were gently scrapped from the theca layer with a fine glass needle, as described previously (Bridges *et al.* 2006). Theca layer were cut into small pieces, distributed at random (3 pieces / well) to 24-well culture plates (Costar, Cambridge, MA) and cultured in 0.5 ml of culture medium (Eagle's MEM supplemented with insulin, transferrin, and cortisol). Granulosa cells were collected by centrifugation, counted with a hemacytometer, and distributed to 24-well Primaria culture plates (200,000 cells / well; Falcon, Becton Dickinson, Lincoln Park, NJ). The cells were cultured at 37 °C in humidified incubation chambers (Billups-Rothenberg, Del Mar, CA) gassed with 5% CO_2 for 72 h. Culture medium was collected (stored frozen for later hormone measurements) and replaced at 12-h intervals. Each experiment was replicated with three follicles. Treatments were applied to duplicate cultures from each follicle and included AngII (0.001, 0.01, 0.1 or 1 μM; Sigma), LH (100 ng/ml, NIH LH-S26). Steroids and PGE_2 and $PGF_2\alpha$ were assayed by Radioimmunoassay as previously described (Komar *et al.* 2001; Bridges *et al.* 2006).

2.5. Statistical analysis

The differences on continuous data between time points (experiment 1) or treatments (experiment 2) were accessed by paired Student's T test using follicle as subject. The AngII and mRNA encoding RAS protein data were analyzed by ANOVA. Multi-comparisons between moments or treatments were performed by least square means. 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, NC) and a $P<0.05$ was considered statistically significant. Data are presented as means ± sem.

3. Results

AngII concentrations and steady-state levels of mRNA expression for AGTR1, AGTR2 and ACE in periovulatory follicles in vivo

To determine whether follicular secretion of AngII is regulated by gonadotropin surge, cows were ovariectomized to obtain pre (at the time of GnRH treatment) and periovulatory follicles at 3, 6, 12, and 24 h after GnRH treatment. The concentration of AngII in follicular

fluid augmented gradually after GnRH treatment (Fig. 1), presenting a significant increase after 6 h and reaching 8-fold increase at 24 h in relation to 0 h.

We examined the mRNA abundance for angiotensin receptors (AGTR1 and AGTR2) and ACE (enzyme that converts AngI to AngII) in theca and granulosa cells obtained at 0, 3, 6, 12, and 24 h after intramuscular injection of GnRH to further characterize the modulation of the renin-angiotensin system induced by the periovulatory gonadotropin surge. All three genes were detected in both cell types. As expected, AGTR1 mRNA expression levels did not change after GnRH treatment in both theca and granulosa cells (Fig. 2a and 2b). On the other hand, the AGTR2 and ACE mRNA expressions were transiently upregulated in theca but not in granulosa cells in response to GnRH treatment (Fig. 2 and 3; $P<0.05$). A 3-fold increase in AGTR2 mRNA was observed in theca cells 3 h after GnRH and returned to the initial levels at 6 h post-GnRH. Similarly, the ACE mRNA expression was upregulated at 3 h post-GnRH (Fig. 3a; $P<0.05$), reaching 30-fold increase by 6 h. After 12 h, the ACE mRNA expression decreased to levels equivalent to those before GnRH treatment ($P<0.05$).

Effect of AngII on follicular secretion of hormones involved in ovulation

Since AngII concentration increases in follicular fluid and components of the renin-angiotensin system are regulated during the periovulatory stage, we hypothesize that AngII modulates secretion of P_4, androstenedione, estradiol, PGE_2 and $PGF_2\alpha$ on periovulatory follicles. To test this hypothesis, using an in vitro culture, we verified whether AngII can modulate hormonal production on healthy dominant follicles obtained from an abattoir and even mimic the LH effects on follicular cells. In the absence of LH, none of the concentrations of AngII tested altered hormonal production on either theca (P_4 and androstenedione) or granulosa cells (P_4, estradiol, PGE_2 and $PGF_2\alpha$; data not shown). As expected, follicular cells treated with LH produced more P_4 and PGs than those in the control medium ($P<0.05$; Fig. 4 and 5). Progesterone secretion increased in theca cell culture more than 6-fold and doubled in granulosa cell culture. When AngII was added to the medium contained LH, the P_4 production by theca cells did not change (Fig. 4a). However, granulosa cells treated with LH and AngII secreted more (3 to 5 fold more than control) P_4 (Fig. 4b), PGE2 (Fig. 5a) and PGF2α (Fig. 5b) than those cultured in the presence of only LH.

4. Discussion

The present study assessed potential modulation of components of the renin-angiotensin system by gonadotropin surge and the role of AngII in the production of P_4, androstenedione, estradiol, PGE2 and PGF2α by follicular cells during the ovulatory process. Our significant findings are: 1) concentration of AngII in follicular fluid increased gradually within a few hours after challenge with GnRH agonist in vivo; 2) AGTR2 receptors and ACE mRNA concentration were rapidly upregulated in theca cells of periovulatory follicles in vivo; and 3) AngII had a synergistic action with LH to induce the production of P_4, PGE_2 and $PGF_2\alpha$ by granulosa cells in vitro. Together, these results provide further support for our hypotheses that AngII has an early role during ovulation, mediating and enhancing modifications induced by preovulatory gonadotropin surge.

Previously, we have shown that an intrafollicular injection of AngII receptor antagonists inhibits ovulation only if performed at the time or soon after GnRH treatment (Ferreira *et al.* 2007). In addition, AngII can rapidly modulate gene expression required for ovulation (Portela *et al.* 2008). Therefore, our hypothesis is that AngII is involved in the initial regulation of the ovulatory process. It is important to highlight that nothing was known up to now about the temporal pattern of AngII in follicular fluid after the preovulatory LH/FSH surge in cattle. Measurement of follicular fluid concentrations of AngII confirmed our hypothesis that intrafollicular AngII increases quickly after the injection of GnRH, but also revealed that AngII synthesis keeps increasing until 24 h later. The 3-fold increase of AngII concentration in follicular fluid observed at 6 h after GnRH could account for its initial effects during ovulation. The second increase in the AngII concentration, between 12 and 24 h after GnRH, implies that it may play a role late in the periovulatory period. These findings corroborate earlier studies that reported the important participation of AngII in the control of the enormous process of tissue remodeling that takes place during the follicle-luteal transition (Portela 2008).

ACE is the main enzyme that cleaves the decapeptide AngI to form the active octapeptide AngII (Peach 1977). ACE mRNA was detected in both theca and granulosa cells at all times examined. Interestingly, GnRH treatment induced a dramatic but transient upregulation of ACE expression on theca cells, reaching a peak of expression at 6 h. However, differential mRNA expression was not observed on granulosa cells during

periovulatory stage. The upregulation of ACE mRNA in theca cells may be coupled to the rise of AngII levels in follicular fluid after GnRH treatment.

Our previous data demonstrated that bovine theca and granulosa cells express AGTR1- and AGTR2-receptor mRNA and protein, and FSH upregulates AGTR2 but not AGTR1 expression in granulosa cells in a dose dependent manner (Portela *et al.* 2008). We also have shown that ovulation is prevented in vivo by AGTR2, but not AGTR1 antagonist (Ferreira *et al.* 2007). Here, we show that AGTR2 receptor mRNA expression is regulated only in theca, and that AGTR1 is not regulated in either cell type after GnRH treatment. Taken together, these data suggest that AGTR2, rather than AGTR1, is the receptor that mediates AngII action during the ovulatory process.

The preovulatory gonadotropin surge induces a shift in follicular steroidogenesis from androgen/estradiol to P_4 (Komar *et al.* 2001) and also leads to the increase in PGE_2 and $PGF_2\alpha$ secretion (Espey *et al.* 1986). These key events were mimicked in vitro, when the follicular cell cultures were treated with LH, validating our model to study the ovulatory process (Jo and Fortune 2002). AngII had no effect on P_4 secretion by theca cells. Although AngII alone had no effect, it augmented the stimulatory effects of LH on P_4 and PG production by granulosa cells in vitro. These data suggest that AngII facilitates or amplifies the action of LH on follicular steroidogenesis and PG production. We also observed a synergic effect of AngII and LH on COX-2, epiregulin, amphiregulin and ADAM17 expression in granulosa cells (Portela 2008). Taken in account that ACE is upregulated in theca cells and, concomitantly, there is an increase of AngII in follicular fluid after GnRH treatment, it is suggestive that theca cells could be producing the AngII that acts on granulosa cells. Further studies are necessary to identify the follicular site of AngII.

The present study reveals that the renin-angiotensin system is intensely controlled by the gonadotropin surge, especially during the initial period of the ovulatory cascade. The early changes in the AngII concentrations in follicular fluid evidence the key role of AngII in the initial process of ovulation. Moreover, the gradual increase in AngII in follicular fluid suggests a role at latter stages of the ovulatory process. Also, the differential mRNA expression of AGTR2 and ACE observed on theca cells after GnRH and the response of granulosa cells to AngII indicates that this peptide is one of the earliest players at the ovulatory cascade, eliciting the increase in P_4 and PG secretion for ovulation.

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Table 1 – Primers used in the expression analysis of candidate gens. Primer sequences and concentrations used to amplify each product are described.

