UNIVERSIDADE FEDERAL DA PARAÍBA
CENTRO DE CIÊNCIAS AGRÁRIAS

MODULAÇÃO DA EXPRESSÃO GÊNICA E PROTEICA CARUNCULAR PELO EMBRIÃO BOVINO DURANTE A PLACENTAÇÃO

Carla Fabiana Gomes de Jesus
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Carla Fabiana Gomes de Jesus
Orientador: Profa. Dra. Danila Barreiro Campos

Dissertação apresentada ao Programa de Pós-Graduação em Ciência Animal do Centro de Ciências Agrárias da Universidade Federal da Paraíba, como parte das exigências para a obtenção do título de Mestre em Ciência Animal.

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DADOS CURRICULARES DO AUTOR

Nascida no dia 12 de dezembro de 1988, em Arapiraca município do estado de Alagoas. No ano de 2006 concluiu o ensino médio no Colégio Normal São Francisco de Assis na mesma cidade, em 2007.2 ingressou no curso de medicina veterinária pela Universidade Federal de Alagoas (UFAL), concluindo o curso em outubro de 2012. No mês março de 2013 ingressou no curso de mestrado no Programa de Pós-Graduação em Ciência Animal, da Universidade Federal da Paraíba.

DEDICATÓRIA

Aos meus pais João Félix de Jesus (*in memorian*) e Marilene Gomes de Jesus, obrigada por me fazerem acreditar que sonhos são possíveis e que as dificuldades fazem parte do nosso amadurecimento.

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RESUMO GERAL

Apesar da existência de vários estudos relacionados à reprodução, alguns eventos sobre a ocorrência da perda do concepto no início da gestação ainda estão sem respostas, devido as variáveis envolvidas. Este estudo teve como objetivo identificar os genes diferencialmente expressos em carúnculas uterinas do corno gravídico (CG) e corno não gravídico (NG) durante a placentação bovina e investigar o papel do embrião produzido por fertilização in vitro (FIV) na expressão desses genes. A verificação de que o embrião de fato influencia também a expressão gênica e proteica caruncular poderá esclarecer quais os sinais do embrião que regulam o desenvolvimento uterino e que os achados poderão estar relacionados com as altas taxas de falhas gestacionais com embriões manipulados. Para execução do estudo foram coletadas carúnculas do CG e NG de fêmeas primíparas *Bos indicus* submetidas à IA ou FIV utilizando sêmen sexado para macho *Bos indicus*. As amostras foram coletadas nos dias 30 (n = 3), 35 (n = 8) e 40 (n = 3) para IA e 35 dias (n=3) e 40 dias (n=3) de FIV, após separação dos cotilédones. Os tecidos foram congelados em nitrogênio líquido e armazenados em freezer -80°C para posterior extração de RNA e proteína, fixados em formaldeído tamponado 4% para realização da imunohistoquímica. O transcriptoma dessas amostras de dia 35 (n=7) foi avaliado por microarranjo utilizando a plataforma Affymetrix, tal análise demonstrou que do total de 23.000 genes investigados, 149 apresentaram-se diferencialmente expressos em carúnculas bovinas do corno gravídico (≥ 1.5 fold, $p<0.05$). Desses, nove potencialmente envolvidos na diferenciação celular foram utilizados na validação dos resultados por PCR em tempo real, sete destes apresentavam-se expressão significativamente aumentada (*ACP5*, *DPP4*, *GJB6*, *IGFBP3*, *INHBA*, *STC1*, *THBS2*)e dois com expressão significativamente diminuída (*CXCR4* e *PTGS2)*. A análise dos resultados do PCR em tempo real demonstrou que a

expressão dos genes foi consistente com os resultados do microarranjo ($p<0,05$). A análise do western blot, realizado em três proteínas (PTGS2, IGFPB3 eTHSB2), indicou aumento na expressão da PTGS2 aos 40 dias e da IGFPB3 e THSB2 aos 35 e 40 dias, ambas gestações IA. Em gestações FIV, a expressão da PTGS2 foi maior aos 35 dias no CG. Em contrapartida, o padrão de expressão da IGFPB3 e THSB2 foi similar ao observado em gestações IA. A imunolocalização das proteínas foi observada no citoplasma de células do epitélio, estroma uterino, células glandulares e vasculares. Observou-se associação entre esses resultados com os observados no western blot, pois a maior intensidade de marcação existiu em células CG aos 35 e 40 dias em gestações obtidas por IA e FIV. Conclui-se que a expressão dos genes diferencialmente expressos nas carúnculas dos cornos gestantes e não gestante, envolvidos no processo de placentação sofrem influencias do embrião e que procedimentos como a produção de embriões *in vitro* e/ou cultivo celular podem influenciar na regulação dessas moléculas, já que a maior expressão na maioria dos genes em gestação FIV ocorreu mais tardiamente, aos 40 dias.

PALAVRAS-CHAVE: Endométrio, fertilização *in vitro*, inseminação artificial,

ABSTRACT

Despite the existence of several studies related to reproduction, some events about embryo loss in early gestation are not understand due the variables involved. This study aimed to identify differentially expressed genes between uterine caruncles of the gravid horns (G) and non-gravid horns (NG) horns during the bovine placentation and investigate the role of embryo produced by in vitro fertilization (IVF) in this gene expression. The finding that the embryo also influences the caruncular gene and protein expression could clarify which embryo signals that regulate uterine development and that the findings may be related to high failure rates of pregnancy in the pregnancies of manipulated embryos. In the present study caruncles from the G and NG were collected from pregnant primiparous cows (*Bos indicus*) undergoing artificial insemination (AI) or transfer of embryos produced by in vitro fertilization (IVF) using *Bos indicus* male sexed sperm. Animals were slaughter at 30 (n = 3), 35 (n = 8) or 40 (n = 3) days of gestation and tissues were collected following separation of the weakly associated cotyledons. Tissues were either frozen in liquid nitrogen or stored at -80°C freezer until RNA or protein extraction or fixed in 4% buffered formaldehyde for immunohistochemical analysis. The transcriptome of samples of 35 days (n = 7) were evaluated by microarray using an Affymetrix microarray platform. Analysis showed that 23.000 genes, 149 were differentially expressed in cattle caruncles from the gravid horn (≥ 1.5 fold, $p<0.05$). Nine genes potentially involved in cell differentiation were used to validate the results of real time PCR: seven upregulated genes (*ACP5*,*DPP4*, *GJB6*, *IGFBP3*, *INHBA*, *STC1*,*THBS2*) and two downregulated genes (*CXCR4* e *PTGS2)*. Quantitative PCR demonstrated that expression ratios of selected genes were consistent with the microarray results ($p<0,05$). Western blot analysis was performed to investigate PTGS2, THSB2 e IGFPB3. Densitometric evaluation indicated an increase in protein content of PTGS2 at 40 days and IGFPB3 and THSB2 at 35

