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Perfil gênico no oviduto bovino de fêmeas Nelore e Aberdeen Angus

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“A amizade multiplica coisas boas e divide as más”

Baltasar Gracián y Morales

RESUMO

O oviduto possui papel essencial na reprodução de mamíferos, promovendo um microambiente favorável para a maturação oocitária, estocagem e capacitação do espermatozoide, fertilização, transporte dos gametas e desenvolvimento inicial do embrião. Anatomicamente e funcionalmente, o oviduto é dividido em três regiões principais: infundíbulo, ampola e istmo. O oócito e o espermatozoide entram nos lados opostos do oviduto, respectivamente no infundíbulo e istmo, e são transportados até a ampola, local onde ocorre a fertilização. O sucesso reprodutivo está diretamente ligado a temporização apropriada do transporte dos gametas ao local da fertilização, bem como, a precisão no tempo de transporte do embrião até o útero, para a aquisição da capacidade de implantação. A coordenação e regulação das funções do oviduto são complexas e estão sob efeitos endócrinos, parácrinos e autócrinos, os quais alteram temporalmente e espacialmente a transcrição e tradução de diversos fatores. Diante disso, o presente trabalho visou avaliar o efeito de biotecnologias reprodutivas, especificamente da superestimulação ovariana, bem como de características genéticas e fisiológicas reprodutivas no perfil transcricional de diversos fatores no oviduto bovino. Para tanto, foram avaliados os efeitos da indução de múltiplas ovulações em vacas da raça Nelore (dados apresentados no primeiro manuscrito), bem como os efeitos da influência da seleção genética de animais com alta contagem folicular em novilhas da raça Nelore e Aberdeen Angus, no período inicial pós ovulação (dados apresentados no segundo manuscrito), na expressão de genes relacionados ao transporte de gametas e fertilização. Os resultados demonstram que a superestimulação ovariana modula a expressão de alguns genes relacionados à contratilidade do oviduto em vacas da raça Nelore e que a ovulação é principal fator responsável por controlar as regulações transcricionais no oviduto bovino, com menor ou inexistente impacto da raça e da contagem folicular ovariana.

ABSTRACT

The oviduct has an important role in mammal reproduction, promoting a favorable microenvironment for oocyte maturation, sperm storage and capacitation, fertilization, transport of gametes and early embryo development. Anatomically and functionally, the oviduct is divided in three regions: infundibulum, ampulla and isthmus. The oocyte and the sperm enter in opposite sides of the oviduct, respectively infundibulum and isthmus, and are transported to the fertilization site, the ampulla. Reproductive success is directly related to appropriate timing of gamete transport to the fertilization site, as well as a precise time of embryo transport to the uterus, to obtain the capacity of implantation. The coordination and regulation of oviductal functions are complex and under endocrine, paracrine and autocrine effects, which temporally and spatially alter the transcription and translation of several factors. Therefore, this study aimed to evaluate the effect of reproductive biotechnologies, specifically ovarian superstimulation, as well as genetic and physiological reproductive characteristics in the transcriptional profile of several factors in the bovine oviduct. To do so, we evaluated the effects of inducing multiple ovulation in Nelore cows (data presented in the first manuscript), and the effects of the influence of genetic selection of animals with high follicle count in Nellore and Aberdeen Angus heifers, in the initial period post-ovulation (data presented in the second manuscript), in gene expression related to gametes transport and fertilization. The results demonstrated that ovarian superstimulation modulates the expression of some genes related to oviductal contractility in Nelore cows and ovulation is the main factor responsible for transcriptional control in bovine oviduct, with less or no impact of breed and ovarian follicle count.

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Formação Complementar

2013 – Estágio de Pesquisa no Exterior – Faculdade de Montreal, Saint Hyacinthe, Quebec, Canadá, 15 Agosto a 15 Dezembro, 2013.

2013 – Treinamento da PCR Quantitativa em Tempo Real (Life Technologies Brasil) – São Paulo, São Paulo, Brasil, 10-12 Abril, 2013.

Participação em eventos

2013 – 46th Annual Meeting of the Society for the Study of Reproduction (SSR) – Montreal, Quebec, Canadá, 22-26 Julho, 2013.

2013 – Análise Genômica 2013 – Botucatu, São Paulo, Brasil, 08-11 Julho, 2013.

2013 – I Curso de Inverno em Farmacologia e Biotecnologia do Programa de Pós Graduação em Ciências Biológicas (Farmacologia) – Botucatu, São Paulo, Brasil, 15-20 Julho, 2013.

2013 – III Simpósio de Farmacologia da UNESP – Botucatu, São Paulo, Brasil, 14-15 Junho, 2013.

2013 – 3º Workshop Internacional: Genômica Aplicada à Pecuária – Araçatuba, São Paulo, Brasil, 24-25 Fevereiro, 2013.

2012 – IV International Symposium on Animal Biology of Reproduction (ISABR) – Campinas, São Paulo, Brasil, 17-20 Outubro, 2012.

2012 – XXVI Reunião Anual da Sociedade Brasileira de Tecnologia de Embriões (SBTE) Foz do Iguaçu, Paraná, Brasil, 30 Agosto a 02 Setembro, 2012.

2012 – II Simpósio de Farmacologia da UNESP – Botucatu, São Paulo, Brasil, 22-23 Junho, 2012.

2012 – XI Workshop da Pós graduação – Botucatu, São Paulo, Brasil, 03-05 Maio, 2012.

2012 – IV Curso de Biologia Molecular Genotyping – Botucatu, São Paulo, Brasil, 27-28 Janeiro, 2012.

Resumos em congressos

2014 – Evento: 40th Annual Meeting of the International Embryo Transfer Society (IETS)
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2012 – Evento: 17th International Congress on Animal Reproduction (ICAR)

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2012 – Evento: XI Workshop da Pós Graduação

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INTRODUÇÃO

INTRODUÇÃO

Com o intuito de maximizar a exploração do potencial genético de fêmeas e consequentemente incrementar a produção animal, diversas biotécnicas reprodutivas, tais como a inseminação artificial (IA), a transferência de embriões (TE) e a produção *in vitro* de embriões (PIV) foram desenvolvidas e têm sido aprimoradas (Renesto 2004). Protocolos de tratamento para indução da ovulação múltipla, visando o melhoramento da produção de embriões bovinos são amplamente difundidos no Brasil (Barros and Nogueira 2001, Baruselli et al. 2006, Bo et al. 2006, Barros et al. 2010), no entanto, o melhoramento do desempenho produtivo e reprodutivo em animais não requer somente a implantação das biotécnicas de reprodução, mas também o conhecimento do grau de variação genética dos animais, como a contagem folicular entre as fêmeas bovinas, positivamente relacionada com a fertilidade (Mossa et al. 2012) e sua relação com o controle farmacológico dessas fêmeas.

Os ovidutos, também denominados tuba uterina, são tubo pares (direito e esquerdo), de natureza predominantemente muscular, ligando os ovários ao útero (Hafez and Hafez 2004), possuem papel essencial na reprodução de mamíferos, promovendo um microambiente favorável para a maturação oocitária, estocagem e capacitação do espermatozoide, fertilização, transporte dos gametas e desenvolvimento inicial do embrião (Buhi 2002). Anatomicamente e funcionalmente, o oviduto é dividido em três regiões principais: infundíbulo, ampola e istmo, com suas respectivas zonas de transição; junção ampola-istmo e junção útero-tubária, sendo o infundíbulo a região mais próxima ao ovário, a ampola; a região média e o istmo, a última região, mais proximal ao útero (Yániz et al. 2000).

O sucesso na fertilização está diretamente ligado à temporização apropriada do transporte dos gametas até o local da fecundação; a ampola (Talbot et al. 2003), bem como à precisão temporal no transporte do embrião até o útero, para a aquisição da capacidade de implantação (Pulkkinen 1995). Para executar tais funções, o oviduto dispõe de camadas de musculatura lisa circular e longitudinal, células ciliares e células não ciliares (células secretoras) na constituição da sua estrutura (Yániz et al. 2000). A regulação por fatores endócrinos, parácrinos e autócrinos (Halbert et al. 1976, Croxatto 2002) está intimamente relacionada às alterações observadas durante cada fase do ciclo estral nos diferentes segmentos do oviduto e compartimentalização de cada segmento (região apical ou basal; Yániz et al. 2000).

Baseando-se no importante papel do oviduto como coordenador de etapas essenciais para o desenvolvimento embrionário *in vivo*, a presente proposta almeja avaliar o impacto de biotécnicas reprodutivas e de diferentes grupos genéticos sobre aspectos moleculares do oviduto em fêmeas das raças Nelore e Aberdeen Angus, promovendo a maximização do entendimento da fisiologia do oviduto bovino.

CAPÍTULO 1

1. REVISÃO DE LITERATURA

1.1 Aspectos fisiológicos do oviduto: da reserva espermática ao transporte do embrião

Milhões de espermatozoides são ejaculados na vagina da vaca no momento do coito, porém apenas centenas ou milhares chegam ao oviduto e dezenas a centenas chegam ao local da fertilização (Suarez and Pacey 2006, Suarez 2007). A passagem do espermatozoide através do trato reprodutivo feminino maximiza as chances de fertilização, assegurando um espermatozoide com morfologia e motilidade normal (Suarez and Pacey 2006). A cérvix é a primeira grande barreira, selecionando os espermatozoides com motilidade adequada para conseguir atravessar o muco da cérvix (Silva et al. 1995, Barros et al. 1984), seguido da junção útero-tubária, que além do muco, possui um lúmen tortuoso e estreito, selecionando os espermatozoides que chegam ao istmo (Yániz et al. 2000).

A formação da reserva espermática em bovinos dá-se pela ligação dos espermatozoides ao epitélio do oviduto, mais especificamente no istmo. O espermatozoide liga-se com a cabeça exclusivamente em células ciliares, formando um ângulo tangencial (Kölle et al. 2009). De modo mais detalhado, sabe-se que em bovinos, proteínas na superfície do espermatozoide, conhecidas como BSP (do inglês, *binder of sperm*), ligam-se a um componente dos receptores conhecidos como fucose (Lefebvre et al. 1997). A presença de fucose foi identificada em receptores conhecidos como anexinas, em bovino foram identificadas quatro anexinas (ANXA1, ANXA2, ANXA4 e ANXA5), todas as quatro estão presentes na superfície apical da mucosa do epitélio do oviduto, especificamente nos cílios (Ignotz et al. 2007).