Gene		Sequence	Conc. (μM)	Reference or accession nº
ACE	F	ACTCCTGGAGGTCCATGTACGA	200	ENSBTAT00000044222
	R	ACGTAGGCGTGCAGGTTCAG	200	
CYCLOPHILIN	F	GGTCATCGGTCTCTTTGGAA	200	Leudoux et al., 2006
	R	TCCTTGATCACACGATGGAA	200	
AGTR1	F	TTGACCGCTACCTGGCTATTG	200	ENSBTAT00000022540
	R	CCTGCCAGCAGCCAAATAAT	200	
AGTR2	F	GACCTGGCACTTCCTTTTGC	200	XM_001249373.1
	R	GGAGCTTCTGCTGGAACCTATTC	200	

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

Figure legends

Figure 1 – Concentration of angiotensin II (pg/ml ± SEM) in follicular fluid obtained from preovulatory follicles at 0, 3, 6, 12, or 24 h after GnRH analogue injection to induce an LH surge (n = at least 5/time point). Bars with no common letters are significantly different ($P < 0.05$).

Figure 2 – Relative mRNA expression of AGTR1 receptors in theca (Fig. 2a), granulosa cells (Fig. 2b), AGTR2 receptors in theca (Fig. 2c) and granulosa cells (Fig. 2d) obtained from periovulatory follicles at 0, 3, 6, 12, or 24 h after GnRH analogue injection to induce an LH surge (n = at least 5/time point). Within a panel, bars with no common letters are significantly different ($P < 0.05$).

Figure 3 - Relative mRNA expression of ACE receptors in theca (Fig. 3a) and granulosa cells (Fig. 3b) obtained from periovulatory follicles at 0, 3, 6, 12, or 24 h after GnRH analogue injection to induce an LH surge (n = at least 5/time point). Within a panel, bars with no common letters are significantly different ($P < 0.05$).

Figure 4 – Cumulative progesterone secretion (ng/ml ± SEM) between 36 and 48 h by theca (Fig. 4a) and granulosa cells (Fig. 4b) obtained from healthy dominant follicles (n = 3), cultured in medium alone, or with LH (100 ng/ml), LH + angiotensin II (ANG, 1 μM). Within a panel, bars with asterisk or with no common letters are significantly different ($P < 0.05$).

Figure 5 – Cumulative secretion (ng/ml ± SEM, between 36 and 48 h) of prostaglandin E_2 (Fig. 5a) and $F_2\alpha$ (Fig. 5b) by granulosa cells obtained from healthy dominant follicles (n = 3), cultured in medium alone, or with LH (100 ng/ml), LH + angiotensin II (ANG, 1 μM). Within a panel, bars with no common letters are significantly different ($P < 0.05$).

Figure 1 –

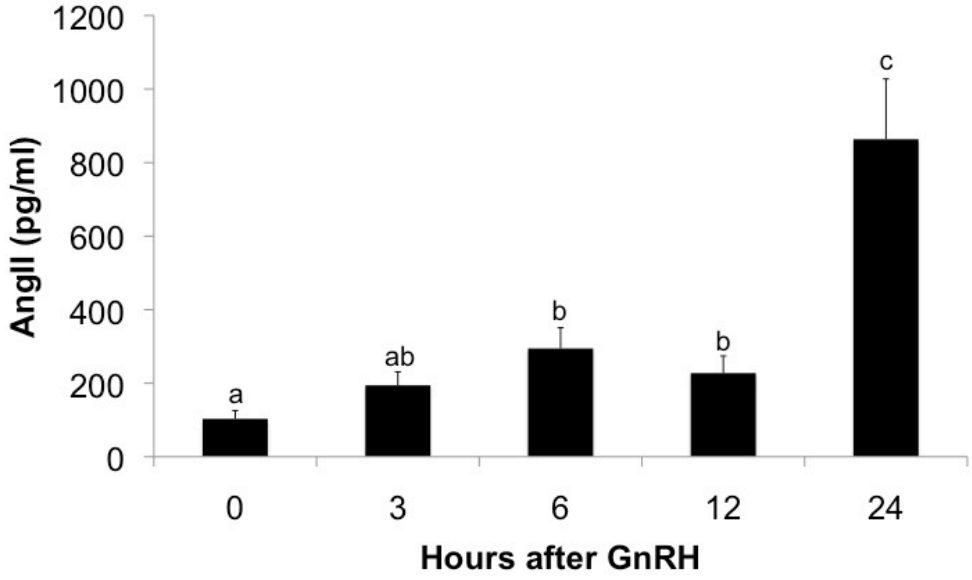
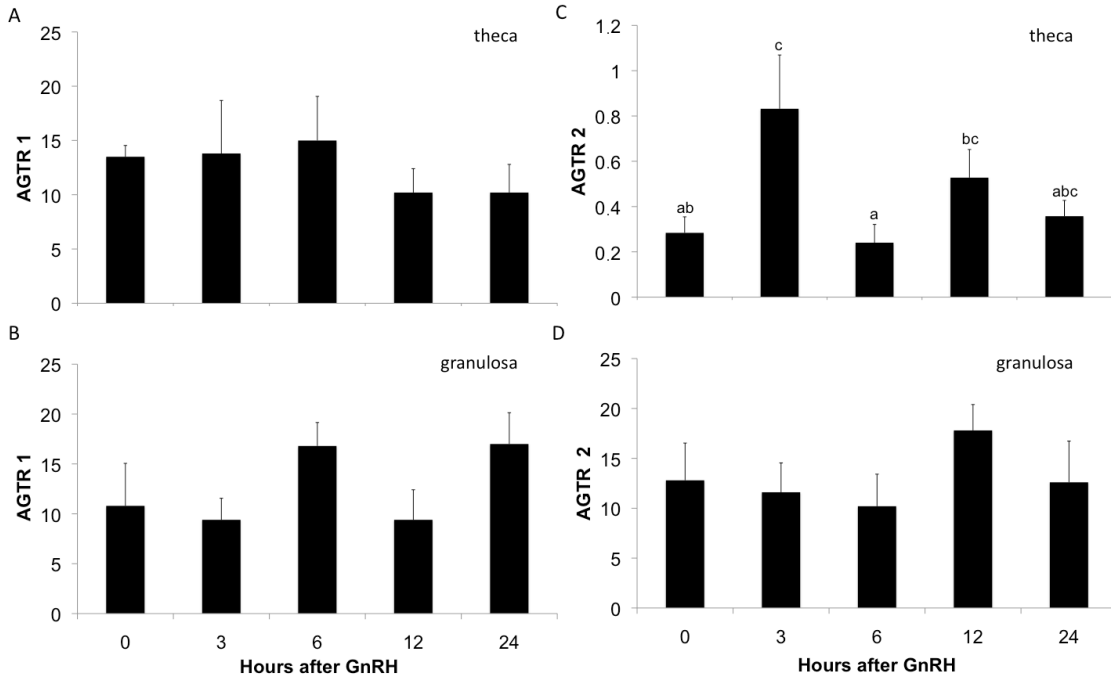

1200
1000
800
600
400
200
0
AngII (pg/ml)
a
ab
b
b
c
0
3
6
12
24
Hours after GnRH

Figure 2 -


A
theca
AGTR 1
25
20
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B
granulosa
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Hours after GnRH
C
theca
AGTR 2
1.2
1
0.8
0.6
0.4
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ab
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a
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abc
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granulosa
AGTR 2
Hours after GnRH

Figure 3 -

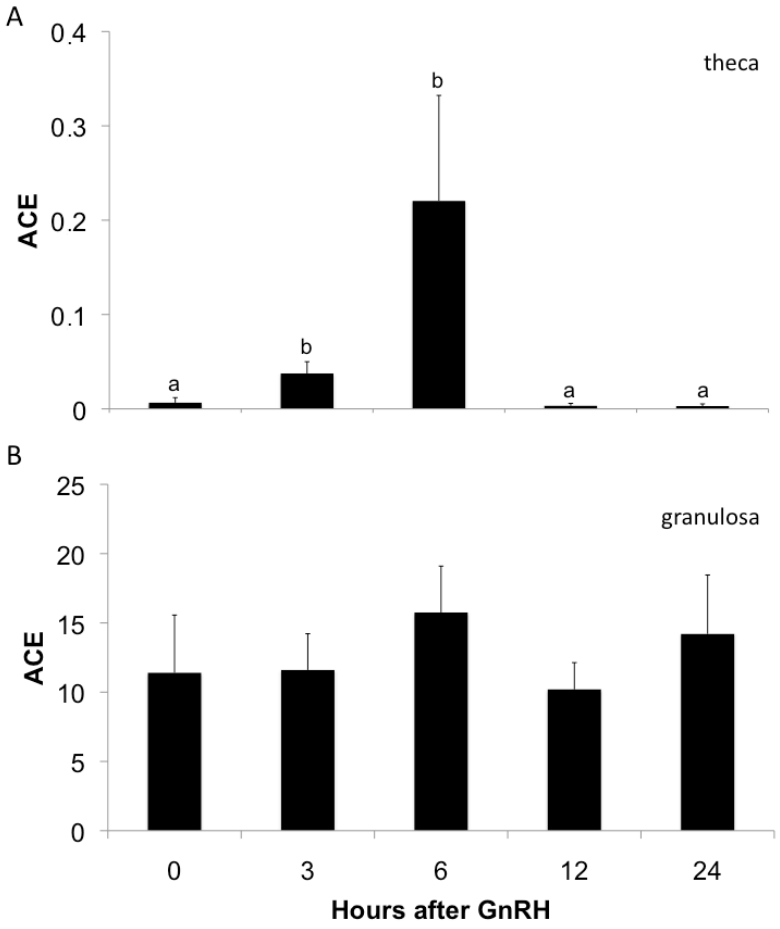

A
theca
ACE
0.4
0.3
0.2
0.1
0
a
b
b
a
a
B
granulosa
ACE
25
20
15
10
5
0
0
3
6
12
24
Hours after GnRH