and 40 days of IA both gestations. In FIV gestations, PTGS2 protein abundance was significantly higher at 35 days in caruncles from the gravid horn. On the other hand, the pattern of IGFPB3 e THSB2 protein expression in FIV gestations was similar to that observed in IA gestations. Protein immunostaining was observed in the cytoplasm of epithelial and stromal uterine cells, glandular uterine cells and endothelial uterine cells. We observed that staining intensity was associated to western blot data, with cells from cotyledon-associated caruncle showing more intensity of staining at 35 and 40 days in both AI and FIV gestations. We conclude that the expression of placentation involved genes differentially expressed in caruncles of pregnant and non-pregnant horns suffer influence of embryo and procedures such as in vitro embryo production and cell culture can influence the regulation of these molecules, since the greatest expression of most genes in IVF pregnancies occurred later after 40 days.

Key words: Artificial Insemination, Endometrium, in vitro Fertilization.

CONSIDERAÇÕES GERAIS

INTRODUÇÃO

Durante a gestação, os vertebrados vivíparos desenvolvem um sistema complexo de membranas que envolvem o feto. No local de união das membranas fetais com a mucosa uterina forma-se a placenta, um órgão transitório formado por tecidos maternos e fetais, que possui a função de transportar substâncias nutritivas do organismo materno para o feto, promover trocas metabólicas e desempenhar funções endócrinas por meio da produção de hormônios importantes na manutenção da gestação (LEISER & KAUFMANN, 1994).

A placentação é a justaposição das vilosidades do córion fetal com as criptas da mucosa uterina (GRUNERT & BIRGEL, 1989) sendo o resultado de numerosas variações entre a adesão do embrião com o endométrio, pois essa interação vai de uma simples, porém firme adesão, até a completa erosão do endométrio (LEFEVRE et al., 2007), iniciando logo após a implantação do embrião, momento em que ocorre o desenvolvimento e associação de membranas fetais e tecido uterino (BJÖRKMAN, 1976), que é o diferencial para caracteriza-la segundo as espécies(MOSSMAN, 1987).

De acordo com Wooding (1992) nos ruminantes a placenta é relativamente não invasiva, o cório ficar em contato com o sincício materno-fetal, sendo caracterizada de sinepiteliocorial (AMOROSO, 1952). A placenta da vaca ainda é classificada como cotiledonária, presença dos vilos coriônicos (cotilédones) que ao

unir-se com as carúnculas uterinas formam os placentomas, pontos de troca materno-fetal. E adeciduada, por apresentar perda mínima de tecido uterino após o parto (BJÖRKMAN, 1982).

A formação dos placentomas inicia-se simultaneamente com a implantação, em torno do 20° dia de gestação na vaca, porém o íntimo contato ocorre após 30 dias de gestação, sendo os mais complexos, observados após o 42° dia de gestação. Com o decorrer da gestação os placentomas aumentam de tamanho, variando em função do local que se encontram no útero (KING et al., 1979; SCHLAFER et al., 2000). No entanto, não são todas as carúnculas que formam placentomas durante a gestação. Nas ovelhas cerca de 70 - 80% das carúnculas participam da placentação, essa redução é compensada com aumento do tamanho dos placentomas (HRADECKY et al., 1988). Em gestações de embriões produzidos *in vitro*, como em clones, são detectados menos placentomas em vacas receptoras, sugerindo um retardo do crescimento feto / placentária e ainda um menor desenvolvimento caruncular (HASHIZUME et al., 2002).

O diálogo mãe-embrião nos bovinos é ainda pouco conhecido (WOLF et al., 2003), pois além da interação dos fatores químicos materno-fetais, Nos estudos sobre implantação, na maioria das vezes, estão focalizados no lado endometrial(WANG & DEY, 2006), os estudos relacionados à modulação destes eventos pelo feto ainda são limitados (LEE & YEUNG, 2006).

PLACENTAÇÃO NOS MAMÍFEROS

Os passos essenciais no estabelecimento da gestação são: desenvolvimento do embrião até a fase de blastocisto, sua implantação no endométrio uterino e a formação de uma placenta funcional. Cada fase desta requer uma interação entre o concepto (trofoblasto) e um ou mais dos tipos de células do endométrio materno, e o sucesso da implantação depende do grau de invasão do trofoblasto no endométrio para estabelecer um adequado suprimento sanguíneo (WOODING & FLINT, 1994).

A placentação inicia-se logo após a implantação do embrião, momento em que ocorre o desenvolvimento e associação de membranas fetais e tecido uterino (BJÖRKMAN, 1976). Há diferenças marcantes na implantação entre as espécies, e estas estão relacionada às diferenças nas formas de placentação (WOODING & FLINT, 1994), que é a justaposição das vilosidades do cório fetal com as criptas da mucosa uterina (GRUNERT & BIRGEL, 1989).

PLACENTAÇÃO NOS BOVINOS

A caracterização placentária difere a medida que os tecidos fetais invadem a parede do útero; sendo classificadas de acordo com a capacidade de invasão (MOSSMAN, 1987). De acordo com Wooding (1992) a placentação dos ruminantes é relativamente não-invasiva, e foi caracterizada como sinepiteliocorial. Tal característica foi atribuída devido o cório ficar em contato com o sincício materno-fetal e não com tecido conjuntivo e a perda do tecido materno durante o parto (AMOROSO, 1952). Santos e Beletti (2013) demonstraram que ultraestruturalmente a placenta da vaca no início da gestação, é uma placenta epiteliocorial e por volta do início do segundo mês aparecem os primeiros sinais da transformação da placenta para sinepiteliocorial, mesmo os trofoblastos atingindo a lâmina própria da carúncula, que ocorre de forma superficial.