Adicionalmente, a relação entre o oviduto e o espermatozoide resulta em modificações na fisiologia do oviduto. De fato, a presença dos gametas no oviduto alterou 32 proteínas do fluido, em sua a maioria, pela presença do gameta masculino (Georgiou et al. 2007). Somado a isso, Kodithuwakku et al. (2007) demonstraram que os espermatozoides são capazes de estimular a biossíntese e secreção de prostaglandinas através do aumento da expressão gênica da *COX2, PGES* e *PGFS*, assim como aumentar, dose dependentemente, a liberação de PGF2a e PGE2 por células do epitélio do oviduto bovino *in vitro*, sugerindo que o espermatozoide estimula o aumento da motilidade do oviduto, facilitando seu transporte para o local da fertilização.

Sinais hormonais que induzam a ovulação ou sinais do folículo pré-ovulatório possivelmente estimulam o epitélio do istmo a secretar fatores que ativam a capacitação e hiperativação espermática (Ho and Suarez 2001). A capacitação envolve mudanças na membrana plasmática, tais como mudanças em proteínas de membrana e colesterol, preparando o espermatozoide para a reação acrossômica e fertilização, assim como, mudanças e perdas de proteínas de membrana, diminuindo a afinidade do espermatozoide ao epitélio do istmo, preparando a liberação do espermatozoide da reserva (De Jonge 2005).

Em animais monovulatórios, como os bovinos, um complexo cumulus-oócito é liberado do folículo pré-ovulatório e então transportado pelo infundíbulo até o lúmen da ampola. O complexo cumulus-oócito (CCO), constituído por um oócito envolto por numerosas camadas de células do cumulus (Familiari et al. 1998), é ovulado na cavidade peritoneal e então transportado pelo infundíbulo do oviduto (Talbot et al. 1999).

Ao chegar na ampola, o CCO adere fortemente ao epitélio do oviduto. Essa ligação é tão forte que apenas destruindo as células do *cumulus* é possível desgrudar o CCO do epitélio (Kölle et al. 2009). O processo de maturação do CCO envolve a expansão

do cumulus e modificação da zona pelúcida (ZP). Ocorre um aumento da acessibilidade da ZP ao fluido do oviduto e modificações ultraestruturais (Funahashi et al. 2001). Proteínas e açúcares ligam-se a ZP, contribuindo para interação do espermatozoide com o oócito (Coy et al. 2012). A produção de prostaglandinas pelas células do oviduto induz a expansão das células do *cumulus* do CCO bovino, assim como fatores de crescimento (fatores de crescimento fibroblástico e fator de crescimento endotelial vascular) são descritos por influenciar a maturação oocitária no oviduto (Einspanier et al. 1999).

A fertilização ocorre quando os gametas feminino e masculino se encontram no local e tempo adequado no oviduto. Em vacas, o CCO liga-se ao epitélio do oviduto assim que chega a ampola, sendo esse o local da fertilização em bovino (Kölle et al. 2009). Assim que o CCO chega a ampola, o espermatozoide proveniente da reserva espermática é hiperativado e move-se em direção ao CCO (Kölle et al. 2009).

Diferentemente da fertilização *in vitro*, na qual o espermatozoide move-se sem direção até encontrar o oócito ao acaso, estudos *in vivo* mostram que a interação do CCO com o oviduto produz agentes de quimiotaxia (Gakamsky et al. 2008, Kaupp et al. 2008) que direciona o espermatozoide ao oócito. Além dos movimentos flagelares do espermatozoide guiado por fatores quimioatrativos, outro fator envolvido no transporte do espermatozoide é a contração da musculatura lisa do oviduto. Devido à forte corrente do fluido do oviduto em direção ao útero formada pelos batimentos ciliares, a contratilidade do oviduto em direção ao ovário é essencial (Kölle et al. 2009). Guidobaldi et al. (2012) demonstraram que a inibição dos movimentos do oviduto ou dos agentes quimioatrativos diminuiu a quantidade de espermatozoide que chegaram ao local da fertilização. Além disso, ao inibir os dois fatores simultaneamente, os espermatozoides ficaram retidos no istmo, não alcançando o local da fertilização, confirmando que o

transporte do gameta masculino é coordenado tanto pela contratilidade da musculatura do oviduto, quanto pelos fatores quimioatrativos.

Antes da fertilização, a velocidade de transporte pelos batimentos ciliares não difere entre os antímeros contralateral e ipslateral a ovulação (133 µm/sec). Porém, após a fertilização, a velocidade de transporte é significantemente menor no lado que em se encontra o embrião (46 µm/sec), quando comparado ao lado sem o embrião (> 150 µm/sec; Kölle et al. 2009). Em vacas, o embrião encontra-se na ampola até dois dias após a fertilização, logo em seguida entra no istmo e 3,5 dias após a fertilização chega no útero (Kölle et al. 2009). Além disso, o embrião é capaz de induzir mudanças locais na vascularização do oviduto e na morfologia da parede do oviduto (o oviduto ipslateral, quando comparado ao contralateral, é mais grosso, mais edematoso e mais transparente; Kölle et al. 2009)

1.2. Fatores reguladores da fisiologia do oviduto bovino

Na espécie bovina, na qual a ovulação é restrita a um dos dois ovidutos, há diversas diferenças entre o oviduto ipsilateral e contralateral a ovulação. Maiores concentrações de estradiol (E_2) durante a fase folicular e de prostaglandinas (PGs) e endotelina-1 (ET-1) durante a fase folicular e pós-ovulatória no oviduto ipsilateral foram descritas por Wijayagunawardane et al. (1998); além disso, os mesmos autores demonstraram altas concentrações de progesterona (P_4) no tecido do oviduto ipsilateral durante a fase luteal do ciclo estral quando comparado ao oviduto contralateral em bovinos. Não obstante, a expressão gênica do oviduto ipsilateral difere da observada no oviduto contralateral. Bauersachs et al. (2003) identificaram 35 genes diferentemente expressos entre os ovidutos ipsilateral e contralateral em bovinos, sendo que 27 genes

tiveram maiores níveis de expressão no oviduto ipsilateral e oito no oviduto contralateral, com funções variadas, tais como, proteínas de superfície celular, proteínas de interação células-célula, membros das vias de transdução, proteínas relacionadas à imunidade e enzimas.

Algumas dessas macromoléculas são de grande importância no controle das funções do oviduto. É o caso da Glicoproteína específica do oviduto-1 (OVGP1, do inglês *oviduct-specific glycoprotein*), uma proteína sintetizada e liberada exclusivamente pelas células secretoras do oviduto (Buhi 2002). Oócitos pré-incubados com OVGP1 demonstraram aumento na taxa de fertilização (Buhi 2002). Além disso, OVGP1 e heparina-like glicosaminoglicanos (GAGs) do fluido do oviduto ligam-se a zona pelúcida e aumentam a resistência a digestão enzimáticas e a ligação e penetração do espermatozoide, diminuindo a ocorrência de polispermia (Coy et al. 2008).

Outra proteína de grande funcionalidade no fluido do oviduto é a proteína regulada por glicose 78-kDa (GPR78, do inglês *78-kDa glucose-regulated protein*). Estudos mostram que a GPR78 tem capacidade de ligar-se ao espermatozoide bovino durante sua passagem pelo trato reprodutivo feminino, participando da proteção da integridade da membrana do espermatozoide e modulando a interação entre espermatozoide e zona pelúcida (Boilard et al. 2004). Além disso, em camundongos desempenha importante função no estágio de desenvolvimento de blastocisto e proteção do embrião contra apoptose (Luo et al. 2006). Lin et al. (2012) mostraram a diferença de expressão de GRP78 durante o ciclo estral em camundongos e observaram uma maior presença da proteína GRP78 no istmo comparado ao infundíbulo e ampola, demonstrando uma possível relação da expressão de GRP78 ao transporte dos gametas, fertilização e desenvolvimento do embrião.

Os sistemas das prostaglandinas e angiotensina-II mostram-se importantes fatores no controle dos batimentos ciliares (Nishimura et al. 2010, Saridogan et al. 1996, Verdugo et al. 1980). Juntamente com os sistemas da endotelina-1 e do VEGF, as PGs e ANGII regulam o contração da musculatura lisa do oviduto, promovendo o controle do deslocamento dos gametas para a fertilização e do embrião em tempo necessário para o sucesso da implantação (Wijayagunawardane et al. 2005, Wijayagunawardane et al. 2001a, Priyadarsana et al. 2004).

As concentrações de PGE_2 e $PGF_{2\alpha}$ são maiores no período da ovulação comparada a fase luteal do ciclo estral em vacas (Wijayagunawardane et al. 1998). O ácido araquidônico é convertido a PGH_2 pela ação das cicloxigenases (COX1 e COX2), essa por sua vez sofre ação de prostaglandinas sintetases, produzindo diversas prostaglandinas. A PGE sintetase (PGES) e a PGF sintetase (PGFS) convergem PGH_2 em PGE_2 e $PGF_{2\alpha}$, respectivamente (Okuda et al. 2002). A expressão de *COX1* durante o período da ovulação é maior quando comparado à fase luteal do ciclo estral bovino, o mesmo não é observado com a COX2, cujos níveis de transcrição não se alteram no decorrer do ciclo estral (Odau et al. 2006). Os efeitos da PGE_2 são mediados pela interação com seus receptores: EP1, EP2, EP3 e EP4 (Sugimoto et al. 2000). A ativação dos receptores EP1 e EP3 geralmente resulta em contração da musculatura lisa, enquanto que a ativação dos receptores EP2 e EP4 resulta em relaxamento (Sugimoto et al. 2000). No oviduto bovino os efeitos da PGE_2 são mediados principalmente pelos receptores EP2 e EP4 através da ativação da adenilato ciclase (Narumiya et al. 1999), o receptor EP1 não é expresso no oviduto bovino (Gabler et al. 2008). Estudos *in vitro* demonstram que PGE_2 e $PGF_{2\alpha}$ aumentam a amplitude da contração do oviduto bovino, mas não a frequência (Wijayagunawardane et al. 2001b). Além disso, as PGs também possuem efeito sobre os batimentos ciliares do oviduto. Verdugo et al. (1980) descreveu o efeito estimulante da

PGE_2 e da $PGF_{2\alpha}$ na frequência dos batimentos ciliares no oviduto de coelhos, do mesmo modo Hermoso et al. (2001) observou o mesmo efeito da PGE_2 no oviduto de hamster.