Figure 4 –

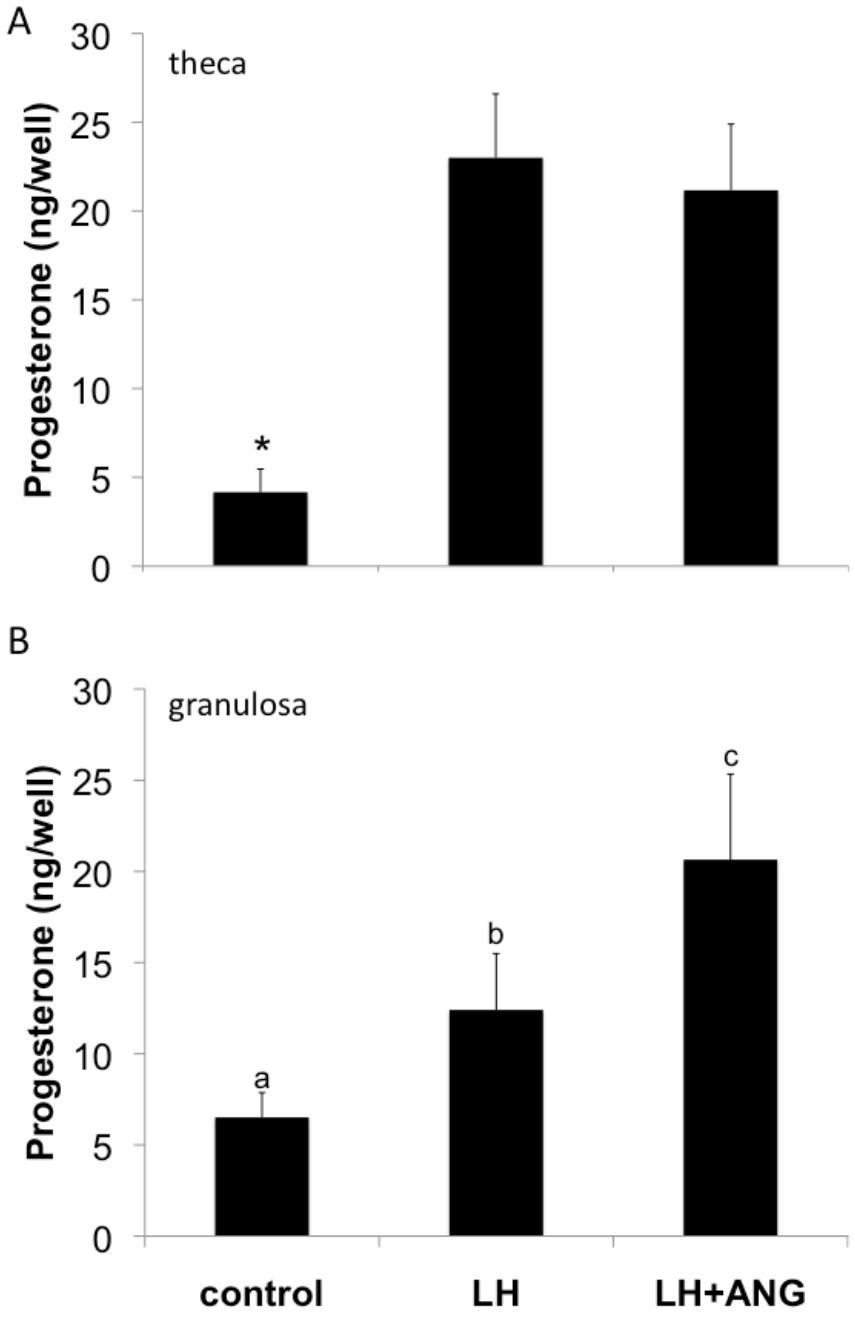
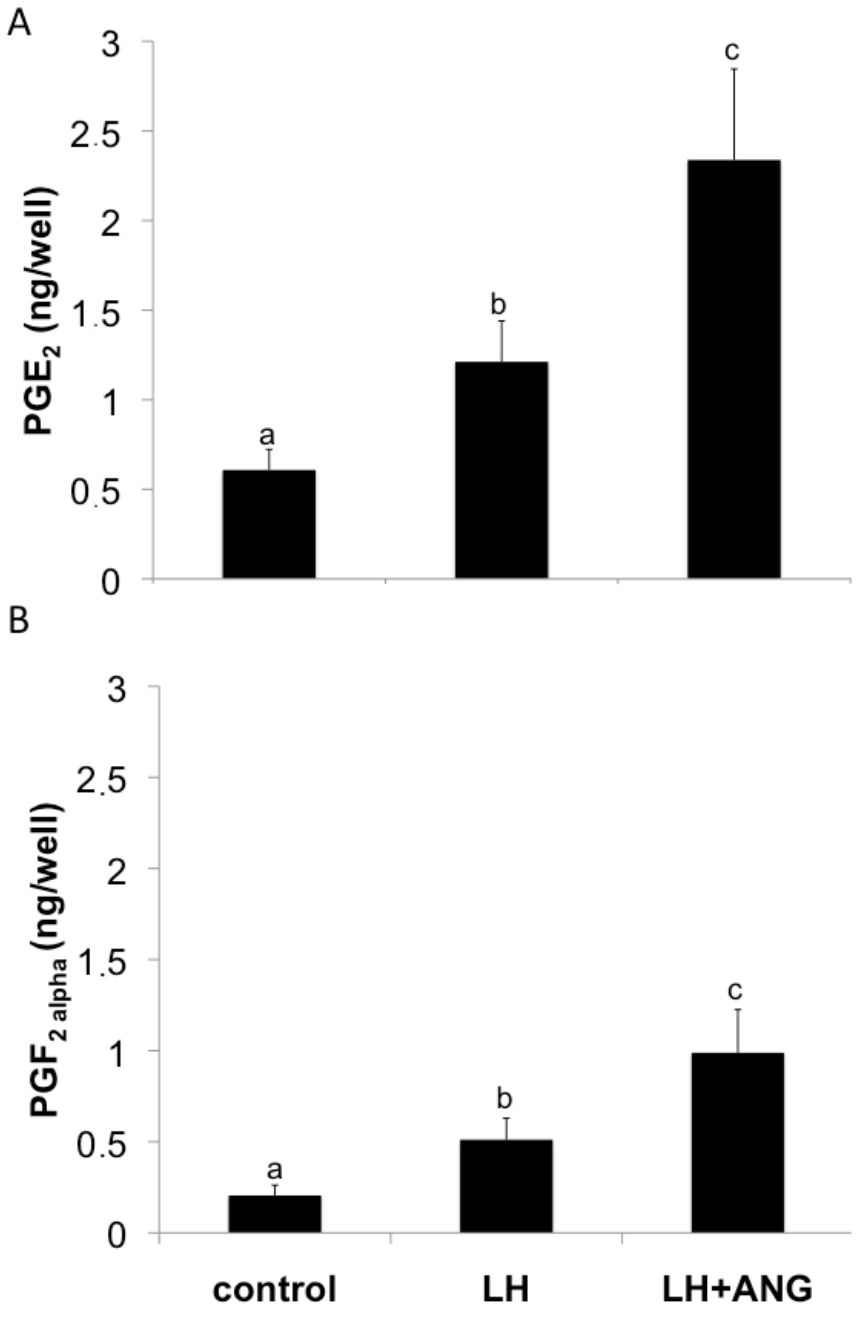

A
theca
Progesterone (ng/well)
30
25
20
15
10
5
0
*
B
granulosa
Progesterone (ng/well)
30
25
20
15
10
5
0
a
b
c
control
LH
LH+ANG

Figure 5 –


A
3
2.5
2
1.5
1
0.5
0
PGE2 (ng/well)
a
b
c
B
PGF2 alpha (ng/well)
a
b
c
control
LH
LH+ANG

4. CAPÍTULO 2

TRABALHO À SER ENVIADO PARA PUBLICAÇÃO:

Angiotensin II, progesterone and prostaglandins are sequential steps in pathway to oocyte nuclear maturation

Lucas Carvalho Siqueira, Marcos Henrique Barreta, Bernardo Gasperin, Rodrigo Bohrer, Joabel Santos, Jose Buratini Junior, João Francisco Oliveira, Paulo Bayard Gonçalves

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Angiotensin II, progesterone and prostaglandins are sequential steps in pathway to oocyte nuclear maturation

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Abstract

The oocyte meiotic resumption is triggered by the ovulatory gonadotropin surge. Angiotensin II (AngII) and prostaglandins (PG) are key mediators of this gonadotropin-induced event in bovines. Here, we tested the hypothesis that progesterone (P_4) is also involved in oocyte meiotic resumption induced by gonadotropin surge. In experiment I, we demonstrated that P_4 induces nuclear maturation in a dose-dependent manner using a co-culture of follicular hemisections and cumulus-oocyte complexes. In the second experiment, using an in vivo model, an injection of Saralasin (AngII receptor antagonist) at the antrum of preovulatory follicles prevented GnRH-induced oocyte meiotic resumption in vivo. In experiment III, with similar co-culture system from experiment I, P_4 receptor antagonist prevented AngII stimulatory effects on resumption of meiosis, but Saralasin (AngII receptor antagonist) did not inhibited P_4 actions. These results evidenced that AngII is upstream to P_4 in a same cascade to induce meiotic resumption. Nothing was known on the role of fibroblast growth factor 10 (FGF10) on in the meiotic resumption of oocytes. In experiments IV and V we demonstrated that FGF10 blocked AngII- but not progesterone-induced oocyte meiotic resumption. This is the first evidence that FGF10 is a follicular factor that maintains oocyte nuclear progression arrested during folliculogenesis. These results also suggest that steroids may counteract FGF10 effects. Previously, we had shown that AngII acts throughout the PGs pathway to modulate nuclear progression. In experiment V, indomethacin inhibited resumption of meiosis induced by P_4, providing further support to the AngII-P_4 sequential effect on meiotic resumption. In conclusion, our study suggests that AngII, P_4 and PGs are sequential steps in the same pathway that culminates with oocyte maturation. Also, it identifies FGF10 as a candidate factor that maintains mammals oocytes arrested at GV stage during folliculogenesis.