A placenta da vaca ainda é classificada como cotiledonária, devido a presença dos vilos coriônicos ramificados, chamados de cotilédones. Que ao unir-se com as carúnculas uterinas formam os placentomas, os únicos pontos de troca materno-fetal. E adeciduada por apresentar uma perda mínima de tecido uterino e a placenta ficar retida no útero sendo eliminada somente após o parto (BJÖRKMAN, 1982).

COMUNICAÇÃO MATERNO-FETAL

Para que ocorra a implantação uma variedade de fatores maternos e embrionários são necessários, como: fatores uterinos (enzimas), citocinas, fatores de crescimento, íons, hormônios, glicose, proteínas de transporte e moléculas de adesão (histotrofo) que são sintetizadas principalmente nas glândulas endometriais (MARTAL et al., 1997). Nos ruminantes, estas glândulas são especificamente localizadas em grandes áreas do endométrio (zonas intercaruncular) enquanto pequenas áreas aglandulares de origem estromal espalhadas pela superfície do endométrio são chamadas de carúnculas. As glândulas endometriais das áreas intercaruncular demonstraram ser determinantes para o desenvolvimento do concepto (GRAY et al., 2001) e as áreas carunculares, presentes no endométrio cíclico, se fundem com os cotilédones fetais para dar origem a placentomas nas fêmeas prenhas (ATKINSON et al., 1984).

Nos ruminantes, a estrutura endometrial difere de outras espécies de mamíferos por apresentar as carúnculas e áreas intercarunculares, essenciais para apoiar a gestação. A partir desses pontos dá-se o contato materno - fetal, realizado pelos placentomas, que são estruturas formadas pelo cotilédone fetal e carúncula materna (SLOSS & DUFTY, 1980; NODEN & LAHUNTA, 1990).

O diálogo mãe-embrião nos bovinos é ainda pouco conhecido (WOLF et al., 2003), pois além da interação de fatores químicos materno-fetais, existe o fator que envolve a ocupação do lúmen uterino ipsilateral ao corpo lúteo, para bloquear efetivamente a luteólise. Para isto o embrião deve estar alongado o suficiente para fazer o contato necessário com a superfície do epitélio endometrial. Embriões subdesenvolvidos que não realizam esta ocupação efetivamente, podem ser eliminados (BINELLI, 2000).

No entanto não são todas as carúnculas que formam placentomas durante a gestação. Nas ovelhas cerca de 70 - 80% das carúnculas participam da placentação, essa redução é compensada com aumento do tamanho dos placentomas para facilitar a eficiência na troca de nutrientes (HRADECKY et al., 1988). No caso de gestações de embriões produzidos *in vitro*, por exemplo, clone, diversas anomalias são detectadas incluindo menos placentomas em vacas receptoras, sugerindo um retardamento do crescimento feto / placentária nestes animais e ainda um menor desenvolvimento caruncular (HASHIZUME et al., 2002).

BIOTECNOLOGIAS REPRODUTIVAS

O Brasil atua como um dos líderes na pesquisa de biotecnologias de reprodução assistida, sendo elas: inseminação artificial (IA), múltipla ovulação e transferência de embriões (MOTE) e a fertilização *in vitro* (FIV) (GARCIA, 2009), criadas com o intuito de melhoria do potencial zootécnico animal.

Dentre essas biotecnologias da reprodução, a inseminação artificial (IA) é a mais difundida e que possui maior impacto nos programas de melhoramento animal, pois permite a rápida difusão do material genético de animais superiores (VARAGO et al., 2009), principalmente quando se utiliza sêmen congelado. Que além disso ainda pode evitar a propagação de doenças infecciosas, uma vez que os antibióticos

adicionados aos meios diluidores proporcionam um controle microbiológico eficiente (XAVIER et al., 2009). Mas apesar dessas vantagens, ainda existem limitações no poder de fecundação dos espermatozoides quando congelados, que se torna menor ao se comparar com sêmen fresco (SALAMON & MAXWELL, 2000; ANEL et al., 2006). Isto por causa das lesões que ocorrem durante o processo de congelamento, muitas vezes irreversíveis, diminuindo a viabilidade dos gametas (HOLT, 2000).

A fertilização in vitro (FIV) tornou-se viável na produção de embriões e ganhou destaque na espécie bovina (VIANA et al., 2012). Porém, os problemas de mortalidade embrionária ficaram mais evidentes, principalmente com o decorrer do aprimoramento da técnica. As taxas de prenhez dos embriões produzidos *in vitro* são menores que os obtidos *in vivo* por causa das variações bioquímicas e metabólicas após a transferência (HASLER et al., 2003) e dos mecanismos de reconhecimento materno ocorrer mais tardiamente (FARINET et al., 2001). Além das dificuldades na obtenção dos ovócitos que a qualidade varia de acordo com a idade da fêmea, dificuldades na preservação dos embriões para mantê-los viáveis e o alto custo na produção para os embriões. Ainda existe os altos índices de abortos no início da gestação, prolongamento do período gestacional e morte perinatal causadas por anormalidades congênitas (Ambrose,, 1999; WAGTENDONK-DELEEW et al., 2000).

SEXAGEM DE SÊMEN

O sêmen sexado tem uma boa aceitação pelos grandes produtores, mesmo tendo o valor superior ao do sêmen convencional, devido a capacidade de maximizar a exploração do potencial produtivo dos rebanhos, diminuindo o intervalo de gerações principalmente com o uso de biotecnias como a PIVE (VARAGO et al.,2008).

Na PIVE, o sistema de cultivo é responsável por desviar a proporção dos sexos para o masculino, devido estes apresentar desenvolvimento mais acelerado durante os sete primeiros dias após a fecundação *in vitro*. Além disso, fatores epigenéticos, como a presença de soro fetal bovino (SFB) no meio de cultura, podem acelerar o desenvolvimento embrionário e também alterar a proporção dos sexos (GUTIÉRREZ-ADÁN et al., 2001). Com o uso do sêmen sexado ocorre uma contribuição para sua eficiência, quando a maior proporção de embriões de determinado sexo (macho ou fêmea) é desejável.

A técnica para sexagem é feita por citometria de fluxo e baseia-se nas diferenças de quantidade de DNA entre espermatozóides portadores de cromossomo X ou Y, determinado por corantes fluorescentes (JOHNSON et al.,1987). O espermatozoide X recebe cerca de 4% mais corante vinculado ao seu DNA do que o espermatozoide Y. A fluorescência é medida por um detector e analisado por computador fornecendo o teor do conteúdo de DNA (GARNER, 2006). Esse complexo causa danos nos espermatozoides (MORTON et al., 2007) podendo ser até irreparáveis, capaz de comprometer a eficiência do espermatozoide no processo de fertilização e produção de embriões (DE LIMA, 2007). Além de alterar o padrão de expressão de genes (MORTON et al., 2007).