A endotelina-1 (ET1), primeiramente identificada como um potente peptídeo vasoconstritor (Yanagisawa et al. 1988), tem sido descrita no controle das funções reprodutivas. A expressão de mRNA para ET1 e seus receptores (ETR-A e ETR-B) no oviduto bovino é diferenciada durante as fases do ciclo estral, sendo mais expressos durante a fase pós-ovulatória (Priyadarsana et al. 2004). Além disso, a ET1 possui função estimulatória na liberação de prostaglandinas no oviduto bovino. Sua atividade no oviduto possivelmente está relacionada ao transporte de gametas e embrião, já que aumenta significativamente a amplitude de contração do oviduto no período ovulatório (Priyadarsana et al. 2004).

O sistema do Fator de Crescimento Vascular Endotelial (VEGF, do inglês *Vascular Endothelial Growth Factor*) também coordena importantes funções do oviduto. Estudos *in vitro* realizados por Wijayagunawardane et al. (2005) indicaram um interessante controle na contratilidade do oviduto pelo sistema VEGF, na qual os níveis pré-ovulatório de LH, juntamente com altos níveis de E_2 secretados pelo folículo pré-ovulatório e baixos níveis de P_4 do corpo lúteo em regressão, aumentam a ação do sistema VEGF no oviduto (aumento da expressão de VEGF e seus receptores), induzindo a produção de fatores de contração (PGE_2, PGF2α e ET-1) e um rápido transporte dos gametas ao local de fertilização. Os níveis crescente de VEGF no oviduto desencadeia uma auto regulação e diminui a sua expressão, contribuindo para a supressão da contratilidade do oviduto e para um transporte seguro e no tempo adequado do embrião até o útero.

A Angiotensina II (AGTII) é amplamente conhecida por regular a pressão sanguínea, mas também possui diversas funções na biologia reprodutiva, tais como,

controle vascular ovariana, formação do corpo lúteo e luteólise (Gonçalves et al. 2012). A enzima conversora de angiotensina (ACE, do inglês *Angiotensin converting enzyme*) converte angiotensina I em angiotensina II. Estudos demonstraram expressão de AGTII e seu receptor na tuba uterina humana (Johnson et al. 1998) e a presença de mRNA da ACE e de AGTII liberado no oviduto foi identificado durante todo o ciclo estral no oviduto bovino, apresentando maiores níveis durante o período pós-ovulatório (Wijayagunawardane et al. 2009), indicando que a AGTII possui algum papel na regulação do oviduto. Saridogan et al. (1996) observaram um efeito estimulatório da AGTII na frequência dos batimentos ciliares na tuba uterina humana, sendo atribuído ao receptor tipo 1 da AGTII o controle dessa atividade. O receptor tipo 1 da AGTII também está associado ao controle do transporte iônico na tuba uterina humana e regulação da composição do fluido do oviduto em humanos (Mahmood et al. 2002). Além disso, estudos mostraram que a AGTII também está relacionada com a contratilidade do oviduto. No oviduto bovino a AGTII estimulou a liberação de PGE_2 e $PGF_{2\alpha}$ e endotelina-1 (ET1), que são indutores da contração no oviduto (Wijayagunawardane et al. 1999a, Wijayagunawardane et al. 1999b, Wijayagunawardane et al. 2001b).

1.3. Funções do oviduto: influência do controle farmacológico e população folicular

Os bovinos, mamíferos pertencentes à família *Bovidae*, são divididos em dois gêneros: *Bos taurus taurus*, que abrange o gado europeu e *Bos taurus indicus*, gado originalmente encontrado na Índia, Ásia e África (Santiago 1985). Animais *Bos indicus* recrutam maior número de folículos por onda de crescimento folicular que animais *Bos taurus* (33,4±3,2 vs 25,4 ± 2,5; Carvalho et al. 2008). Essa característica tem influência direta na eficiência da técnica de transferência de embriões e na aspiração folicular para

fertilização *in vitro* (FIV), indicando vantagem de fêmeas zebuínas sobre taurinas. Relatos indicam que número de folículos recrutados por onda de crescimento folicular apresenta diferença entre indivíduos e essa característica possui alta repetibilidade durante a vida reprodutiva da fêmea (Boni et al. 1997, Mossa et al. 2010a, Mossa et al. 2010b, Jimenez-Krassel et al. 2009). Devido a essa característica, pode-se dizer que há animais com alta contagem folicular (ACF) e baixa contagem folicular (BCF).

A quantidade de folículos que um animal apresenta em cada recrutamento permanece constante até que as fêmeas bovinas atinjam a idade de 8 a 10 anos, havendo mudanças após esse período, provavelmente influenciada pela depleção das reservas foliculares ovarianas (Ireland et al., 2007). Alguns estágios fisiológicos podem interferir negativamente no recrutamento, tais como: lactação (Lucy 2001), estresse térmico (Wolfenson et al. 1995), gestação (Ginther et al. 1996) e nutrição inadequada (Lucy 2001).

Animais BCF possuem menor fertilidade comparada a animais ACF (Mossa et al. 2012). Os menores níveis plasmáticos de progesterona em vacas BCF (Jimenez-Krassel et al. 2009) podem estar associados a essa menor fertilidade, já que baixos níveis desse hormônio estão associados a maior taxa de mortalidade embrionária em bovinos (Stronge et al. 2005, McNeill et al. 2006). Além disso, vacas com ACF apresentam ovários maiores, maior número de folículos e oócitos morfologicamente saudáveis e uma reserva ovariana maior quando comparado a vacas BCF (Ireland et al. 2008).

Especificamente em bovinos, devido ao interesse crescente em se obter uma maior exploração do potencial genético de fêmeas para incremento da produção animal, diversas biotécnicas, tais como a inseminação artificial (IA), a transferência de embriões (TE) e a produção *in vitro* de embriões (PIV), têm sido desenvolvidas e aprimoradas (Renesto 2004). O conhecimento detalhado da dinâmica folicular possibilitou o desenvolvimento

de tratamentos hormonais capazes de regular o crescimento folicular e o momento da ovulação. Deste modo, diferentes gonadotrofinas, doses, vias de administração e variadas combinações de hormônios são aplicados para desenhar inúmeros protocolos de tratamento para indução da ovulação múltipla, visando o melhoramento da produção de embriões bovinos (Barros and Nogueira 2001, Baruselli et al. 2006, Bo et al. 2006, Barros et al. 2010). No entanto, o melhoramento da performance produtiva e reprodutiva em animais não requer somente a implantação das biotécnicas de reprodução, mas também o conhecimento do grau de variação genética dos animais. Trabalhos recentes do nosso grupo de pesquisa demonstraram que a taxa de blastocisto dos grupos de vacas superestimuladas e não-superestimuladas não diferiram, 40% e 37%, respectivamente (Barros et al. 2013). Adicionalmente, dados ainda não divulgados, demonstraram o aumento da expressão de genes relacionados à melhor competência embrionária (PLAC8, NANOG e OCT4) em embriões produzidos in vitro a partir de oócitos de vacas submetidas à superestimulação ovariana quando comparado às vacas não superestimuladas.

Adicionalmente, postula-se que a diferença na contagem folicular entre os bovinos está correlacionada com a fertilidade do animal. Vacas que têm maior quantidade de folículos emergentes apresentam oócitos de melhor qualidade, vida reprodutiva mais longeva e maior fertilidade. Essas fêmeas quando tratadas com protocolos de superestimulação ovariana, geraram maior porcentagem de embriões transferíveis em relação às fêmeas com menor quantidade de folículos recrutados (Ireland et al. 2007).

Em suma, o oviduto possui papel chave na promoção de um microambiente adequado para a maturação do oócito, capacitação do espermatozoide, fertilização e transporte do embrião. A coordenação dos movimentos opostos para transporte dos gametas ao local de fertilização, especificidade das funções de cada um dos segmentos e

adequada temporização dos acontecimentos no oviduto requer um controle complexo e refinado. A regulação por fatores parácrinos, endócrinos e autócrinos são continuamente pesquisados e descritos para compreensão da regulação das funções do oviduto. Diante disso, o presente trabalho visou avaliar os efeitos do uso de biotecnologias reprodutivas na regulação da expressão gênica no oviduto bovino. Mais especificamente avaliou-se os efeitos do uso de protocolos de indução de múltiplas ovulações em vacas Nelore e os efeitos da seleção genética de animais com alta contagem folicular em novilhas da raça Nelore e Aberdeen Angus, diante da presença ou ausência da ovulação no período inicial pós-ovulatório, sobre a regulação da expressão de genes relacionados ao transporte dos gametas e fertilização.

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CAPÍTULO 2

Prostaglandin receptors (*EP2* and *EP4*) and angiotensin receptor (*AGTR2)* mRNA expression increases in the oviducts of Nelore cows submitted to ovarian superstimulation

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ABSTRACT

Many peptides are responsible for the coordination of muscle contraction, secretion and ciliary beating of the oviduct epithelium to allow the transport of gametes and embryos, including vascular endothelial growth factors (VEGF), prostaglandins (PGs), endotelin-1 (ET-1) and angiotensin II (Ang II). The effect of reproductive biotechnologies used to improve embryo yield on oviduct gene expression is poorly understood. Thus, the aim of the present study was to evaluate the effect of ovarian superstimulation on the mRNA expression of the genes encoding the major peptides involved in oviduct contraction in bovine. Therefore, Nelore cows were submitted to P-36 (n=5) or P-36/eCG (n=5) ovarian superstimulatory protocols and a control group of cows was not submitted to any superstimulatory protocol (n=5). The relative expression of VEGF *(VEGF, Flk1, Flt1),* Ang II *(AGTR2, ACE1),* ET1 *(ET1, ECE1)* and PG pathway members *(PGES, EP2, EP4, COX1, COX2)* was analyzed using real time RT-PCR in each of oviduct segment (infundibulum, ampulla and isthmus). All target genes were expressed in the three segments of the bovine oviduct; however, specific genes were regulated by

ovarian superstimulation: *EP2* and *EP4* receptors mRNA was affected by P-36/eCG protocol, in the ampulla and infundibulum, respectively; and *AGTR2* mRNA was up-regulated by both the P-36/eCG and P-36 protocols in the isthmus. The upregulation of *EP2*, *EP4* and *AGTR2* expression in the superstimulated cows suggests a suitable effect of FSH and eCG on bovine oviduct physiology, coordinating the contraction in Nelore cows.

INTRODUCTION

In the oviduct, endocrine and paracrine factors induce morphological, biochemical and physiological changes in the infundibulum, ampulla and isthmus to provide an ideal microenvironment for oocyte transport and maturation, sperm capacitation and transport, fertilization and early embryonic development. Thus, the temporal and spatial organization of each of these events is fundamental to reproductive efficiency (Ruckebusch and Bayard, 1975; Wijayagunawardane et al., 2001b).