Key words: ovulation; meiotic resumption; Angiotensin; steroid; eicosanoid.

1. Introduction

The preovulatory gonadotropin surge triggers a cascade of events that culminates with ovulation and nuclear oocyte maturation. Recently, Angiotensin II (AngII) has been recognized as one of the earliest mediators of gonadotropin-induced ovulation and oocyte maturation [1-3]. The positive effect of AngII in these processes is mediated through the type 2 receptor [2,4]. Furthermore, it was demonstrated that concentration of AngII and expression of its receptors (AT2) increase intrafollicularly during the time between gonadotropin surge and ovulation (Siqueira et al., unpublished data). Other studies provided additional evidence that AngII regulates secretion of progesterone (P_4) and prostaglandins (PGs), hormones involved in the ovulatory process [5,6]. In granulosa cell culture, AngII upregulated the expression of cyclooxigenase 2 (COX-2), the rate-limiting enzyme for PGs production [7].

Bovine oocytes remain arrested at the prophase of the first meiotic division, during follicle development and resume meiosis after preovulatory luteinizing hormone (LH) surge [8] or after removal from follicular environment [9]. The presence of follicular wall fragments in a co-culture system with cumulus-oocyte complexes (COCs) prevents meiotic resumption [10]. This co-culture system has proven to be a good model to study the role of factors that act through follicular cells on oocyte nuclear maturation [11,12]. Using this co-culture system, we have shown that AngII acts through PGs pathway to mediate gonadotropin-induced oocyte meiotic resumption [1].

The cyclooxygenase pathway is a classical mediator of LH-induced ovulation and nuclear oocyte maturation in bovines. Progesterone is another key element in the ovulatory cascade. Indeed, there are evidences that PGs are downstream factors to this steroid, where gonadotropin surge stimulates an increase in intrafollicular P_4 and that P_4 acts by binding to its nuclear receptor and increasing the abundance of mRNA for COX2. The role of P_4 on oocyte nuclear maturation in cattle is still controversial. Although Sirotkin [13] reported a stimulatory effect on oocyte meiotic resumption, more recent studies concluded that P_4 is not necessary to promote nuclear maturation, cumulus expansion and early embryo development [14,15].

Follicular cells secrete factors that prevent the oocyte meiotic resumption before the LH surge in vivo. The family of fibroblast growth factors (FGFs) is composed of more than 20 factors, largely studied on embryogenesis, oogenesis. Buratini et al. [16] showed that the bovine theca cells and oocytes express FGF10. The expression of FGF10 receptor (FGFR2IIIb) was identified in theca [17], granulosa [16] and cumulus cells [18]. FGF10 in

the granulosa cell culture inhibits steroidogenesis [16] and AT2 expression [3]. Although the activation of FGFs receptors (FGFRs) appears to be involved in the inhibition of germinal vesicle breakdown in mice (GVBD; [19], nothing was known about the role of FGF10 on oocyte nuclear maturation in mammals until recently. Zhang et al. [20] reported that FGF10 improves oocyte maturation, cumulus expansion and subsequent embryo development when added to the maturation medium with estradiol.

In the present study, we examined the hypotheses that P_4 are involved in the cascade, with participates of oocyte meiotic progression induced by gonadotropin surge in concert with AngII, PGs and FGF10 is an inhibitory factor in the oocyte meiotic resumption process.

2. Materials and methods

All experimental procedures were reviewed and approved by the Federal University of Santa Maria Animal Care and Use Committee.

2.1. Preparation of follicular hemisections, oocyte recovery and nuclear 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 ^{o}C containing 100 IU/ml penicillin and 50 μg/ml streptomycin sulfate (All chemicals used were purchased from Sigma Chemical Company, unless otherwise indicated in the text). The dissection and follicle culture procedures have been previously validated in our laboratory [1,11,12]. Briefly, transparent follicles measuring 2–5 mm in diameter were selected and dissected from ovarian stromal tissue, and sectioned into halves. Follicular hemisections were washed in TCM 199 containing 0.4% bovine serum albumin (BSA), randomly distributed into 4-well culture dishes containing culture medium with desired treatment (Nunc®, Roskilde, Denmark; eight follicular halves per 200 μl of medium) and incubated for 2 h before adding the COCs.

COCs were aspirated from follicles between 3 to 8 mm in diameter, recovered under a stereomicroscope and selected according to Leibfred & First [21]. Grade 1 and 2 COCs (n=10–30) were randomly distributed into treatments and cultured in an incubator at 39°C in a saturated humidity atmosphere containing 5% CO2 and 95% air, for either 7, 15 or 24 h depending on the experiment. The culture medium used was TCM 199 containing Earle's salts and L-glutamine (Gibco Labs) supplemented with 25 mM HEPES, 0.2 mM pyruvic acid, 2.2 mg/ml sodium bicarbonate, 5.0 μg/ml LH (Lutropin-V, Bioniche, Ontario, CA, USA), 0.5 μg/ml FSH (Folltropin-V, Bioniche), 0.4% fatty acid-free BSA, 100 IU/ml penicillin, and 50

μg/ml streptomycin sulfate. At the end of the culture period, the cumulus cells were removed by vortexing and oocytes fixed with triton 100x, and classified according to their nuclear chromatin configuration using a fluorescent microscope (Hoechst staining) as germinal vesicle (GV), GV breakdown (GVBD), metaphase I (MI), anaphase I (AI), telophase I (TI), and metaphase II (MII). In all experiments, all treatments were repeated three times.

2.2. Animals, superovulation protocol and ultrasound-guided intrafollicular injection

The superovulation protocol and intrafollicular injection procedures were previously described by [1]. Five cycling cows (Bos taurus taurus), multiparous, with body condition scores of 3 and 4 (1-thin, 5-obese) were submitted to the 9-days "progesterone/FSH-based" superovulation protocol. On day 9 of the progesterone treatment, the number of follicles in the ovary was evaluated by ultrasound scanning and all follicles with size from 5 to 11 mm were aspirated using a vacuum pump, remaining no more than the three largest follicles in each ovary. In the afternoon of day 10, after the intravaginal device had been removed, an ultrasound image of each ovary was processed to produce a map of the follicles in the ovary and follicles greater than 12 mm in diameter were intrafollicularly injected.

The intrafollicular injections were guided by ultrasound equipped with a 7.5 MHz transducer attached to a biopsy guide and a scanner (AquilaVet Scanner; Pie Medical Equipment BV, Maastricht, The Netherlands). A system with two sterile needles was used as previously described by [2]. Briefly, the ovary was manipulated to introduce the needle into the follicle via the ovarian stroma at the base of the follicle. When the ovary and follicle were in position, the outer needle was advanced until the image of its tip became visible on the screen, 3–5 mm from the follicle. At this moment, a second operator pushed the inner needle forward until the image of the needle tip was visible within the follicle. Treatments were then injected into the follicle. The amount of treatment for the injection was calculated based on the volume of follicular fluid, to obtain an adequate final concentration inside the follicle. The follicular fluid volume was estimated by the linear regression equation as described by [2].

Experiment I - Progesterone induced oocyte nuclear maturation

The first experiment was designed to assess the P_4 effect on nuclear maturation of oocytes (n = 565) cultured with follicular hemisections treated with 0, 10, 100, 1,000 or 10,000 ng/ml of P_4. After 22 h of culture, oocytes were considered mature when classified as AI, TI or MII.

Experiment II - Effect of progesterone antagonist on LH-induced meiotic resumption in vivo

As described above, five cows were primed for superovulation and manipulated to present at the time of injection no more than three follicles (>12mm) in each ovary. Follicles from the right ovaries were treated to obtain a final concentration in follicular fluid of 1 μM of mifepristone (Mife group; n=10) and those from the left ovaries were treated with 0.9% saline (control group; n=10). Immediately after the intrafollicular injections, the cows received 100 μg of gonadorelin acetate intramuscularly (GnRH agonist; Profertil, Tortuga, Brazil). Fifteen hours after GnRH injection, the animals were ovariectomized by colpotomy. COCs were recovered, processed as described above. Oocytes at GVBD or MI stages were considered as having resumed meiosis.