PERDAS GESTACIONAIS

As perdas fetais no início da gestação são provenientes de anormalidades no embrião ou na placenta, alterações no microambiente uterino, ou ainda falhas na interação materno-fetal (WILMUT et al., 1986). Em animais oriundos de clonagem ou cultura *in vitro*, essas anormalidades ocorrem com mais frequência (THOMPSON et al., 1995; STICE et al., 1996; WAKAYAMA et al., 1998; HILL et al., 1999).

Para o ideal estabelecimento da gestação é preciso de algumas fases: desenvolvimento do embrião até a fase de blastocisto, implantação deste no endométrio uterino e a formação de uma placenta funcional. Cada fase desta requer uma interação do concepto (trofoblasto) junto com um ou mais tipos de células do endométrio materno, invasão deste no endométrio para promover um adequado suprimento sanguíneo (WOODING & FLINT, 1994). Em fetos ovinos clonados ocorre um atraso no desenvolvimento, segundo McMillan & Peterson (1999), está associada a pouca ou a ausência de vascularização placentária, que impede o crescimento e diferenciação fetal e é provável que esta deficiência no início da gestação esteja relacionada com o limitado desenvolvimento fetal ao longo da gestação e a ocorrência de patologias feto/placentárias.

Ainda existe uma variedade de outros fatores como: enzimas uterinas, citocinas, fatores de crescimento, íons, hormônios, glicose, proteínas de transporte e moléculas de adesão que são sintetizadas principalmente nas glândulas

endometriais (MARTAL et al., 1997), que em ruminantes, ficam espalhadas pela superfície do endométrio (GRAY et al., 2001). E o evento que envolve a ocupação do lúmen uterino ipsilateral ao corpo lúteo, para bloquear a luteólise. Para isto, é preciso que o embrião esteja alongado o suficiente para que possa fazer o contato necessário com a superfície do epitélio endometrial. Embriões que não realizam esta ocupação efetivamente, tem grandes chances de serem eliminados (BINELLI., 2000).

EXPRESSÃO GÊNICA

As interações materno-fetal entre as espécies mesmo sendo diferentes, por meio da expressão gênica foi possível manter algumas características (LEISER & KAUFMANN,1994; REIK & WALTER,2001).

Na maioria das espécies as bases moleculares da comunicação materno-fetal não estão totalmente definidas, sendo o papel do embrião como modulador da expressão gênica uterina demonstrado em várias espécies. A evolução dos mecanismos de implantação, invasão e formação da placenta foi essencial para o sucesso da viviparidade, a característica que define os mamíferos Eutheria (LEFEVRE et al., 2007). Essa evolução foi acompanhada pelo recrutamento de uma rede de mais de 1500 genes endometriais, além de genes adicionais ativados devido às numerosas variações evolucionárias que resultaram em diferentes formas de placentação (LYNCH et al., 2011).

EXPRESSÃO DA CICLOXIGENASE - 2 (COX-2) NOS TECIDOS

A proteína cicloxigenase (COX) juntamente com a fosfolipase, dá origem ao ácido araquidônico (AA) que é um limitante da taxa de enzimas envolvidas na biossíntese das prostaglandinas (PGs) (POYSER, 1995; SMITH,1992). Os diversos efeitos da PGs são mediados por uma família de receptores de membrana acoplados à proteína G (COLEMAN et al.,1994). As PGs (PGF-2α e PGE-2) influenciam nas alterações morfológicas que o endométrio sofre durante o ciclo estral e prenhez (MOELJONO et al 1976; MCCRACKEN et al. 1999) e ainda estão envolvidas em vários aspectos da reprodução, incluindo a ovulação, fertilização, implantação, decidualização (LIM et al., 1997a), parturição (CHALLIS & LYE, 1994) e junto com esteróides sexuais, formam os reguladores mais importantes no sucesso da gravidez (POYSER, 1995).

Três isoformas da enzima COX foram identificadas, a COX-1 (HEMLER et al. 1976; MIYAMOTO et al., 1976), a COX-2 (KUJUBU et al., 1991; XIE et al., 1991) e a COX-3 sem papel funcional ainda estabelecido (CHANDRASEKHARAN et al., 2002). A COX-1 é considerada como uma enzima cuja expressão é constitutiva com desenvolvimento regulado e a COX-2 é expressa em estímulos a resposta inflamatória e é ativa em respostas fisiológicas aos fatores de crescimento e glicocorticóides (DE WITT & MEADE, 1993). A isoforma constitutiva COX-1, é expressa numa variedade de tecidos (NEEDLEMAN, et al., 1986). Enquanto que a isoforma COX-2, é induzida por uma variedade de fatores, tais como: gonadotrofina

coriônica, citocinas ou promotores tumorais (SIROIS & RICHARDS, 1992; KUJUBU et al, 1991; O'BANION et al., 1992). A COX-2 foi implicada em eventos reprodutivos tais como ovulação (SIROIS & RICHARDS 1992; SIROIS, 1994; SIROIS & DORÉ 1997), a luteólise (CHARPIGNY et al., 1997a;. AROSH et al., 2002), alongamento do concepto (CHARPIGNY et al,, 1997b.; WILSON et al., 2002) implantação (SONG et al., 2000; SCHERLE et al., 2000), e ainda no parto (ZHANG et al., 1996; GYOMOREY et al., 2000). Apesar de extensos estudos sobre a COX-1 e COX-2, em relação à localização e função nos processos reprodutivos, há pouca informação disponível (BLITEK et al., 2006).

Arosh et al., (2002) não conseguiram detectar COX-1 RNAm no endométrio bovino. Resultados semelhantes foram obtidos em vacas prenhas, onde a COX-1 RNAm foi detectada apenas no miométrio e carúnculas (FUCHS et al., 1999). As células do estroma do endométrio babuíno não expressaram COX-1 durante o ciclo estral, nem durante a gravidez (KIM et al., 1999). Por outro lado, em roedores, a expressão desta isoforma aumenta à medida que a gestação avança (CHAKR-ABORTY et al., 1996; DONG et al., 1996). Mesmo camundongos fêmeas deficientes de COX-1 são férteis (LANGENBACH et ai., 1995).