The smooth muscle contraction, flow of tubal secretions and ciliary beating of the oviduct epithelium are responsible for the transport of gametes and embryos (Jansen, 1984; Lyons et al., 2006b). It is known that some peptides are responsible for the orchestration of these processes, including vascular endothelial growth factors (VEGF) (Gabler et al., 1999; Wijayagunawardane et al., 2005), prostaglandins (PGs) (Bridges and Fortune, 2007; Gabler et al., 2008), endotelin-1 (ET-1) (Priyadarsana et al., 2004; Bridges et al., 2011) and angiotensin II (Ang II) (Wijayagunawardane et al., 2001a; Wijayagunawardane et al., 2009). Indeed, PGs increase the contractility of smooth muscles in the bovine oviduct (Lindblom et al., 1978; Weber et al., 1991) and stimulate ciliary beating in the human oviduct (Lyons et al., 2006a), and PG release is

stimulated by VEGF (Wijayagunawardane et al., 2005), ET1 (Wijayagunawardane et al., 2001b; Priyadarsana et al., 2004) and Ang II (Wijayagunawardane et al., 2001a). Moreover, studies have shown an increase in the amplitude of contraction in the bovine oviduct caused by ET1 (Wijayagunawardane et al., 2001b) and Ang II (Wijayagunawardane et al., 2001a).

Reproductive biotechnologies, such as ovarian superstimulation, artificial insemination and embryo transfer, are very important for the improvement of cattle reproduction (Barros et al., 2000; Barros and Nogueira, 2001; Nogueira et al., 2004; Baruselli et al., 2006). Several protocols to induce multiple ovulations in cattle have been proposed to improve embryo yield (Barros and Nogueira, 2001; Baruselli et al., 2006; Barros et al., 2010); however, the effects of ovarian superstimulation on oviduct physiology is poorly understood.

Thus, the aim of the present study was to evaluate the effect of ovarian superstimulation on the mRNA expression of the genes encoding the major peptides involved in oviduct contraction in Nelore cows. Therefore, the mRNA abundance of VEGF, Ang II, ET1 and PG pathway genes was assessed in each segment of the bovine oviduct (infundibulum, ampulla and isthmus) from unstimulated controls and Nelore cows that underwent P-36 or P-36/eCG ovarian superstimulatory protocols.

MATERIAL AND METHODS

Ovarian superstimulation

All experimental animals were treated according to the animal protection law of Brazil. This study was conducted on a farm located in Santa Cruz do Rio Pardo (São Paulo, Brazil; latitude 22º 53’ 56”; longitude 49º 37’ 57”; altitude 467 m). The cows

were maintained on pasture (*Brachiaria brizantha*) with *ad libitum* access to water and a mineral supplement.

Nelore non-lactating multiparous cows ranging from 5 to 7 years of age with body condition scores ranging from 2.0 to 3.5 were submitted to P-36 (n=5) or P-36/eCG (n=5) ovarian superstimulatory protocols; a control group of cows was not subjected to any superstimulatory protocol (n=5; fig. 1). At a random stage of the estrous cycle, all cows received a Progesterone-releasing vaginal insert (1.0 g, PRIMER®, Tecnopec, São Paulo, Brazil) and estradiol benzoate (2.5 mg, i.m., Estrogin®, Farmavet, São Paulo, Brazil) on Day 0. The P-36 protocol was performed by administration of pFSH (Folltropin-V®, Bioniche Animal Health, Belleville, ON, Canada) twice daily from Days 5 to 8; a total of 200 mg was given with a decreasing dose regimen: 40% on Day 5, 30% on Day 6, 20% on Day 7 and 10% on Day 8. All cows received 150 mg of d-cloprostenol (Prolise®, Tecnopec, São Paulo, SP, Brazil) i.m. twice on Day 7 (7 h and 19 h). The progesterone-releasing vaginal inserts were removed at 19 h on Day 8 and the cows were slaughtered at 7 h on Day 9. For the P-36/eCG protocol, the last two doses of FSH were replaced by two doses of eCG (total dose = 400 IU, i.m., Novormon®, Syntex, Buenos Aires, Argentina; fig. 1). Additionally, blood samples were collected from the jugular vein on Day 8 (19:00) and Day 9 (7:00) to quantify the plasmatic concentration of LH and to ensure that no cow had undergone an endogenous LH surge.

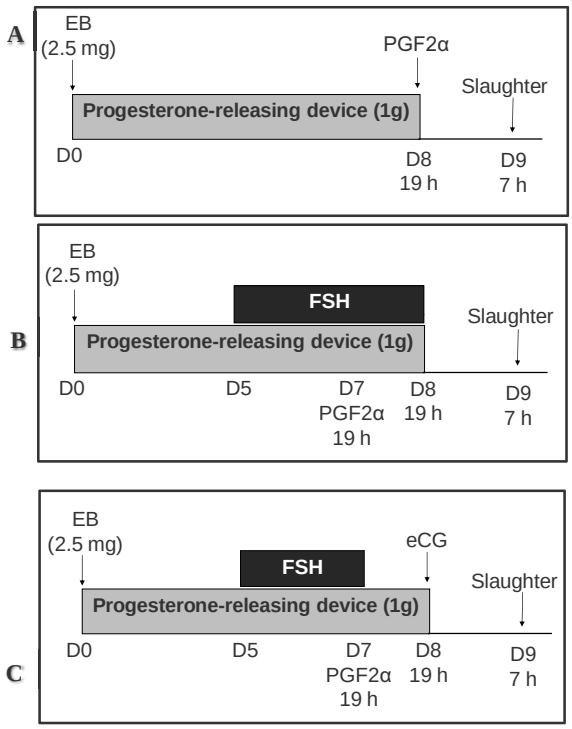

Figure 1. Experimental design of the ovarian superstimulatory protocols used in Nelore cows. Panel (A): control group, non-superstimulated cows. Panel (B): P-36 protocol. Panel (C): P-36/eCG protocol. EB: Estradiol Benzoate, PGF2α: Prostaglandin F2 alpha, D: Day.

Sample collection

The reproductive tracts of the cows were transported to the laboratory in saline solution (0.9%) at 4º C. The oviducts were isolated and the surrounding connective tissues were trimmed. Each segment of the oviduct was analyzed separately (infundibulum, ampulla and isthmus) and the transition regions were discarded. The samples were placed in Trizol® (Invitrogen, São Paulo, SP, Brazil) and homogenized with Polytron (Ultraturrax®, Luzern, Switzerland). The total RNA was extracted according to the manufacturer's protocol and stored at -80 °C.

110 *Real-time RT-PCR*

Total RNA (1 µg) from each segment of the oviduct (infundibulum, ampulla and
112 isthmus) was incubated with DNAse I (Invitrogen®) and then reverse transcribed with SuperScript III (Invitrogen) using Oligo-d(T) primer. Relative real-time RT-PCR
114 analysis was performed with a StepOne Plus thermo cycler using Power Sybr Green PCR Master Mix (Applied Biosystems) with bovine-specific primers. The primers and
116 reaction conditions used for the amplification of the VEGF pathway genes (*VEGF* and its receptors *Flk1* and *Flt1*), endothelin-converting enzyme 1 *(ECE1)*,
118 angiotensinconverting enzyme 1 (*ACE1)* and prostaglandin E synthase (*PGES)* were previously published by (Wijayagunawardane et al., 2005). The primers and conditions
120 used for the amplification of the angiotensin II receptor (*AGTR2*) and cyclooxygenase 2 (*COX2*) were previously published by (Portela et al., 2008) and (Silva et al., 2013),
122 respectively. For the other target genes, the primers were designed using available bovine sequences and are shown in table 1.

124

Table 1. Details of bovine-specific primers.

Gene	Sequence	NCBI RefSeq
ET1 (sense)	5'-CCTCGTGGAAGTCTGTCTAATG-3'	NM_181010.2
ET1 (antisense)	5'-AAGTGAGGGAAACTCCTGATTC-3'	NM_181010.2
EP2 (sense)	5'-CTCTGCTGTCGGGTTTCATTA-3'	NM_174588.2
EP2 (antisense)	5'-CTACCCTCCTCAAAGGTCAATC-3'	NM_174588.2
EP4 (sense)	5'-CGAGATCCAGATGGTCATCTTAC-3'	NM_174589.2
EP4 (antisense)	5'-CTCCAGTTGTGGCCGATATAA-3'	NM_174589.2
COX1 (sense)	5'-GTAGACCTCGGCCACATTTAT-3'	NM_001105323.1
COX1 (antisense)	5'-CTCCATTGAGCATCTGGTACTT-3'	NM_001105323.1

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The PCR reactions were carried out in 25 µl volumes with 1 µl of each sample, and the PCR cycling conditions were 95° C for 10 min then 40 cycles of 95° C for 10 sec followed by annealing at 60° C for 1 minute. The reactions were optimized to achieve maximum amplification efficiency for each gene (90-110%). Each sample was analyzed in duplicate, and the specificity of each PCR product was determined by melting curve analysis and amplicon size determination in agarose gels. Positive controls (bovine fetal ovary extracts) and negative controls (water replacing cDNA) were run on every plate.

The relative expression of VEGF *(VEGF, Flk1, Flt1),* Ang II *(AGTR2, ACE1),* ET1 *(ET1, ECE1)* and PG pathway members *(PGES, EP2, EP4, COX1, COX2)* was calculated using the ΔΔCt method with efficiency correction (Pfaffl, 2001). To select the most stable housekeeping gene for detailed analyses of the oviduct, peptidylprolyl isomerase A (*PPIA*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and histone H2A (*H2AFZ*) gene expression and amplification profiles were tested and compared using the geNorm applet for Microsoft Excel (medgen.ugent.be/genorm; (Ramakers et al., 2003). Based on this analysis, the most stable housekeeping gene was *PPIA*. Primers previously published by (Machado et al., 2009) were used for amplification of the housekeeping genes.

Statistical analysis

The effect of ovarian superstimulation (P-36 and P-36/eCG) on the mRNA abundance of the target genes in each oviduct region was tested by ANOVA analysis, and means comparisons were performed with the Tukey–Kramer HSD test. The data are presented as the means ± S.E.M. The analyses were performed using JMP software

(SAS Institute, Cary, NC, USA). The differences were considered significant when P<0.05; P values between 0.05 and 0.10 were considered tendencies.