Experiment III - Progesterone mediates AngII-induced meiotic resumption

COC (n=540) were selected and distributed among the following seven groups for 15h of culture: positive and negative controls; AngII (10^{-11}M); AngII plus Mifepristone (MIFE; 1μM; P_4 antagonist); P_4 (100ng/ml), P_4 plus saralasin (10^{-5}M; AngII antagonist); and AngII plus saralasin. In all groups, except positive control, follicular hemisections and COCs were co-cultured. Oocytes in MI or latter stages were considered to have a normal resumption of meiosis.

To verify a possible toxic effect of P_4 antagonist, COCs were cultured for 22h, without follicular hemisections, in absence or presence of Mife (1μM). Oocytes were considered mature when classified as AI, TI or MII.

Experiment IV - Effect of FGF10 on AngII-induced meiotic resumption

Control COCs were cultured in medium in the absence (positive control; n=84) or presence (negative control; n=88) of follicular hemisections for 7 hours. Four treatment groups were established. COCs were cultured in presence of either: a) AngII (10^{-11}M; n=83) with follicular hemisections; b) AngII and FGF10 (100 ng/ml) with follicular hemisections (AngII+FGF10 group; n=82); c) FGF10 with follicular hemisections (FGF10+cells group; n=80) and d) FGF10 without follicular hemisections (FGF10 group; n=88). Oocyte nuclear chromatin configuration was classified as germinal vesicle (GV) or germinal vesicle breakdown (GVBD).

Experiment V - Effect of FGF10 or indomethacin on progesterone-induced meiotic

resumption

Control COCs were cultured in culture medium in the absence (positive control; n=85) or presence (negative control; n=82) of follicular hemisections. Three treatment groups were established. COCs were co-cultured with follicular cells in presence of either: a) progesterone (100 ng/ml; P_4 group; n=84); b) P_4 plus FGF10 (100 ng/ml; P_4+FGF10 group; n=80) and c) P_4 plus indomethacin (a COX nonselective inhibitor; 10μM, P_4+indo group; n=85). Oocyte nuclear chromatin configuration was classified as germinal vesicle (GV) or germinal vesicle breakdown (GVBD).

2.3. Statistical analysis

The results of the experiments I, III, IV and V were analyzed using the ANOVA test in a statistical model for categorical data, using the PROC CATMOD (Categorical Data Analysis Procedures). All in vitro experiments were performed in triplicate. On detecting statistical differences, the independent variables were compared using the contrast test. Data were analyzed using the statistical analysis software (SAS; SAS Institute Inc., Cary, NC, USA). In experiment II, meiotic resumption was compared using the generalized linear models from JMP software (SAS Institute Inc., Cary, NC).

3. Results

Experiment I - Progesterone induced oocyte nuclear maturation

The hypothesis tested in this experiment was that P_4 induces nuclear maturation in bovine oocytes. Bovine COCs, recovered from abattoir ovaries, were co-cultured with follicular hemisections for 22 h with P_4 at concentrations of 0, 10, 100, 1.000 or 10.000 ng/ml. Progesterone induced the nuclear maturation in bovine oocytes cultured with follicular cells in a dose-dependent manner (Fig. 1). The highest MII rate was observed when oocytes were cultured with follicular cells treated with 100 ng/ml of P_4 ($P<0.01$).

Experiment II - Effect of progesterone antagonist on LH-induced meiotic resumption

Once P_4 stimulated nuclear maturation in vitro, we tested whether the LH-induced resumption of meiosis is mediated by progesterone using an in vivo model. Oocytes were recovered at 15 h after GnRH treatment and 24 follicles were intrafollicularly injected with either saline or progesterone receptor antagonist (Mife). The injected follicles were localized

by visual inspection after ovariectomy and 20 oocytes were recovered and evaluated (10 from each group). As expected, GnRH induced 90% of meiotic resumption in oocytes from saline treated follicles (10% of GV, 10% of GVBD and 80% of MI). On the other hand, most of the oocytes were arrested in GV stage when Mife was intrafollicularly injected (70% of GV, 10% of GVBD and 20% of MI; $P<0.01$; Fig. 2).

Experiment III - Progesterone mediated AngII-induced meiotic resumption

Considering that the role of AngII in resumption of meiosis and ovulation is well established, we tested the hypothesis that AngII is an upstream factor to P_4 in the cascade of meiotic resumption. As expected, the meiotic resumption was inhibited when the COCs were co-cultured with follicular hemissections (Fig. 3; positive vs. negative controls). With this model, we observed that AngII or P_4 induced the meiotic resumption (61% and 66%, respectively, compared with 32% of the negative control; $P<0.01$). However, AngII did not induce the resumption of meiosis when saralasin (AngII antagonist) or mifepristone (P_4 antagonist) was present in the maturation medium. Independently of the presence of saralasin, the majority of the oocytes reached MI stage in the presence of P_4. A further experiment was done, culturing COCs without follicular hemisections for 22 h with or without mife to exclude a detrimental effect on oocyte maturation. The oocytes treated with MIFE reached a similar rate of nuclear maturation (88%) to that of oocytes cultured in the control medium (85%). Thus, these experiments revealed that AngII acts early than P_4 on the signaling event cascade that regulates meiotic resumption.

Experiment IV - Effect of FGF10 on AngII-induced meiotic resumption

FGF10 is an anti-steroidogenic factor that regulates negatively AngII type 2 receptor expression on granulosa cells [3,16]. Our hypothesis was that FGF10 has a negative role in the resumption of meiosis induced by AngII. In the absence of follicular cells, the meiotic resumption rate was not different between positive control and FGF10-treated COCs after 7 h of culture (Fig. 4). Also, FGF10 did not affect follicular cells ability to prevent oocytes from resuming meiosis. However, FGF10 inhibited the AngII effect in follicular cells. Oocytes cultured simultaneously with AngII and FGF10 reached 32% of GVBD while those cultured only with AngII achieved 62% ($P<0.01$; Fig. 4).

Experiment V - Effect of FGF10 or indomethacin on P_4-induced meiotic resumption

Finally, we examined the role of FGF10 in the oocyte meiotic resumption induced by

P_4 and tested the hypothesis that P_4 acts through PGs to induce resumption of meiosis. The number of oocytes that resumed meiosis in response to P_4 treatment was not affected by FGF10. However, indomethacin (a nonselective PG antagonist) inhibited P_4 effect (Fig. 5).

4. Discussion

In the present study, we tested the hypotheses that P_4 is an intermediate factor between AngII and PGs in the meiotic resumption stimulatory cascade and that FGF10 is an inhibitory factor to this event. The main findings are: 1) Progesterone induced bovine oocyte nuclear maturation in a dose dependent manner; 2) a P_4 receptor antagonist (mife) inhibited GnRH-induced oocyte meiotic resumption in vivo; 3) Mife inhibited oocyte meiotic resumption induced by AngII, whereas an AngII receptor antagonist did not interfere on P_4 stimulatory effect; 4) P_4-induced oocyte meiotic resumption was blocked by Indomethacin (cox non-selective inhibitor); and 5) FGF10 inhibited AngII- but not P_4-induced oocyte meiotic resumption. Previously, we have shown that AngII acts through PGs to mediate LH-induced oocyte meiotic resumption [1] and that AngII in synergism with LH induces P_4 and PG synthesis in the bovine dominant follicle (Siqueira et al. unpublished). Taken together, these results suggest that AngII is up stream to progesterone in pathway to induce oocyte nuclear maturation that is initiated by gonadotropin surge and stimulate PGs.

In this study, we used two experimental models already established. In the first approach, the spontaneous meiotic progression was inhibited in a co-culture system with oocyte and follicular hemisections [10,11]. With this model, we found that P_4 stimulates oocyte nuclear maturation in a dose-dependent manner. In the second model, the cows were superovulated and, after GnRH challenge, intrafollicular injections guided by ultrasound were performed in the right (treatment) and left (control) ovaries [1,2]. With this in vivo experiment, we demonstrated that P_4 receptor antagonist (Mife) inhibited oocyte meiotic resumption. Progesterone also participates in the oocyte nuclear maturation in primates and swine [22,23]. In monkeys the inhibition of follicular progesterone production by trilostane (steroid synthesis inhibitor) did not reduce gonadotropin-induced oocyte maturation, but augmented the percentage of degenerated oocytes [23]. In pigs, the treatment of COCs with mife modified pattern of expression of P_4 receptors in cumulus and reduced progesterone synthesis.

We showed that P_4 is in the pathway of oocyte meiotic resumption mediated by AngII [1] and PGs [24,25]. Herein, we confirmed the hypothesis that AngII is upstream to P_4 in the

cascade of resumption of meiosis. In experiment III, a P_4 receptor antagonist prevented AngII stimulatory effects on resumption of meiosis, but saralasin (AngII receptor antagonist) did not inhibited P_4 actions. There are evidences that AngII stimulatory effects on oocyte nuclear maturation are mediate by PGs [1]. In experiment V, we observed that indomethacin inhibited resumption of meiosis induced by P_4, suggesting that PGs also mediate this steroid effect. Progesterone is essential to induce PG secretion during the ovulatory process [6] and recently, we have demonstrated that AngII has a synergistic action with LH to induce the production of P_4 and PGs by granulosa cells from large dominant follicles (SIQUEIRA, et al., unpublished). Together these data evidence that AngII, P_4 and PGs are sequential steps from the same pathway.