Chakraborty et al., (1996) usando útero do rato sugeriram que o gene da COX-2 é regulada pelo blastocisto implantado durante o início gravidez. Camundongos deficientes de COX-2 são inférteis, tendo diminuição da ovulação, fertilização, implantação e decidualização (LIM et al., 1997a). Foi observada expressão aumentada da COX-2 após tratamento de ocitocina em células epiteliais de bovino in vitro (PARENT et al., 2003). O efeito da estimulação de hCG

sobre a COX-2 em células do endométrio foi relatado em mulheres (HAN et al., 1996).

A exigência da COX-2 para a implantação normal do blastocisto e decidualização em camundongos é devido o papel das PGs derivadas de COX-2 na regulação vascular e fator de crescimento endotelial (VEGF) que influenciam na permeabilidade vascular e uterina (LIM et al., 1999, MATSUMOTO et al., 2002). Durante a implantação, um aumento da permeabilidade vascular uterina ocorre e é detectada pela localização de corantes ligados às proteínas ao redor do blastocisto implantado (PSY- CHOYOS, 1973).

EXPRESSÃO DA INIBINA - BA (INHBA) NOS TECIDOS

A Inibina foi isolada a partir do fluido folicular dos bovinos em 1985 (ROBERTSON et al., 1985) depois em suínos, ovinos e humanos (LING et al., 1985). Logo em seguida foi descoberta uma proteína antagonista, que estimula a produção e secreção do hormônio folículo estimulante (FSH), que foi caracterizada como ativina (VALE et al., 1986,. YING, 1988).

Inibinas e ativinas (nome gene: inhibinβ A) são glicoproteínas que pertencem à superfamília do fator transformador de crescimento - β (TGF-β) (KINGSLEY, 1994), sendo este fator o percursor envolvido numa gama de processos celulares, incluindo a apoptose, morte celular, proliferação e diferenciação celular (MASSAGUE, 1990) e ainda podem estimular a atividade das células do endométrio (YAMASHITA et al., 2004; FUMAGALLI et al., 2007). Além disso, inhibinβ A, é uma

combinação de três subunidades de proteínas relacionadas (α, βA e βB), codificadas por genes distintos (VALE et al., 1988). Inibina A e B são compostos por uma subunidade α e uma das duas subunidades β , enquanto as activinas são a montagem combinatória das subunidades β (βA -βA , βB - βB , βA - βB) (DEBIEVEL et al, 2000, SHIMASAKI et al., 2004).

Primeiramente pensava-se que inhibinβA atuavam exclusivamente na glândula pituitária em um feedback endócrino clássico; no entanto, o RNAm para esses fatores tem sido descrita numa série de outros tecidos (MEUNIER et al., 1988), testículos (FUJIMURA et al 1998.; TANAKA et al., 2002), ovários (TANAKA et al., 2003), placenta (ARAI et al., 2006; KANDIEL et al., 2010) e útero de suíno (VAN de PAVERT et al., 2001), vaca (SUGAWARA et al., 2010), égua (YAMANOUCHI et al., 1997) e ovelhas (HAYASHI et al., 2003) com suas ações exercidas por meio de mecanismos paracrinos (WOODRUFF, 1998).

Embora inhibinβA seja expressa no útero de diferentes espécies, existem diferenças entre os tipos de células em que se localizam (HAYASHI et al., 2003; VAN de PAVERT et al., 2001; YAMAGUCHI et al., 1996). As subunidades ativina (βA e βB) se localizam no epitélio uterino e células deciduais de camundongos, durante o estabelecimento de gravidez (YAMAGUCHI et al. 1996), e no lúmen de células glandulares epiteliais de éguas no dia 25 de gestação (ZHANG et al., 2013).

Um estudo sugeriu que a tal gene coordena a remodelação do endométrio em bovinos (ISHIWATA et al., 2003). Nos seres humanos, tem um papel estimulador na decidualização e invasão de células do endométrio (DAS et al., 1994; CANIGGIA et al., 1997; JONES et al., 2006).

EXPRESSÃO DA PROTEÍNA LIGADORA DO FATOR DE CRESCIMENTO SEMELHANTE À INSULINA 1 TIPO 3 (IGFBP3) NOS TECIDOS

Em 1989, um encontro internacional de pesquisadores em fatores de crescimento semelhante a insulina (IGF) chegou a um consenso no sistema de nomenclatura para IGF - proteínas ligadoras (IGFBPs), uma família de proteínas (IGFBP-1, IGFBP-2. IGFBP-3, IGFBP-4, IGFBP-5 e IGFBP-6) que está sob estudo em vários laboratórios devido a afinidade com o fator de crescimento semelhante a insulina-I (IGF-I) e fator de crescimento semelhante a insulina-II (IGF-II) (BALLARD et al., 1989).

Originalmente o fator de crescimento semelhante à insulina (IGF), foi descoberto durante as investigações do controle de crescimento ósseo e desde então continua sendo estudado, por mais de 50 anos (DAUGHADAY & SALMON, 1999). Estes possuem funções importantes na regulação do crescimento celular, diferenciação e metabolismo (SHIMASAKI & LING, 1991), funções reprodutivas voltadas à placenta e desenvolvimento dos folículos ovarianos (FORBES & WESTWOOD, 2008; SILVA et al., 2009). Os IGFs também estão envolvidos no crescimento de muitos tipos de câncer (POLLAK, 2008a,b; VARDATSIKOS et al., 2009), o que o tornou uma via alvo para terapias contra câncer (GUALBERTO & POLLAK, 2009).

As funções das IGFBPs incluem (CHARD, 1994) aumentar a semivida de IGFs circulantes (HILL et al., 1998), transportar IGFs na circulação (O'DELL & DAY,

1998), localizar células específicas para IGFs (DeCHIARA, 1990) e modular as ações de promoção de crescimento (CLEMMONS, 1993).