RESULTS

All the target genes were expressed in the three regions of the bovine oviduct; however, a few specific genes were regulated by ovarian superstimulation. In the ampulla, only *EP2* receptor mRNA was affected by ovarian superstimulatory treatment: it showed higher levels in cows submitted to the P-36/eCG protocol (Figure 2). Similarly, *EP4* receptor mRNA was also upregulated in the infundibulum from Nelore cows submitted to the P-36/eCG treatment (Figure 3). Moreover, the expression of *PGES* and *EP2* mRNA tended to increase in the ampulla (Figure 2) and infundibulum (Figure 3), respectively, in cows submitted to ovarian superstimulation on the P-36/eCG protocol. Although, ovarian superstimulatory treatment seems to, in general, mostly affect genes of the PG pathway, the *AGTR2* mRNA was also upregulated by both the P-36/eCG and P-36 protocols in the isthmus (Figure 4).

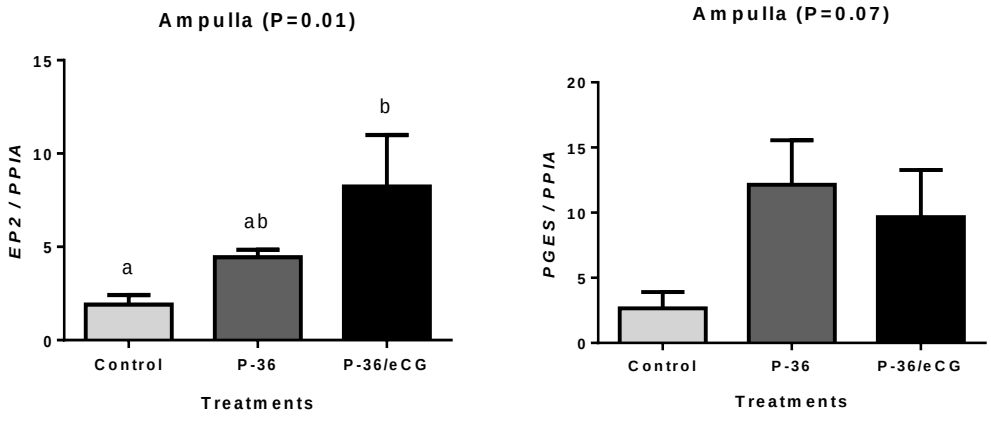

Figure 2. Effects of ovarian superstimulation on the abundance of *EP2* and *PGES* mRNA (mean ± S.E.M) in the ampulla of oviducts from Nelore cows. The relative mRNA levels (target gene/PPIA by Pfaffl's equation) were analyzed by ANOVA and the means were compared with a Tukey-Kramer test. The differences (a,b) were considered significant when P<0.05, and P values between 0.05 and 0.10 were considered tendencies. Control group, non-superstimulated cows (n=5 cows), P-36 protocol (n=5 cows) and P-36/eCG protocol (n=5 cows).

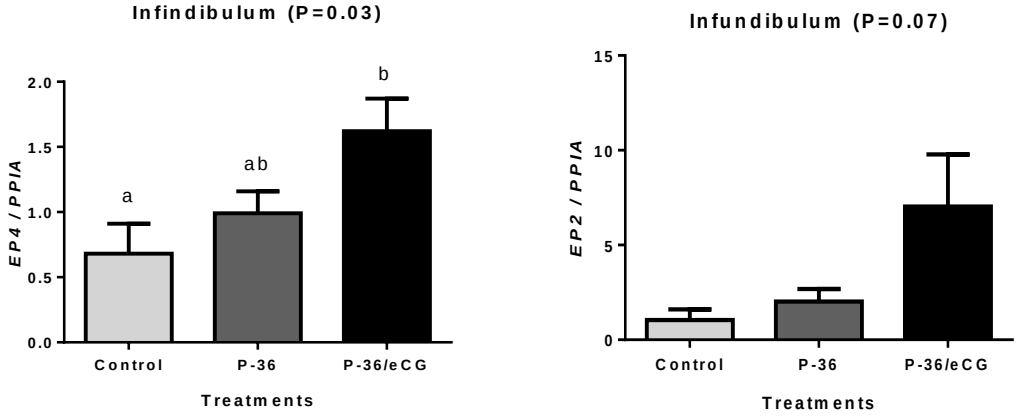

Figure 3. Effects of ovarian superstimulation on the abundance of *EP2* and *EP4* mRNA (mean ± S.E.M) in the infundibulum of oviducts from Nelore cows. The relative mRNA levels (target gene/PPIA by Pfaffl's equation) were analyzed by ANOVA and the means were compared with a Tukey-Kramer test. The differences (a,b) were considered significant when P<0.05, and P values between 0.05 and 0.10 were considered tendencies. Control group, non-superstimulated cows (n=5 cows), P-36 protocol (n=5 cows) and P-36/eCG protocol (n=5 cows).

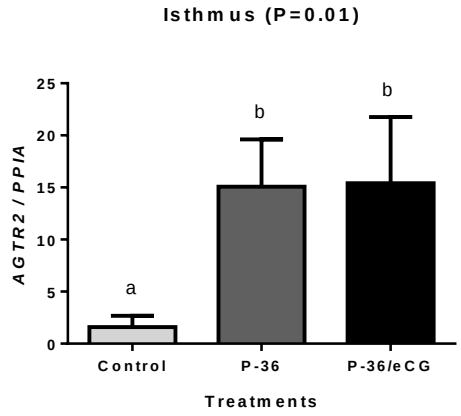

Figure 4. Effects of ovarian superstimulation on the abundance of *AGTR2* mRNA (mean ± S.E.M) in the isthmus of oviducts from Nelore cows. The relative mRNA levels (target gene/PPIA by Pfaffl's equation) were analyzed by ANOVA and the means were compared with a Tukey-Kramer test. The differences (a,b) were considered significant when $P<0.05$. Control group, non-superstimulated cows (n=5 cows), P-36 protocol (n=5 cows) and P-36/eCG protocol (n=5 cows).

DISCUSSION

The impact on oviduct physiology, specifically on initial embryo development, of exogenous gonadotropin used in ovarian superstimulation protocols remains unclear. The effect of ovarian superstimulation on the rate of embryo production is controversial: while some studies have shown no difference in blastocyst production between unstimulated and superstimulated cows (Mapletoft and Bó, 2011; Barros et al., 2012), others (Gad et al., 2011) have demonstrated a decrease in embryos that developed to the blastocyst stage in superovulated heifers when compared with unstimulated heifers. However, recent findings of our research group have demonstrated an upregulation of pluripotency genes (*NANOG* and *OCT4*) and genes involved in placental development

(*PLAC8*) in the embryos of cows submitted to ovarian superstimulation when compared to unstimulated cows (unpublished data), reinforcing a positive role of ovarian superstimulation (P-36 and P36/eCG) on embryo competence in Nelore cows.

In this study, we demonstrated for the first time the effect of two different protocols of ovarian superstimulation on mRNA expression of the main genes involved in gamete transport in the bovine oviduct. This study revealed tissue-specific regulation (infundibulum, ampulla or isthmus) of the mRNAs encoding prostaglandin (*EP2* and *EP4*) and angiotensin receptors (*AGTR2*) in the bovine oviduct. Additionally, the mRNA expression levels of several members of the VEGF, angiotensin II, endothelin 1 and prostaglandin pathways were determined separately for each portion of the bovine oviduct.

Endothelins were first identified as potent vasoactive peptides, regulating vascular tone and blood pressure (Yanagisawa et al., 1988). Three isoforms are known, ET1, ET2 and ET3, but ET1 is the most important in the oviduct (Priyadarsana et al., 2004; Bridges et al., 2011). The endothelin converting enzymes (mostly represented by ECE1) are responsible for producing the active isoform, ET1. The evidence indicates a possible function of ET1 during the periovulatory period in the oviduct because the highest level of ET1 secretion occurs during the periovulatory period (Priyadarsana et al., 2004). Moreover, ET1 stimulates PGE_2 and $PGF_{2\alpha}$ release, and increases oviductal contraction during the periovulatory period in the bovine oviduct (Wijayagunawardane et al., 2001b; Priyadarsana et al., 2004). Along with ET1, VEGF was shown to stimulate the biosynthesis and release of PGE_2 and $PGF_{2\alpha}$ in the bovine oviduct by Wijayagunawardane et al. (2005), indicating that VEGF participates in oviduct contraction. Indeed, the increased levels of *VEGF* mRNA during the periovulatory

period in the human oviduct (Lam et al., 2003) and of the receptor, *Flt-1,* during the preovulatory period in the bovine oviduct (Gabler et al., 1999) confirm the importance of the VEGF pathway in the physiology of oviduct contraction. Furthermore, VEGF stimulates ET1 release and mRNA expression in the bovine oviduct (Wijayagunawardane et al., 2005). Thus, the activities of endothelin and VEGF in the bovine oviduct are essential to promote oviduct contractions. In the present study, the expression of transcripts encoding components of all of these pathways in the oviducts of cows submitted to ovarian superstimulation protocols and the lack of changes in *ET1*, *ECE*, *VEGF*, *Flk1* and *Flt1* mRNA upon ovarian superstimulation indicate normal functioning of the processes controlled by these factors in bovine oviduct physiology.

The prostaglandins are initially produced from arachidonic acid (liberated from phospholipids) by cyclooxygenases with conversion to PGH_2, which is post-converted to several prostaglandins by the prostaglandin synthases. The PGE synthase (PGES) and PGF synthase (PGFS) convert PGH_2 to PGE_2 and $PGF_{2\alpha}$, respectively (Okuda et al., 2002). The F series are known to cause contraction in smooth muscle, and the E series are known to cause relaxation (Siemieniuch et al., 2009). PGE_2 has four receptor subtypes: EP1, EP2, EP3 and EP4 (Sugimoto et al., 2000). In the bovine oviduct, the most important are EP2 and EP4, which act activation of adenylate cyclase (Narumiya et al., 1999). In the present study, we showed an upregulation of *EP2* mRNA in the ampulla and *EP4* mRNA in the infundibulum, as well as a tendency of *EP2 mRNA* levels to increase in the infundibulum of the oviduct from cows submitted to the P36/eCG protocol. These results agree with Sayasith et al. (2009) and Segi et al. (2003), who reported an upregulation of EP2 and EP4 by hCG in the preovulatory follicles of mares and mice, respectively.