Toxic effects should be accountable for none of the inhibitory effects caused by the antagonists used. Saralasin, mife and indomethacin have been proven to be safe for cell viability and function [1,6]. Indeed, in the present study, saralasin did not affected P_4-induced meiotic resumption. Also, mife in absence of follicular hemisections (Exp. III) did not impaired oocyte nuclear maturation.

FGF10 inhibited the positive effect of AngII but not of progesterone on oocyte meiotic resumption. To our knowledge, this is the first evidence to indicate that FGF10 is an inhibitor of meiotic resumption in mammals. Despite cumulus cells also express FGF10 receptors [18], FGF10 did not affect meiotic resumption rate in the absence of follicular cells. These results suggest that FGF10 inhibited meiotic progression by acting on the follicular wall. Indeed, FGF10 may be acting on AngII-induced meiotic resumption by modulating steroid production in follicular cells. Type II receptors for AngII (AT2) seem to transduce AngII positive signal for resumption of meiosis in oocytes and ovulation [2,4]. FGF10 down-regulates the expression of AT2 receptors in follicular cells [3] and inhibits steroidogenesis [16]. Activation of FGFR2IIIb (FGF10 receptor) inhibits gonadotropin-induced progesterone secretion in granulosa cells [26]. Therefore, FGF10 could be exerting its negative effect through a downregulation of AT2 expression and, consequently, decreasing AngII-stimulated progesterone synthesis or directly inhibiting follicular cell steroidogenesis.

It seems important to point out that the addition of estradiol to culture media could be detrimental to early embryo development [15]. Therefore, the improvement in oocyte competency induced by FGF10 when CCOs were cultured in presence of estradiol reported by Zhang et al. [20] could be due to its anti-esteroidogenic effects. In their culture system, FGF10 may be counteracting the negative effects caused by estradiol. Nevertheless, further studies are necessary to elucidate the role of FGF10 on bovine oocyte nuclear maturation.

In summary, the results from the present work demonstrated that in bovine P_4, lsimilarly AngII and PGs, mediates the resumption of meiotic progression induced by gonadotropin surge. Indeed, our study suggests that AngII, P_4 and PGs are sequential steps in the same pathway that culminates with oocyte maturation. Also, we identify FGF10 as a candidate factor that maintains mammalian oocytes arrested at GV stage during folliculogenesis. Taken together with other studies from our group, it is possible to propose a model (Fig. 6) in which the gonadotropin surge stimulates a single cascade of events to induce ovulation and nuclear oocyte maturation. In this model, gonadotropin surge stimulates AngII secretion and upregulates AT2 expression in follicular cells. AngII increases follicular cells secretion of P_4 that in the sequence stimulates PGs. Therefore this sequence of events culminates with the ovulation of a fertile oocyte.

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Figure Legends

Figure 1 - Effect of progesterone on oocyte nuclear maturation. Metaphase II rates (solid bars) and predicted regression line after co-culture of bovine oocytes (n=565) and follicular hemisections treated for 22h with different concentrations of P_4 (0, 10, 100, 1.000 or 10.000 ng/mL; $P<0.01$). The experiment was performed in triplicate.

Figure 2 – Effect of progesterone antagonist on LH-induced meiotic resumption in vivo. When the follicles reached 12mm, cows were challenged with GnRH (100 mg gonadorelin acetate, i.m) and follicles were ultrasound-mediated intrafollicular injected with saline (n=10) or progesterone receptor antagonist (Mife; n=10). Ovaries were obtained by ovariectomy 15 h after intrafollicular injection and oocytes were collected by follicular aspiration to determine the stage of nuclear maturation. *indicates statistical difference between groups ($P<0.01$).

Figure 3- Angiotensin II (AngII) and Progesterone (P_4) in the cascade of oocyte meiotic resumption. The cumulus–oocyte complexes (n=540) were co-cultured for 15 h with follicular cells and AngII, AngII plus Mifepristone (Mife), P_4, P_4 plus saralasin, and AngII plus saralasin. The experiment was performed in triplicate. Significant differences ($P<0.01$) are indicated by different letters.

Figure 4 – Effect of FGF-10 on AngII-induced meiotic resumption. Cumulus-oocyte complexes (n=505) were cultured for 7h with or without follicular hemisections treated with fibroblast growth factor 10 (FGF10) and/or angiotensin II (AngII). The experiment was performed in triplicate. Bars with no common letters are significantly different ($P\leq0.05$) between groups.

Figure 5 - Meiotic resumption after co-culture of bovine oocytes (n=416) and follicular hemisections treated with progesterone (P_4), P_4+fibroblast growth factor 10 (FGF10) or P_4+indomethacin (indo) for 7h. The experiment was performed in triplicate. Different letters indicate statistical significance ($P\leq0.05$) between groups

Figure 6 - Proposed model for a single cascade of events to induce ovulation and nuclear oocyte maturation.

Figure 1.

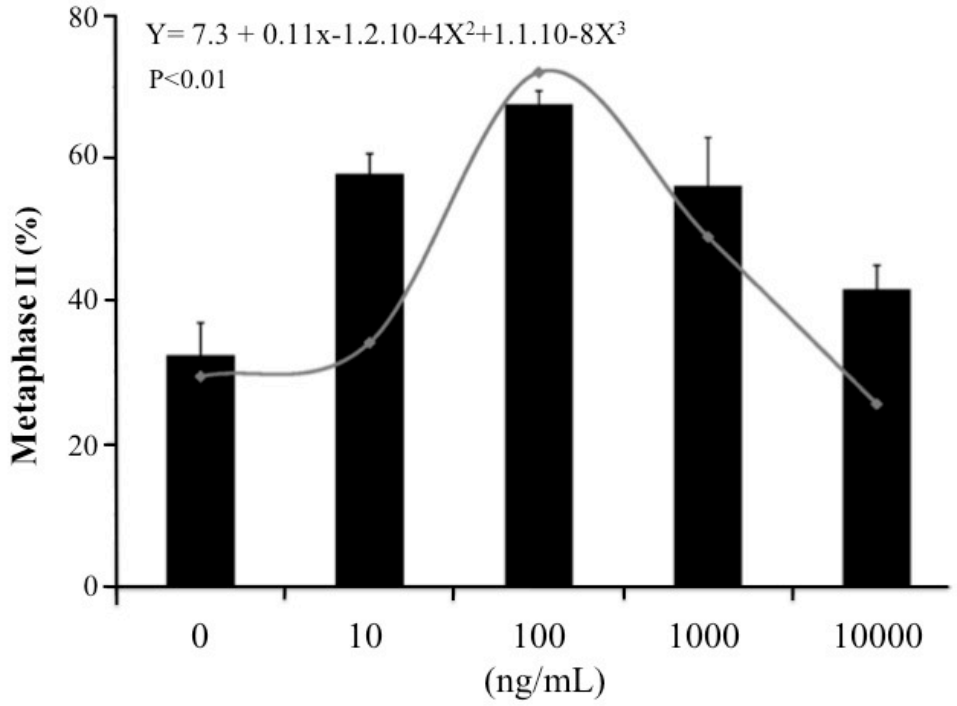

Y= 7.3 + 0.11x-1.2.10-4X2+1.1.10-8X3
P<0.01
Metaphase II (%)
80
60
40
20
0
0
10
100
1000
10000
(ng/mL)

Figure 2.

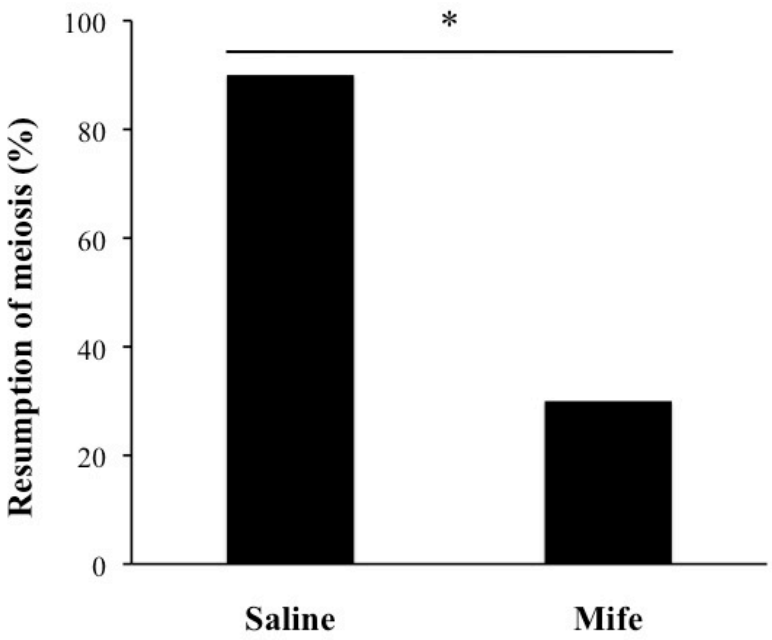


Figure 3.