Kirby et al., (1996) e Keller et al., (1998) relataram uma maior expressão de IGPB-3 no miométrio de vacas prenhas do que no endométrio. O tecido caruncular e intercaruncular continham mais IGFPB-2 e menor atividade de IGFBP-3 segundo Keller et al., (1998). A detecção do RNAm de IGFBP-3 no miométrio evidencia a produção local, podendo essa produção ter um papel na regulação do transporte de IGFs circulantes no miométrio e subsequente distribuição para os tecidos circundantes (OH et al., 1993). Ao comparar a expressão de IGFPB-3 com outras espécies foi encontrado maior semelhança com o ser humano (ZHOU et al., 1994) do que com o rato (GIRVIGIAN et al, 1994). No tecido uterino de ovelhas, quando hibridazado com a IGFBP-3, o RNAm foi maior no epitélio luminal, moderada no miométrio e pouco no endométrio durante 13-15 dias de gestação (REYNOLDS et al 1997).

EXPRESSÃO TROMBOSPONDINA - 2 (THSB-2) NOS TECIDOS

Trombospondina (THSB) foi inicialmente descritos em grânulos alfa de plaquetas (LAWLER et al.,1978), agindo na agregação plaquetária para formação do coágulo (ASCH & NACHMAN,1989; MOSHER, 1990; FRAZIER,1991). Mas outros inquéritos estabeleceu que THSB também seja secretada em outras células que inclui: células endoteliais (McPHERSON et al., 1981; MOSHER et al., 1982; CANFIELD et al., 1990), as células musculares lisas e fibroblastos (RAUGI et al.,

1982; JAFFE et al., 1983), queratinócitos (WIKNER et al., 1987), macrófagos (JAFFE et al., 1985),e células da glia (ASCH et al., 1986).

A trombospondina (THSB) é uma família de cinco componentes (THSB-1, THSB-2, THSB-3, THSB-4, THSB-5), são segregados de glicoproteínas modulares, cujas funções na matriz extracelular são diversificadas e pouco compreendidas (FRAZIER, 1991; ADAMS & LAWLER, 1993; BORNSTEIN & SAGE, 1994; BORNSTEIN, 1995). É considerado que THSB-1 e THSB-2 são estruturalmente mais semelhantes entre si do que a THSB 3-5 que formam uma subfamília (BORNSTEIN & SAGE, 1994).

O papel biológico de THSB-2 permanece indefinido, porém seu RNAm já foi detectado por meio de hibridização *in situ* em camundongos (IRUELA-ARISPE et al., 1993) e embriões de galinha (TUCKER, 1993), predominando em tecidos conjuntivos, e em regiões de proliferação e migração no embrião de rato (O'SHEA & DIXIT,1988). A distribuição de THSB2 em tecidos de bovino adulto parece ser generalizada. A maioria dos tecidos endócrinos como o córtex adrenal , testículo , ovário e placenta com exceção da glândula tireóide expressam THSB2. Não é detectável no músculo esquelético , intestino e soro , mas é fracamente expresso na medula supra-renal e no fígado. O córtex supra-renal, coração, baço , cérebro e rim são os que contêm os maiores níveis da TSHB (WIGHT et al., 1985).

A síntese THSB está associado com todas as fases do desenvolvimento embrionário (O'SHEA & LIU, 1990). A expressão THSB é geralmente maior nos tecidos do que nas células estão a sofrer proliferação e migração ativamente

declinando e chegando a ser restrito em tecidos adultos (O'SHEA & DIXIT,1988, WIGHT et al., 1985).

EXPRESSÃO DE FOSFATASE ÁCIDA TIPO 5 (ACP5) NOS TECIDOS

Fosfatase Ácida Tipo 5 (ACP5) ou Fosfatase Ácida Resistente ao Tartarato (TRAP) é homóloga a uteroferrina, uma proteína que é secretada pelo útero gravídico de porcas e está envolvido no transporte de ferro da mãe para o feto (BUHI et al. 1982). ACP5 já foi localizada em várias espécies, no epitélio endometrial glandular, de éguas prenhas, ovelhas e búfalos de água (ELLENBERG et al.,2008). Uma característica que envolve todas estas espécies é o tipo de placenta que apresentam, epiteliocorial ou syndesmocorial, um tipo de placentação que contrapõe o epitélio corial mas não invade o epitélio endometrial (PADUA et al.,2012). A expressão de *ACP5* no endométrio é regulado hormonalmente, principalmente pela progesterona e modulado pelo estrógeno, prolactina, e interferon-τ (GAO et al.,2010).

EXPRESSÃO DO RECEPTOR DE QUIMIOCINA 4 (CXCR4) NOS TECIDOS

As quimiocinas fazem parte de uma superfamília das citocinas estruturais e são funcionalmente relacionados com a atividade quimiotática, dirigindo populações de leucócitos específicos (BACON et al., 2002). Dentro dessa superfamília existe

quatro subfamílias de quimiocinas (CC, CXC, C e CX3C) (LUSTER, 1998), e receptores de superfície de células, as proteínas G-acoplados (CCR e CXCR) (DIMITRIADIS et al., 2005).

O receptor de quimiocina 4 (CXCR4), é especificamente regulada no endométrio humano durante a janela de implantação. As quimiocinas e os seus receptores são fatores importantes na implantação e vascularização da placenta (DOMINGUEZ et al., 2003). Outros receptores de quimiocinas (CXCR1, CCR5 e CCR2B) são também encontrados no epitélio do endométrio humano durante a fase receptiva, o que permite supor que as quimiocinas liberadas no lúmen uterino, ou na superfície do epitélio luminal, desempenham papel também nas fases de adesão em seres humanos (DIMITRIADIS et al., 2005).

EXPRESSÃO DE STANNIOCALCINA 1 (STC1) NOS TECIDOS

Stanniocalcina (STC) é uma hormona primeiramente descoberto em peixes ósseos onde é segregados pelos Corpúsculos de Stannius, uma glândula endócrina não encontrada em outros vertebrados (STANNIUS, 1839). Nos mamíferos o STC1 é expresso em vários tecidos, incluindo coração, pulmão, fígado, glândulas supra-renais, rins, próstata e ovários (CHANG et al., 1998; VARGHESE et al., 1998) sugerindo uma ação autócrina e parácrina, pois além da função de regulação da homeostase de cálcio e fósforo, podem está envolvidos processos do desenvolvimento do concepto como batimentos cardíacos, morfogênese óssea e respiração mitocondrial (KIKUCHI et al., 2011). A expressão STC1 aumenta no

ovário durante a gestação e lactação em ratas (MADSEN et al., 1998). Em experimentos realizados *in vitro* o STC1 foi encontrado em ossos e músculos esqueléticos fetais de ratos atuando como um regulador parácrino da condrogenese (JIANG et al., 2000).Nas observações feitas em ratos transgênicos com expressão de STC1, apresentaram menor peso ao nascer e menor tamanho quando adulto. Já em ratos knockout para STC1 mostraram-se fenotipicamente normais, o que indica que o STC1 não é essencial para o crescimento ou reprodução (FILVAROFF et al., 2002; VARGHESE et al., 2002; CHANG et al., 2005).