Furthermore, the ovarian superstimulatory protocols tended to stimulate *PGES* expression. This observation may be due to the increased serum E_2 concentration in these cows, because PGE_2 synthesis is upregulated by E_2 in bovine oviductal epithelial cells *in vitro* (Wijayagunawardane et al., 1999). It is known that PGE_2 is involved in the relaxation of oviduct smooth muscle (Siemieniuch et al., 2009), and the upregulation of PGE_2 receptors maybe increase the effect of PGE_2 and reduce the oviductal muscle contraction in cows submitted to the P36/eCG protocol, most likely through eCG-mediated effects on the LH receptor (Murphy and Martinuk, 1991). This hypothesis is corroborated by the work of Gawronska et al. (1999) who demonstrated an inhibitory effect of LH on spontaneous contractions *in vitro* in the isthmus and ampulla of swine during the periovulatory period, suggesting a deleterious effect of eCG due to decreased oviduct contractions. However, some studies have suggested that the ciliary activity is able to transport the ovum to the site of fertilization, independent of oviduct contraction (Halbert et al., 1976; Halbert et al., 1989), and that PGE_2 has a stimulatory effect on the ciliary beat frequency (CBF) in hamster oviducts (Hermoso et al., 2001), human Fallopian tubes *in vitro* (Lyons et al., 2006a) and cultured human nasal mucosa (Haxel et al., 2001). A reduced efficacy of ovum pick-up is associated with decreased fimbrial CBF and increased infertility rates (Lyons et al., 2006b). Therefore, the upregulation of *EP2* and *EP4* in the present work may also suggest a role for this system in CBF improvement and ovum transport in cows submitted to the P36/eCG protocol. However, more studies are needed to identify the specific location where the PGE_2 receptors are upregulated, the smooth muscle, the ciliary cells or both, which will provide a better understanding of the effects of the ovarian superstimulation protocols.

Angiotensin II is known for its role in blood pressure regulation, but this factor has many functions in reproductive biology, i.e., vascular control of ovarian function, corpus luteum formation and luteolysis (Gonçalves et al., 2012). Angiotensin converting enzyme (ACE) converts the Ang I into Ang II. The differences between species reveal different functions of Ang II; expression of Ang II type 2 receptor (*ATGR2*) mRNA has been observed in atresic follicles in rats (de Gooyer et al., 2004). In contrast, *ATGR2* expression in bovine granulosa cells was significantly higher in healthy follicles than in atretic follicles (Portela et al., 2008). Furthermore, Ferreira et al. (2007) demonstrated that Ang II is essential for ovulation in cattle, because an intrafollicular injection of Ang II antagonist led to the inhibition of ovulation. Evidence suggests that Ang II is involved in the contraction systems in the oviduct. Wijayagunawardane et al. (2001a) demonstrated a stimulatory effect of Ang II on oviduct contraction, as well as a positive effect of Ang II on PGE_2, $PGF_{2\alpha}$ and ET1 release in bovine oviducts *in vitro*. The isthmus is known to be a storage reservoir for sperm (Hunter and Wilmut, 1984), and studies have found ACE activity in ejaculate, which may modulate the local activity of Ang II and smooth muscle tonus of the oviduct and facilitate sperm transport (Jentzsch et al., 1989). Therefore, the increased *AGTR*2 expression observed in the present work may increase the Ang II activity and facilitate sperm transport to the site of fertilization. Portela et al. (2008) observed increased levels of *AGTR2* mRNA in bovine granulosa cells cultured with FSH. Similarly, Barros et al. (2012) demonstrated that the P-36 and P-36/eCG protocols increased the abundance of *AGTR2* mRNA in bovine granulosa cells *in vivo*. Based on these observations, the upregulation of *AGTR2* expression in the isthmus observed in the present study following both ovarian superstimulation protocols may stimulate oviduct contraction and suggests an important role for the Ang II

pathway in several reproductive tissues, which likely ensures fertility in cows submitted to the P-36 and P-36/eCG ovarian superstimulatory protocols.

Thus, in conclusion, the mRNA expression of all genes related to oviduct contraction were analyzed in Nelore cows submitted to ovarian superstimulation or unstimulated controls, and the upregulation of *EP2*, *EP4* and *AGTR2* expression in the superstimulated cows suggests a suitable effect of exogenous hormones from the protocols on bovine oviduct contractions while optimal conditions for embryonic development are maintained.

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CAPÍTULO 3

Influence of ovulation on gene expression in the oviduct from Nelore (Bos taurus indicus) and Aberdeen (Bos taurus taurus) heifers.

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ABSTRACT

The differences of reproductive biotechnologies responsiveness and physiological characteristics between *Bos taurus indicus* and *Bos taurus taurus* breeds are largely described. Particularly, the antral follicle count (AFC) seems to be related to a better fertility and is an important factor for bovine reproduction. However, the information about impact of AFC on the bovine oviduct physiology is still poorly understood. Therefore, this present work focused to evaluate the differences of gene expression in the bovine ipsilateral and contralateral oviduct from Nelore and Aberdeen Angus heifers with different AFC during early post ovulation time. For this, Nelore heifers (High Follicle Count, HFC; n=4 and Low Follicle Count, LFC; n=4) and Aberdeen Angus (HFC, n=4; LFC, n=4) were slaughtered one day after the ovulation. The ipsilateral and contralateral oviducts were isolated and the total RNA was extracted of each oviductal segment (infundibulum, ampulla and isthmus). The mRNA abundance of genes involved with gametes transport and fertilization was analyzed by relative RT-qPCR using the TaqMan® Low Density Array (TLDA, Life Technologies, USA). The ampulla from Aberdeen Angus heifers demonstrated higher levels of AGTR1 mRNA compared

to Nelore heifers. However, the most important effect was observed on the regulation of target genes between ipsilateral and contralateral antimere. Unfortunately, the AFC had no effect in the gene expression in the oviduct. These finds suggest that functions of the bovine oviduct are mainly regulated by the ovulation, and that breed and AFC have minimal effect in the oviductal molecular physiology during early post ovulation time in cattle.

INTRODUCTION

The oviduct is responsible to provide the microenvironment for final gametes maturation and transport, fertilization and early embryo development and these events occur in a specific oviductal segment: infundibulum, ampulla and isthmus. Even more, the oviduct is the local where occur the first communication between embryo and maternal reproductive tract (Buhi 2002).

The infundibulum is responsible to cumulus-oocyte complexes (COC) pickup and transport to ampulla (Talbot *et al.* 1999), where occurs fertilization and early embryo development (Kölle *et al.* 2009). In isthmus occurs the formation of sperm reservoir, capacitation and hyperactivation (Hunter and Wilmut 1984; Suarez 2002; Suarez and Pacey 2006). The temporal and spatial organization of these events is fundamental to reproductive efficiency (Ruckebusch and Bayard 1975; Wijayagunawardane *et al.* 2001b) and is under control of complex regulation.

Reproductive differences between *Bos taurus indicus* and *Bos taurus taurus* cattle are largely known, e.g., the number of follicles per wave. *Bos taurus indicus* cows recruit a bigger number of follicles than *Bos taurus taurus* cows (Carvalho *et al.* 2008), thus, there are animals with high follicle count (HFC) and low follicle count (LFC). There are evidences that antral follicle count (AFC) is highly variable among animals,

but is highly repeatable within individuals (Boni *et al.* 1997; Jimenez-Krassel *et al.* 2009; Mossa *et al.* 2010a; Mossa *et al.* 2010b). A high number of follicles per wave is directly associated with a better efficiency in reproductive biotechnologies, such as, embryo transfer, *in vitro* embryo production and ovarian superstimulatory protocols (Burns *et al.* 2005; Mossa *et al.* 2010a). Indeed, the low AFC is associated with impaired fertility, specifically with reduced conception rates and with a longer interval from calving to conception (Mossa *et al.* 2012). However, the effect of breed and AFC in the profile of gene expression in bovine oviduct is still unknown.

Thus, the aim of this study was investigate the differences on the expression of genes involved with gametes transport and fertilization in the ipsilateral and contralateral oviduct between animals with HFC and LFC in *Bos taurus indicus* and *Bos taurus taurus* heifers during early post ovulation time.

MATERIAL AND METHODS

Animals

This study was conducted on a farm located in Ribeirao do Sul (Sao Paulo, Brazil). Eight Nelore heifers (HFC, n=4; LFC, n=4) and eight Aberdeen Angus (HFC, n=4; LFC, n=4) were used in this study. Heifers were maintained on pasture (Brachiaria brizantha), with *ad libitum* access to water and a mineral supplement. Nelore (*Bos taurus indicus*) and Aberdeen Angus (*Bos taurus taurus*) heifers ranged from 24 to 30 months were submitted to two doses of prostaglandin $F_2\alpha$ with an interval of 11 days to synchronized the estrous cycle. After estrus detection, the ovarian ultrassonography was performed to characterize the ovulation time. One day after ovulation, the heifers were slaughtered. All experimental animals were treated according to the animal protection law of Brazil.

78

80 *Sample collection*

The reproductive tracts of the cows were transported to the laboratory (about 2
82 hours) in saline solution (0.9%) at 4º C. The two oviducts (ipsilateral and contralateral to the ovulation) of each animal were isolated and the surrounding connective tissues
84 were trimmed. The length of the oviducts was measure (data are showed in mean±EPM; cm). To gene expression, each segment of the oviduct was analyzed separately
86 (infundibulum, ampulla and isthmus) and the transition regions were discarded. The samples were storage in -80ºC. The total RNA was extracted using Illustra TriplePrep
88 Kit (GE Healthcare, USA), according to the manufacter's protocol. Tissue sample were homogenized separately using Precellys® (Bertin Technologies) with 500ul of lyses
90 buffer according to protocol: three cycles of 50 seconds at 6500 rpm.

92 *Real-time RT-PCR*

Total RNA (1.2 µg) from each segment of the oviduct (infundibulum, ampulla
94 and isthmus) was incubated with DNAse I (Invitrogen®) and then reverse transcribed within High Capacity cDNA kit (Life Technologies, USA), according to the
96 manufacturer's protocols. Relative RT-qPCR analysis was performed with TaqMan® Low Density Array (TLDA, Life Technologies, USA). The genes analyzed in this study
98 are details in table 1.

100

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Table 1. Genes analyzed in bovine oviduct using TLDA system.