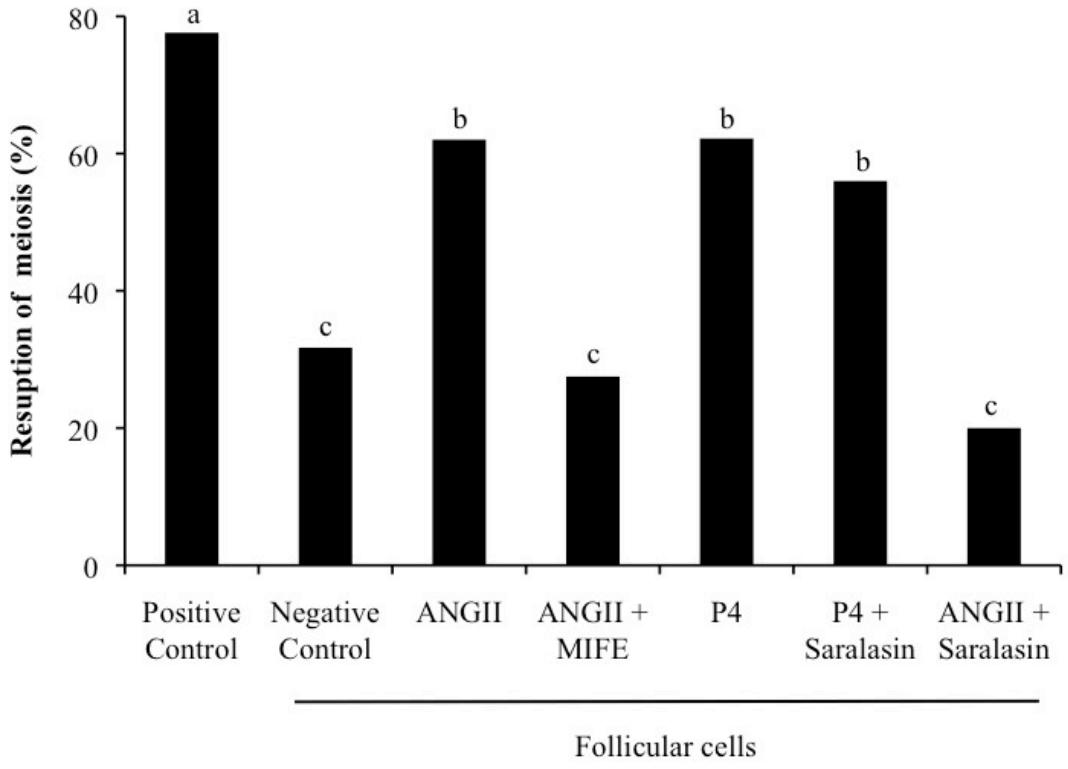

Resuption of meiosis (%)
80
60
40
20
0
a
c
b
c
b
b
c
Positive Control
Negative Control
ANGII
ANGII + MIFE
P4
P4 + Saralasin
ANGII + Saralasin
Follicular cells

Figure 4.

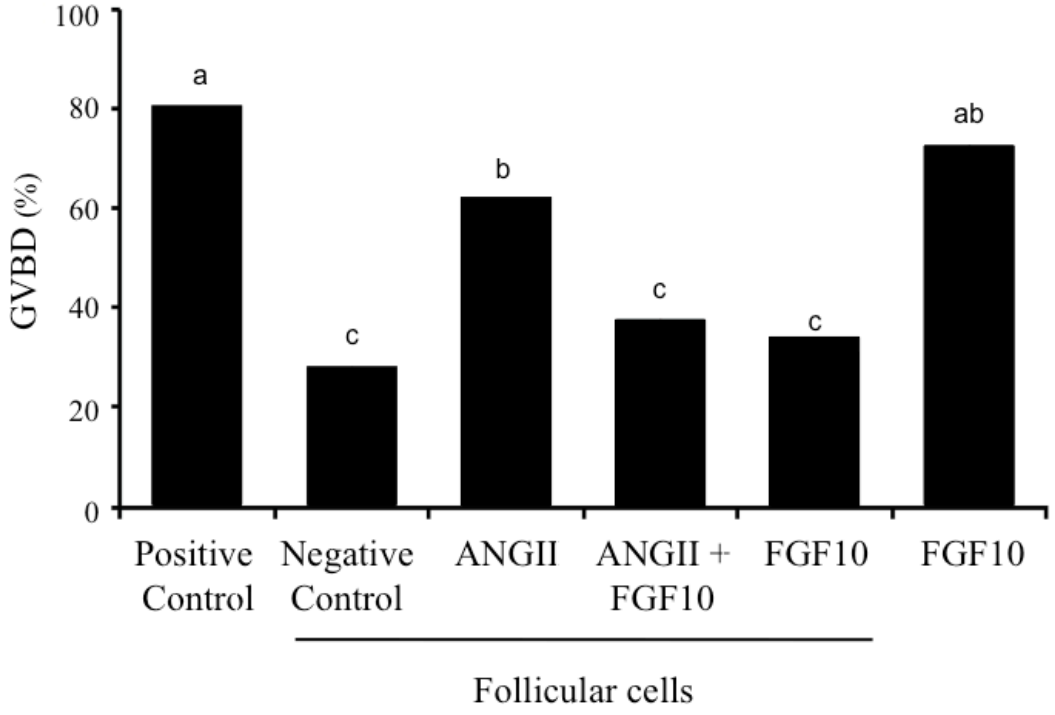

100
80
60
40
20
0
GVBD (%)
a
c
b
c
c
ab
Positive Control
Negative Control
ANGII
ANGII + FGF10
FGF10
FGF10
Follicular cells

Figure 5.

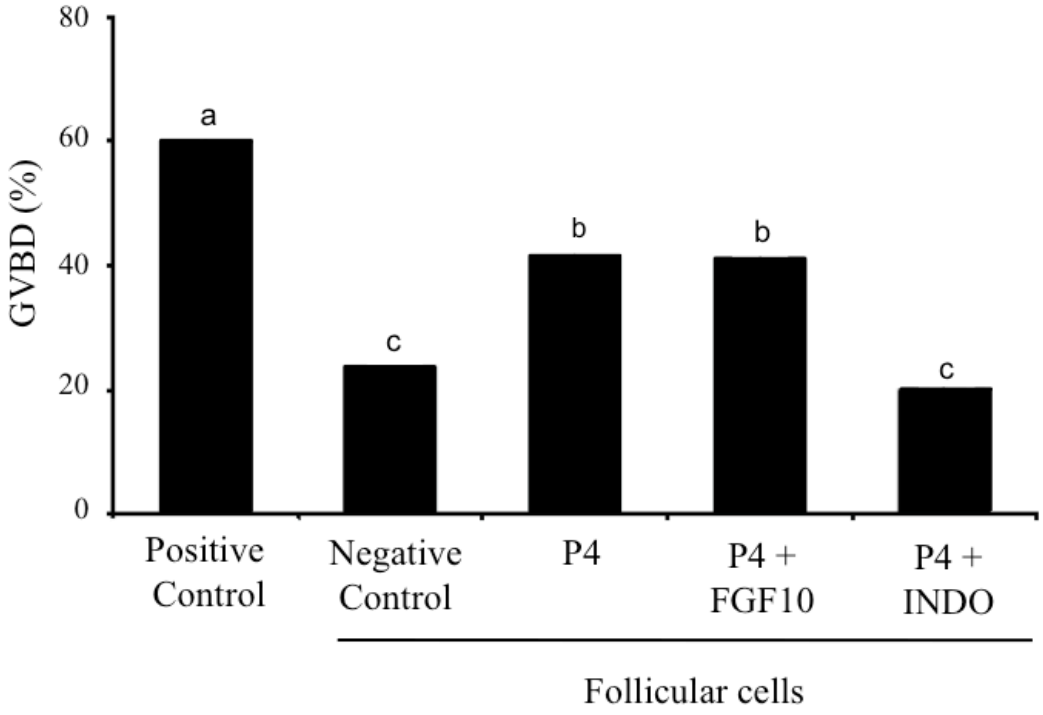
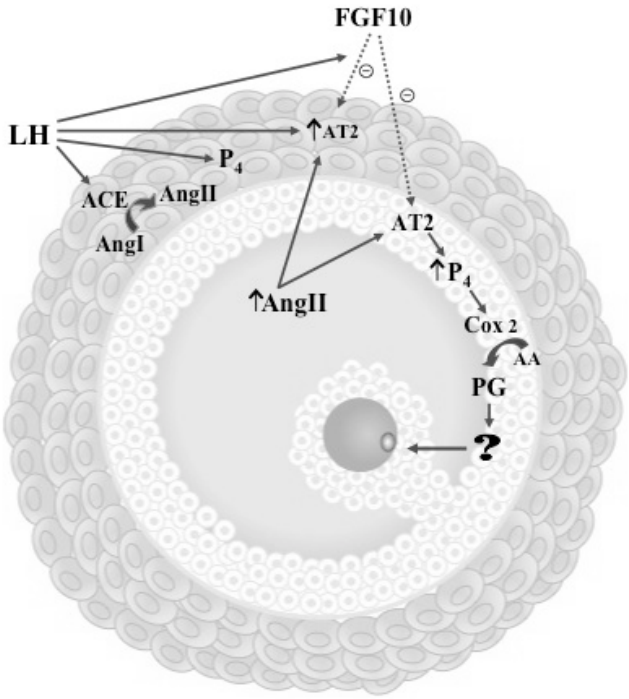

80
60
40
20
0
GVBD (%)
a
c
b
b
c
Positive Control
Negative Control
P4
P4 + FGF10
P4 + INDO
Follicular cells

Figure 6.