EXPRESSÃO DA CONEXINA BETA 6 (*GJB6*) DURANTE A GESTAÇÃO

Em 1996 foi identificada uma nova conexina de camundongo pela equipe do pesquisador Dahl, com massa molecular de 30,366kDa sendo denominada connexin30 (Cx30) e possui o símbolo *GJB6* para sua designação (SCHWARZ et al, 1994).

A partir da comparação de sequências de nucleotídeos e aminoácidos, sugerem que Cx30 é similar ao Cx26 (VALIUNAS et al, 1999). Pois compartilha 77% de identidade na sequência, presumivelmente, surgiram por meio da duplicação de genes (SCHWARZ et al., 1992).

A conexina Cx30 é expressa em tecidos de camundongo como: cérebro de adulto, pele e é pouco expresso no útero, pulmão, tecido do olho, testículo e nervos. Nenhum mRNA Cx30 foi encontrado no fígado e pâncreas os quais expressa níveis relativamente altos de Cx26 (ZHANG & NICHOLSON, 1989). A comparação

quantitativa de mRNA Cx30 expresso em tecidos diferentes demonstrou que a expressão era mais abundante no cérebro após 4 semanas de desenvolvimento pós-natal, já o gene Cx26, é mais expresso no cérebro pré-natal e diminui após o nascimento (DERMIETZEL et al., 1989)

OBJETIVO E JUSTIFICATIVA

O presente estudo tem como objetivos identificar os genes diferencialmente expressos entre carúnculas uterinas do corno gestante e não gestante durante a placentação bovina e investigar o papel do embrião produzido por fertilização in vitro (FIV) na expressão desses genes. Verificar que o embrião de fato influencia além da expressão gênica, também a expressão proteica caruncular, poderá abrir um leque de questões ainda não elucidadas, como por exemplo, quais os sinais do embrião que regulam esse desenvolvimento uterino; e que os achados estão relacionados com as altas taxas de falhas gestacionais observadas em gestações de embriões manipulados em laboratório.

CAPÍTULO 1.

The Bovine Embryo Modulates Caruncular Gene Expression during Placentation1

[1]Manuscrito submetido à revista Reproduction, Fertility and Development

The Bovine Embryo Modulates Caruncular Gene Expression During Placentation

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SUMMARY

Endometrial remodeling is essential for placentation in all mammalian species. Embryo growth factors may affect the endometrial development during placental establishment. Our objective was to elucidate the modulation exerted by the bovine embryo on the caruncular gene expression as well as determine the influence of in vitro fertilization (IVF) in this gene expression. For this purpose, caruncles were collected from the gravid horn of the uterus from cows submitted to artificial insemination (AI) or FIV at days 30, 35 and 40 of gestation following separation of the weakly associated cotyledons. Caruncles were simultaneously collected from the non-gravid horns of the uterus. Transcriptomes of AI caruncles were evaluated by microarray analysis at day 35 of gestation and the expression of selected genes was investigated by qPCR, western blot and immunohistochemistry, comparing the caruncles from days 30, 35 and 40 of AI and FIV gestations. A library of 149 differentially expressed genes between cotyledon-associated caruncles and those from the non-gravid horn was generated. Among these transcripts, 114 were upregulated

and 35 were downregulated in caruncles from the pregnant horn (±1.5-fold, $p<0.05$). Functional analysis demonstrated that many expressed genes were involved in cell motility (30.2%), cell morphology (29.5%) and cell differentiation (26.1%). Subsequent qPCR analysis of nine genes (*ACP5*, *DPP4,GJB6*, *IGFBP3*, *INHBA*, *STC1*, *THBS2*, *CXCR4* and *PTSG2*) involved in cell differentiation demonstrated that only one transcript *GJB6* showed the same pattern of expression in AI and IFV gestations. In IVF animals, upregulation of six transcripts (*ACP5, DPP4, INHBA, STC1, THBS2* and *PTGS2*) occurred at 40 days of pregnancy and expression of two genes (*CXCR4, IGFBP3*) did not vary between caruncles from gravid and non-gravid horns. Protein analysis showed that IGFPB3 e THSB2 protein expression was similar in AI and FIV gestations, but distinct in relation to PTGS2. Protein immunostaining was observed in the cytoplasm of epithelial and stromal uterine cells, glandular cells and endothelial cells and staining intensity was associated to western blot data, with cells from cotyledon-associated caruncle showing more intensity of staining at 35 and 40 days in both AI and FIV gestations. Our data provide new insight into the mechanisms by which embryos control uterine development, suggesting that in vitro manipulation may alter the embryo's capability to modulate the uterine microenvironment. These findings indicate that embryo influences, or lack thereof, may contribute to high levels of loss of IVF gestations.

Key words: Artificial Insemination, Endometrium, in vitro Fertilization.

INTRODUCTION

The evolution of implantation, invasion and placental formation was essential for the success of viviparity, the defining characteristic of Eutheria mammals (Lefevre et al., 2007).This evolution was associated with a rewiring of a network of over 1500 endometrial genes as well as additional genes activated due to numerous evolutionary changes that resulted in different forms of placentation (Lynch et al., 2011).

A complex and coordinated maternal-fetal communication is required for a successful blastocyst implantation and for establishment of the placenta and pregnancy (Mamo et al., 2012). In most species, the molecular bases of maternal-fetal cross talk are not totally understood. The embryo role in the modulation of uterine gene expression has been demonstrated in several species. In ruminants is described the more known and studied embryo signaling mechanism to the uterus. In these animals, the embryonic interferon-tau initiates a cascade of uterine molecular events that lead to maternal recognition of pregnancy (Thatcher et al., 2001). The equine embryo regulates the expression of uterine genes that are essential to its own development (Kikuchi et al., 2011). Human embryo drives the modulation of maternal uterine environment shortly post-fertilization. Viable embryo secretes preimplantation factor (PIF), which plays a critical role during implantation, priming the uterus and creating a receptive milieu for embryo development (Barnea et al., 2012). Furthermore, it was demonstrated an active role of mouse embryo in the establishment of uterine environment during implantation. In this case, the embryo regulates genes involved not only in its own development but also in the development and differentiation of maternal endometrium (Kashiwagi et al., 2007).