Gene	Gene description	Code
ANXA1	Annexin 1	Bt03224459_g1
ANXA2	Annexin 2	Bt03215891_g1
ANXA4	Annexin 4	Bt03210021_m1
ANXA5	Annexin 5	Bt03252080_g1
FUCA1	Fucosidase, alpha-L-1	Bt03238509_g1
FUCA2	Fucosidase, alpha-L-2	Bt04285945_m1
FLT1	VEGF receptor, type I receptor tyrosine kinase	Bt04302190_m1
FLK1	VEGF receptor, type III receptor tyrosine kinase	Bt03258885_m1
VEGF	Vascular Endothelial Growth Factor	Bt03213282_m1
COX1	Prostaglandin-endoperoxidase synthase/Cyclooxygenase 1	Bt03817775_m1
COX2	Prostaglandin-endoperoxidase synthase/Cyclooxygenase 2	Bt03214492_m1
EP2	Prostaglandin E receptor 2	Bt03223848_m1
EP4	Prostaglandin E receptor 4	Bt03223849_m1
ET1	Endothelin 1	Bt03217446_m1
ECE1	Endothelin Converting Enzyme 1	Bt03217439_m1
AGTR1	Angiotensin II receptor, type 1	Bt03213473_m1
ACE	Angiotensin Converting Enzyme	Bt04300007_g1
LHR	Lutropin Hormone receptor	Bt03213972_m1
OVGP1	Oviductal glycoprotein 1	Bt03253683_g1
GRP78	Glucose-regulated protein 78kDa (HSP 70kDa protein 5)	Bt03244880_m1
PPIA	Peptidylprolyl Isomerase A/Cyclophilin A	Bt03224615_g1
ACTB	Actin, Beta	Bt03279174_g1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Bt03210913_g1
18S	18S Ribosomal RNA	Hs99999901_s1

104 The white lines are target genes, and the gray lines are references genes.

106 The relative expression of target genes was calculated using the ΔΔCt method with efficiency correction (Pfaffl 2001). To select the most stable reference gene for 108 analysis of the oviduct: peptidylprolyl isomerase A (*PPIA*), Actin Beta (*ACTB*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and 18s Ribosomal RNA (*18S*) 110 gene expression and amplification profiles were tested and compared using the geNorm

applet for Microsoft Excel (medgen.ugent.be/genorm; (Ramakers *et al.* 2003). Based on this analysis, the stables references genes *PPIA* and *18S* were used to normalization.

Statistical analysis

All results were transformed to logarithmic to be a normal distribution. The mRNA abundance of the target genes and the length of oviduct were tested by ANOVA, using PROC GLM procedure of SAS (SAS, 9.2, SAS Inst., Cary, NC, USA). Individual differences were analyzed through pair-wise comparisons (SAS). The mRNA abundance were compared in each segment (ampulla, infundibulum and isthmus), no comparisons were performed between segments. The differences were considered significant when $P<0.05$. Data are showed in mean ± S.E.M.

RESULTS

The first interesting finding was the difference on the total oviduct length between breeds. The total oviduct from Aberdeen Angus heifers (29.59±0.68 cm; n=16) was longer when compared with Nelore heifers (21.37±1.17 cm; n=16). No effects of segment, ovulation or AFC were found in our analysis.

All target genes were expressed in the three regions of the bovine oviduct, except the *LHR* expression, which was not detected in 47% of the samples, therefore, the mRNA abundance of *LHR* was not conclusive. No difference in gene expression was found between groups of different ovarian follicular count (HFC and LFC).

In general, the gene expression was different between the antimere of ovulation. In the infundibulum, the ipsilateral oviduct presents a higher expression of *EP4*, *GRP78, FUCA2* and *FUCA1* (Fig. 1). The same effect of ipsilateral oviduct was observed in the ampulla; the abundance of *COX2, OVGP1, GRP78, FUCA1* and *ANXA4*

136 was higher in the ipsilateral oviduct (Fig. 2). In the isthmus the results were different; the expression of *VEGF*, *FLK1* and *FUCA2* was higher in the contralateral oviduct (Fig. 138 3). About breed effect, only a higher abundance of *AGTR1* mRNA was observed in Aberdeen Angus compared to Nelore heifers (Fig. 4).

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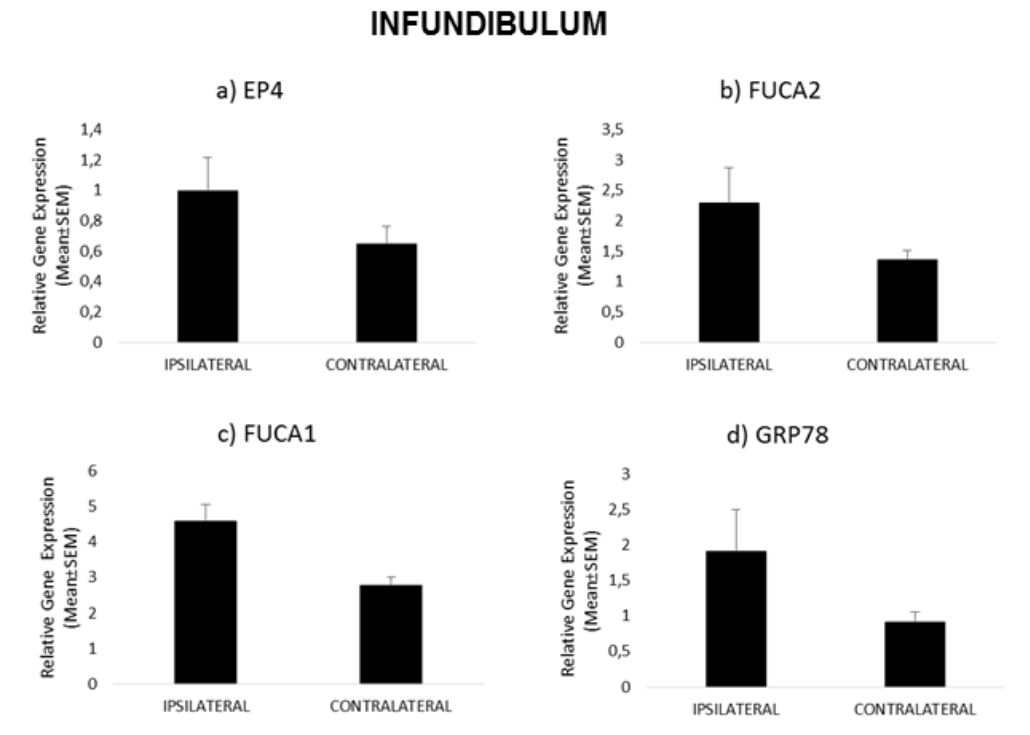

142

Figure 1. Difference in gene expression in the ipsilateral and contralateral antimere in 144 the infundibulum of bovine oviduct (mean ± S.E.M). a. *ER4*, b. *FUCA2*, c. *FUCA1*, d. *GRP78*. The relative mRNA levels were analyzed by ANOVA. The differences were 146 considered significant when $P<0.05$. Ipsilateral antimere (n=16) and contralateral antimere (n=16).

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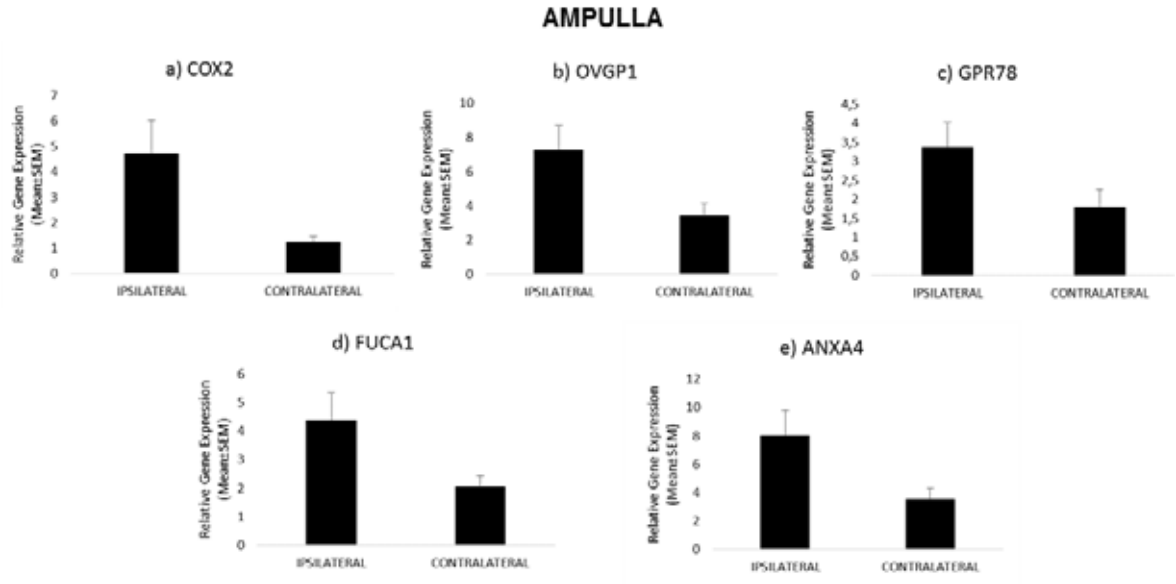

Figure 2. Difference in gene expression in the ipsilateral and contralateral antimere in the ampulla of bovine oviduct (mean ± S.E.M). a. *COX2*, b. *OVGP1*, c. *GRP78*, d. *FUCA1*, e. *ANXA4.* The relative mRNA levels were analyzed by ANOVA. The differences were considered significant when P<0.05. Ipsilateral antimere (n=16) and contralateral antimere (n=16).

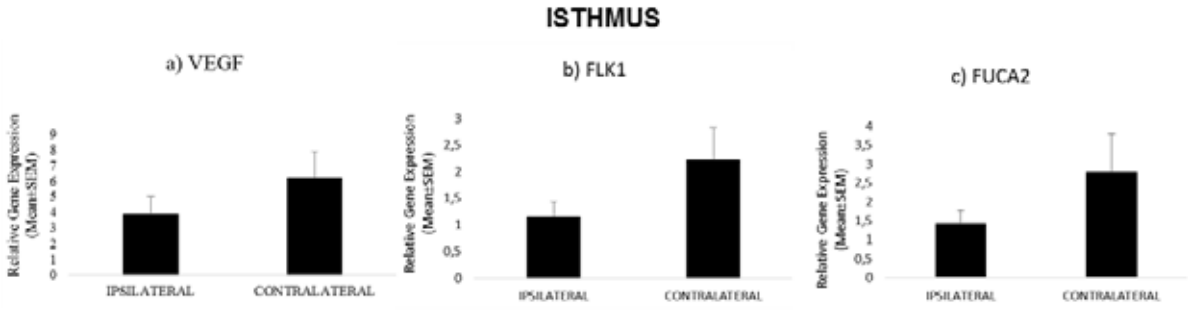

Figure 3. Difference in gene expression in the ipsilateral and contralateral antimere in the isthmus of bovine oviduct (mean ± S.E.M). , a. *VEGF*, b. *FLK1*, c. *FUCA2.* The relative mRNA levels were analyzed by ANOVA. The differences were considered significant when P<0.05. Ipsilateral antimere (n=16) and contralateral antimere (n=16).