AA: Arachidonic acid; ACE: Angiotensin-converting enzyme; AngI: Angiotensin I; AngII: Angiotensin II; AGTR2: AngII type II receptors; FGF10: Fibroblast growth factor 10; LH: Luteinizing Hormone; PG prostaglandins; P_4: Progesterone.

5. DISCUSSÃO

Experimentos prévios sugerem que a AngII, a P_4 e as PGS são fatores essenciais para que ocorra a ovulação com liberação de um oócito fértil. Esse trabalho buscou entender como estes dois sistemas se encadeiam durante o processo ovulatório. Os principais achados foram: 1) o tratamento com GnRH estimula a produção de AngII no líquido folicular e a expressão de RNAm para receptores do tipo AGTR2 nas células da teca; 2) AngII em sinergismo com LH estimula a síntese de P_4 e PGs nas células da granulosa; 3) P_4 estimula a maturação nuclear de oócitos bovinos; 4) AngII antecede a P_4 que por sua vez antecede as PGs no processo de estímulo à progressão meiótica de oócitos; e 5) FGF10 é um possível inibidor do processo de maturação nuclear de oócitos durante a foliculogênese.

Diversos achados evidenciam a participação da AngII no processo ovulatório de bovinos. No entanto, apesar de dados de literatura sugerirem que as concentrações foliculares de AngII aumentariam de forma modesta em períodos próximos a ovulação (ACOSTA et al., 2000) os resultados de nosso laboratório evidenciam uma participação precoce de AngII na cascata ovulatória (FERREIRA et al., 2007; PORTELA et al., 2008b). Buscando uma melhor identificação do envolvimento da AngII nessa cascata, realizamos um estudo de caracterização da expressão de genes de interesse em células de folículos pré-ovulatórios e avaliamos a concentração de AngII no fluido folicular em diversos momentos após a administração de GnRH. Neste sistema, GnRH induz o pico de gonadotrofinas em até duas horas e a ovulação em aproximadamente 29 horas (KOMAR et al., 2001). Corroborando com nossa hipótese de envolvimento precoce da AngII, a concentração do peptídeo no líquido folicular e a expressão de RNAm para seus receptores AGTR2 e da enzima ECA (converte AngI em AngII) aumentaram logo após o momento esperado do pico de gonadotrofinas. Estes achados evidenciam um sistema ativo durante a peri-ovulação e com resposta rápida às gonadotrofinas.

No segundo experimento buscávamos entender se a AngII é capaz de modular a secreção de esteróides e PGs pelas células foliculares, bem como identificar as células alvo dessa possível ação. Confirmando achados anteriores de nosso laboratório, os resultados deste estudo sugerem que as ação da AngII são moduladas por gonadotrofinas. Nenhuma das concentrações de AngII adicionadas (0,001, 0,01, 0,1 ou 1 μM) aos cultivos de células da teca e granulosa alteraram o padrão de secreção de P_4, androstenediona, estrógeno, PGE_2 ou

$PGF_2\alpha$. No entanto, as células da granulosa secretaram 2 vezes mais P_4 e PGs no meio de cultivo, quando em presença simultânea de AngII (1 μM) e altas concentrações de LH (100 ng/ml), do que quando tratadas somente com a gonadotrofina.

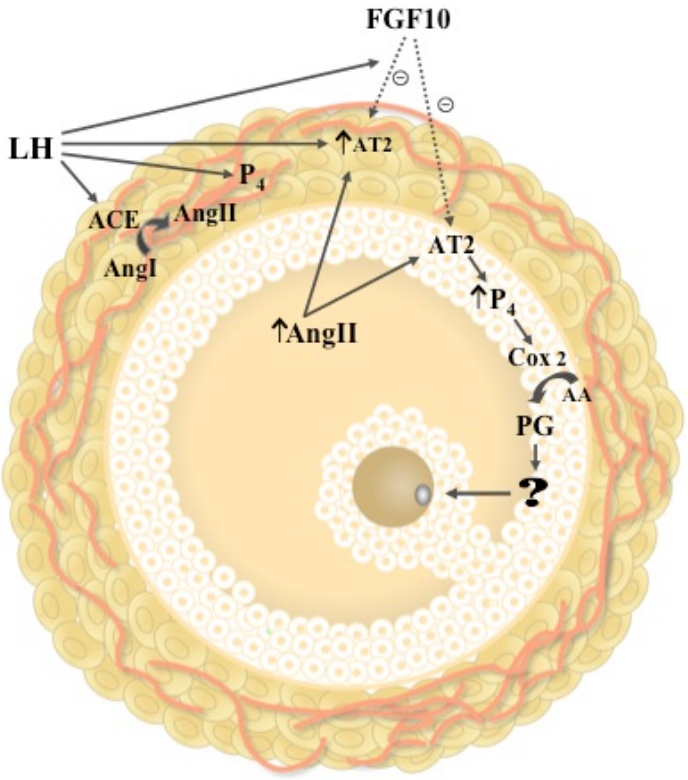
O papel da AngII e PGs como mediadores da maturação nuclear de oócitos em ruminantes induzida por gonadotrofinas já havia sido demonstrado (BARRETA et al., 2008). No entanto uma ação similar realizada pela P_4 ainda possuía caráter questionável em bovinos (SILVA & KNIGHT, 2000; WANG et al., 2006). No presente trabalho, utilizando modelos *in vitro* e *in vivo*, nos evidenciamos que a P_4 não só participa desse processo, mas também é um fator intermediário entre AngII e PGs na cascata de eventos. Em nosso conhecimento, esta é a primeira vez que as ações desses três hormônios são associadas na indução da maturação nuclear de oócitos. Onde temos uma rota sequencial, que se inicia com o pico de gonadotrofinas e passa por AngII, P_4 e depois PGs.

Até recentemente, nada havia na literatura sobre o papel do FGF10 na maturação nuclear de oócitos bovinos. No entanto, ZHANG et al. (2011), utilizando o cultivo de Complexos cúmulos-oócito (CCOs) concluíram que esse fator possui efeito estimulatório nos na maturação nuclear, atuando diretamente nos CCOs, aumentando a competência de embriões bovinos produzidos *in vitro* na presença de estradiol. Em contrariedade a isso, os resultados do nosso estudo sugerem que, atuando através das células foliculares, o FGF10 inibe o reinício da meiose induzido pela angiotensina II, mas não interfere o efeito estimulatório da P_4. Além disso, não observamos nenhum efeito quando CCOs foram cultivados na ausência de células foliculares, ou mesmo na sua presença, mas sem um fator estimulante da maturação como a angiotensina. O incremento nas taxas de maturação nuclear observados por ZHANG et al. (2011) podem ser devidos a efeitos anti-esteroidogênicos do FGF10 (PARROTT & SKINNER, 1998). Lembramos que a adição de estradiol ao cultivo de CCOs bovinos pode ser prejudicial a maturação nuclear e ao desenvolvimento embrionário (SILVA & KNIGHT, 2000; WANG et al., 2006;). No entanto, mais estudos precisam ser feitos para elucidar o papel desse fator na maturação nuclear de oócitos.

De acordo com nossos resultados, é possível sugerir que da mesma forma que a AngII, as ações do FGF10 também são anteriores a sinalização da P_4. Nossa hipótese é que durante a foliculogênese, o FGF10 participe como um fator inibidor da retomada da maturação nuclear, por modular negativamente a expressão de receptores para AngII nas células foliculares (PORTELA et al., 2008a). Com isso, o pico de gonadotrofinas, o qual nós evidenciamos que estimula a expressão desses receptores, permita a AngII estimular a síntese de P_4 e desencadear assim o processo de reinicio da meiose.

Em conjunto este estudo nos permite propor um modelo unificado de eventos tanto para a ocorrência da ovulação quanto para a retomada da maturação nuclear do oócito (Figura 1). Neste modelo, o pico de gonadotrofinas estimula a secreção de a AngII e o aumento na expressão de receptores AGTR2 nas células da teca. Estes receptores induzem o aumento na secreção de P_4, a qual estimulará a produção de PGE_2 e $PGF_2\alpha$ pelas células da granulosa. Por fim, PGs desencadeariam tanto a ovulação quanto a retomada da maturação nuclear do oócito. Ainda, neste modelo, o FGF10 participaria inibindo a expressão de receptores AGTR2, prevenindo o oócito de retomar sua progressão meiótica, até que o aumento abrupto de LH/FSH estimule o aumento da expressão esse receptor e permita a AngII desempenhar suas funções.

Figura 1. Modelo proposto para uma única cascata de eventos para ovulação e retomada da maturação nuclear do oócito



6. CONCLUSÕES

Em conjunto este estudo nos permite concluir que:

O pico de gonadotrofinas estimula a secreção de a AngII e o aumento na expressão de receptores AGTR2 e de ECA nas células da teca;

A AngII é capaz de modular a secreção de P_4 e PGs nas células da granulosa;

A P_4 é um fator intermediário entre AngII e PGs no processo de maturação nuclear de oócitos;

Atuando através das células foliculares, o FGF10 inibe o reinício da meiose induzido pela angiotensina II, mas não interfere o efeito estimulatório da P_4.

Por fim, a angiotensina II, P_4 e prostaglandinas são passos sequenciais de uma mesma rota, atuando simultaneamente nos processos de ovulação e maturação nuclear de oócitos bovinos.

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