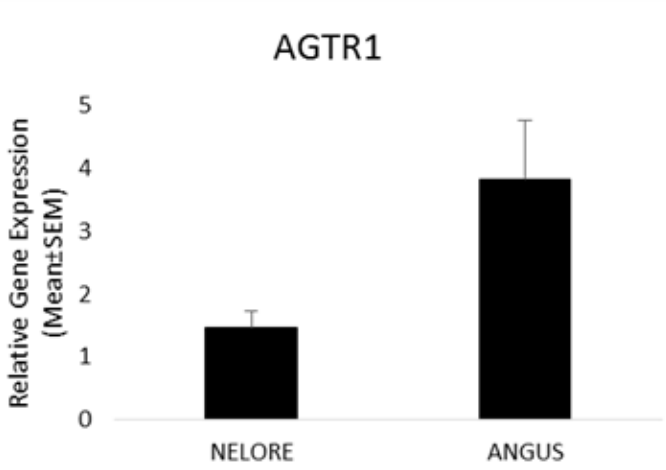

Figure 4. Difference in gene expression of bovine oviduct from Nelore and Aberdeen Angus (Angus). mRNA abundance of *AGTR1* in the ampulla (mean ± S.E.M). The relative mRNA levels were analyzed by ANOVA. The differences were considered significant when $P<0.05$. Nelore (n=16) and Aberdeen Angus (n=16).

DISCUSSION

The present study was focused on the gene expression in bovine oviduct, comparing *Bos taurus indicus* and *Bos taurus taurus* cattle with HFC and LFC in both antimere oviduct: ipsilateral and contralateral to ovulation. The results showed that AFC there was no effect in oviductal gene expression and that breed factor only influenced the difference in *AGTR1* expression in the ampulla. Moreover, ovulation seems to be the major factor that regulates gene expression in all three segments in the oviduct.

The 78-kDa glucose-regulated protein (GRP78) is a endoplasmic reticular component, well characterized to assist the folding and assembly of newly-synthesized proteins and regulation of degradation of aberrant polypeptides (Gething 1999). In the reproduction system, experiments *in vivo* and *in vitro* show that GRP78 interact with sperm and bind to them (Anderson and Killian 1994; Grippo *et al.* 1995; Boilard *et al.* 2004), and this interaction is beneficial to sperm by improving sperm viability,

acrosomal integrity and sperm movements evaluation (Boilard *et al.* 2004). In the female gamete, GRP78 is present on the surface of mature mouse oocyte (Calvert *et al.* 2003) and may act in a coordinated manner to activate fusion machinery on the surface of the oocyte (Bromfield and Nixon 2013). Furthermore, the GRP78 modulates sperm interaction with *zona pellucida* (ZP) in human (Marín-Briggiler *et al.* 2010). The lack of GRP78 increases the inner cell mass (ICM) apoptosis in mouse embryos and its presence is absolutely required for mouse embryos development (Luo *et al.* 2006). In our study, all samples presented *GRP78* expression, but the mRNA abundance was higher in ipsilateral ampulla and infundibulum, suggesting an effect of ovulation to regulate gene expression to promote a better microenvironment to gametes interaction and early embryo development in the ampulla (Kölle *et al.* 2009).

The α-L-fucosidases are involved in the hydrolytic degradation of α-L-fucose (Michalski and Klein 1999). There are two genes encoding α-L-fucosidase, *FUCA1* and *FUCA2* (Sobkowicz *et al.* 2014), and a great difference between them is the ideal pH of activity (Sobkowicz *et al.* 2014). The α-L-fucose is present in annexins, and has been proposed to be the binding sites of bovine sperm in the formation of sperm reservoir in the isthmus (Lefebvre *et al.* 1997). At the time of ovulation, the spermatozoa is released from the reservoir and move toward the ampulla, where the oocytes have just arrived. Studies suggest the α-L-fucosidase regulates sperm binding to α-L-fucose in the oviductal epithelium (Lefebvre *et al.* 1997; Carrasco *et al.* 2008). Furthermore, the α-L-fucosidase is involved in sperm-ZP interaction in mouse (Phopin *et al.* 2013), hamster (Venditti *et al.* 2010) and in cattle (Tanghe *et al.* 2004) and is present in higher levels in the epipidymal fluid of high-fertility bull than low-fertility bull (Moura *et al.* 2006). The present study shows the presence of both genes that encoding α-L-fucosidase (*FUCA1* and *FUCA2*) throughout the bovine oviduct. The up-regulation of these genes in the

206 ipsilateral infundibulum and ampulla maybe is involved in the preparation of fertilization, to improve the sperm-oocyte interaction. Contrarily to expectations, 208 *FUCA2* expression was down regulated in the ipsilateral oviduct, suggesting that is necessary more functional experiments to indicate FUCA2 function in the bovine 210 oviduct, whereas we expected a higher expression of *FUCA* to allow sperm release from isthmus to move toward ampulla. Our study also showed other up-regulated gene in the 212 ipsilateral ampulla, the *ANXA4*. More experiments are necessary to understand the effect of this protein in the bovine oviduct. The possible role in the oviduct could be the 214 regulation of ion and water movement across the oviductal epithelium, as demonstrated by Ponnampalam and Rogers (2006) in human endometrium.

216 Other factor involved in the fertilization is the OVGP1 (Oviductal glycoprotein 1). Oocytes incubated with OVGP1 become more resistant to sperm penetration. This 218 protein, in association to heparin, modifies ZP solubility and consequently makes it more resistant to sperm penetration (Coy *et al.* 2008; Mondéjar *et al.* 2013). Studies *in* 220 *vitro* demonstrated an increase in the incidence of monospermy in porcine (Coy *et al.* 2008) and bovine fertilization (Coy *et al.* 2008; Cebrian-Serrano *et al.* 2013). 222 Additionally, the OVGP1 has a positive effect in embryo development (Boice *et al.* 1992; Kouba *et al.* 2000; Killian 2004). The up-regulation in the *OVGP1* expression in 224 the ipsilateral ampulla suggests a role on the regulation of the monospermy fertilization.

In our experiments, the *COX2* mRNA abundance was higher in the ipsilateral 226 ampulla compared to contralateral, that disagrees to the results described by Odau *et al.* (2006) and Gauvreau *et al.* (2010), which demonstrated that *COX2* expression had no 228 difference in mRNA expression during the whole estrous cycle in the ipsi- and contralateral ampulla and isthmus from bovine oviducts (Odau *et al.* 2006; Gauvreau *et* 230 *al.* 2010). The cyclooxygenases (COX) catalyze the conversion of arachidonic acid to

PHG_2, which is post-converted to several prostaglandins by the prostaglandin synthases. The PGE synthase (PGES) and PGF synthase (PGFS) convert PGH_2 to PGE_2 and $PGF_{2\alpha}$, respectively (Okuda *et al.* 2002). Wijayagunawardane *et al.* (1998) demonstrated higher concentrations of PGE_2 and $PGF_{2\alpha}$ in the ipsilateral oviduct during the follicular and post ovulation stage in the estrous cycle in cattle. So, in our study, the up-regulation of *COX2* expression in ipsilateral oviduct is essential to provide the increase in PGE_2 and $PGF_{2\alpha}$ concentrations. The PGE_2 is known to cause oviductal smooth muscle relaxation by the interaction within its receptors, EP2 and EP4 (Sugimoto *et al.* 2000) and the $PGF_{2\alpha}$ cause the contraction in smooth muscle in the oviduct (Siemieniuch *et al.* 2009). Furthermore, PGE_2 has a stimulatory effect on the ciliary beat frequency (CBF) in hamster oviducts (Hermoso *et al.* 2001), human Fallopian tubes *in vitro* (Lyons *et al.* 2006) and cultured human nasal mucosa (Haxel *et al.* 2001). In the ipsilateral infundibulum, the up-regulation of *EP4* suggests a possibly action of PGE_2 in the CBF, since the smooth muscle has not been found in the cows infundibulum (Lombard *et al.* 1950).

Additionally, in the ipsilateral isthmus, we observed a down-regulation in the *VEGF* and *FLK1* expression. Wijayagunawardane *et al.* (2005) demonstrated the *in vitro* function of VEGF system in the bovine oviduct, these authors suggest that the preovulatory conditions (high levels of LH and E_2 and basal P_4 levels) stimulates the VEGF system, that induces the maximum oviductal production of contraction substances to rapid transportation of gametes to the fertilization site. A negative feedback mechanism of VEGF promoting a down-regulation on their system after higher levels of *VEGF* and its receptors expression, to contribute to suppress oviductal contraction to secure slow transport of the embryo to the uterus (Wijayagunawardane *et al.* 2005). In present study, the lowest levels of *VEGF* and *FLK1* expression in

ipsilateral oviduct corroborates with this negative feedback of VEGF, to promote a secure transport of the embryo only in the isthmus.

Unfortunately, in the present work, no great differences were found between *Bos taurus taurus* and *Bos taurus indicus* heifers, only a higher expression of *AGTR1* in Aberdeen Angus compared to Nelore oviducts was demonstrated. The angiotensin II (AngII) has several functions in reproductive biology, e.g. vascular control of ovarian function, corpus luteum formation, luteolysis (Gonçalves *et al.* 2012) and control of ovulation (Ferreira *et al.* 2007). The AngII type 1 and 2 receptors was demonstrated in the oviduct (Saridogan *et al.* 1996). The AngII has been demonstrated as a stimulator of oviduct smooth muscle contraction (Wijayagunawardane *et al.* 2001a) and a regulator of the composition of oviduct secretions (Mahmood *et al.* 2002). Additionally, Saridogan *et al.* (1996) show a stimulatory increase in the CBF in the human oviduct caused by AngII, and observed that this effect is selective to AGTR1. Based on this, we suggest a compensatory effect in Aberdeen Angus by overexpression of *AGTR1* to provide an increase in CBF to appropriate time transport due to longer length of total oviduct from Aberdeen Angus heifers when compared with Nelore heifers.

In conclusion, the ovulation seems to be the most important regulatory factor of genes involved with gametes transport and fertilization in heifers with HFC and LFC from *Bos taurus indicus* and *Bos taurus taurus breeds* during early post ovulation time.

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