The understanding of embryonic role in endometrial remodeling during implantation and placentation become even more important when considering in vitro produced embryos. In this case, processes of manipulation and cell culture can alter embryo's profile of gene expression and, consequently, its

development and signaling the endometrium (Zhou et al., 2008). Indeed, a large part of embryonic losses in natural bovine pregnancies occur between 8 and 17 days of gestation and, in most cases, due to insufficient communication between mother and fetus and consequent failure in maternal recognition of pregnancy (Thatcher et al., 2001). Considering pregnancies of in vitro produced embryos, the losses are even more expressive. Recovery and detection rates of embryonic discs at 16 days of gestation were 86% and 56% for control embryos, and 37 and 35% for in vitro produced embryos (Bertolini & Anderson, 2002). In addition, pregnancy rates at 30 days of gestation are lower, but comparable, for embryos produced by in vitro fertilization (IVF) compared with embryos produced by superovulation and artificial insemination. However, the losses between days 30 and 60 of gestation are significantly greater in embryos produced by IVF(Bertolini et al., 2007). Several studies indicate that one of the most common causes of pregnancy loss is the development of placental abnormalities. These abnormalities include deficiencies in the establishment of vascularization (Hill et al., 2000), disruption of chorionic villi and insufficient development of caruncles (Hashizume et al., 2002), and may be associated with the establishment of an improper epigenetic pattern in the in vitro produced embryos (Bertolini et al., 2007).

Considering that embryo growth factors may affect the endometrial development during placental establishment and that the process of in vitro production may alter embryo ability to signal to the endometrium, the aim of this study was elucidate the modulation exerted by the bovine embryo on the caruncular gene expression as well as determine the influence of IVF in this gene expression.

MATERIAL AND METHODS

Samples

Caruncles from the gravid horn were collected from pregnant primiparous cows (*Bos indicus*) undergoing artificial insemination (AI) or transfer of embryos produced by *in vitro* fertilization (IVF) using *Bos indicus* male sexed sperm. Animals were slaughter at 30 (n = 3), 35 (n = 8) or 40 (n = 3) AI and 35 (n = 3) or 40 (n = 3) days IVF of gestation and samples were collected following separation of the weakly associated cotyledons. Caruncles were simultaneously collected from the non-gravid horns of the uterus. Tissues were both frozen in liquid nitrogen and stored at -80°C freezer until RNA or protein extraction or fixed in 4% buffered formaldehyde for immunohistochemical analysis. The experiments and animal procedures were approved by Ethics Committee on Animal Use of the Biotechnology Center (CEUA-CBiotec) of the Federal University of Paraíba.

Extraction of RNA, Purification and Reverse Transcription

Caruncular tissues were homogenized and purified using the RNeasy Mini Kit (Qiagen, Valencia, CA). DNase I (Qiagen, Valencia, CA) treatment was performed to avoid contamination by genomic DNA. Concentration and purity of the RNA samples were accessed by absorbance readings at 260 and 280 nm in Nanodrop spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA).

RNA integrity was verified on agarose gel followed byanalysis in the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Only samples with RIN ≥ 8 were used in the experiments. For analysis of differentially expressed genes by quantitative PCR, 1 µg sample of total RNA was reverse transcribedwith the SuperScript III kit and oligo-dT primer (Invitrogen, Carlsbad, CA). All procedures were conducted according to the manufacturers' recommendations.

Global transcriptome analysis of uterine caruncles

Caruncular transcriptomes were evaluated by microarray in seven AI animals at day 35 of gestation. Microarray was performed using the GeneChip® Bovine Genome Array (Affymetrix, Santa Clara, CA), containing 24,072 probe sets representing over 23,000 transcripts. Global analysis of microarray data was performed with the Flex Array version 1.6 (Genome Quebec Innovation Center,Montreal,QC,Canada;http://www.gqinnovationcenter.com/services/bioinformatics/flexarray/index.aspx?l=e). Raw fluorescence data were processed and normalized using the *affx* Bio conductor package version 1.1.0 (http://bioconductor.org/). Genes were considered differentially expressed at a fold change ≥ 1.5 with $p \leq 0.05$.In order to identify relationships between the genes and to uncover common processes and pathways, the list of differentially expressed genes was uploaded into Ingenuity Pathway Analysis 7 software (IPA, Ingenuity Systems, San Francisco, CA;www.ingenuity.com/).The IPA software interprets the data using information from the literature. The “Functional Analysis” tool of IPA software was used to identify the biological functions that were most significant in the data set. In addition, the "Network Analysis" tool was used to determine the interrelationship between genes and to choose genes for microarray validation.

Validation of results and analysis of differentially expressed genes in uterine caruncles using quantitative real-time PCR

Expression of acid phosphatase 5 (*ACP5*), dipeptidyl peptidase 4 (*DPP4*), gap junction protein beta 6 (*GJB6*), insulin-like growth factor binding protein 3 (*IGFBP3*), inhibin beta A (*INHBA*), stanniocalcin 1 (*STC1*), thrombospondin 2 (*THBS2*), chemokine (C-X-C motif) receptor 4 (*CXCR4*) and prostaglandin-endoperoxide synthase 2 (*PTGS2*) in uterine caruncles was assessed by quantitative PCR using an ABIPrism 7500 Sequence Detector (Applied Biosystems®, Thermo Fisher Scientific Inc., Waltham, MA). Tubulin beta (*TUBB*) was used as endogenous control. Expression of selected genes was validated comparing caruncles from gestations collected at days 30 (n = 3), 35 (n = 8) or 40 (n = 3) AI and 35 (n = 3) or 40 (n = 3) days IVF. Oligonucleotides were obtained from bovine sequences previously published (Table 1). PCR reactions were performed using Power SYBR Green PCR Master Mix (Applied Biosystems®, Thermo Fisher Scientific Inc., Waltham, MA). Amplification conditions were 2 minutes at 50°C and 10 minutes at 95°C, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Analysis of melting curve was performed to assess PCR products specificity. Samples were run in triplicate for each sample, and the expression was determined by relative quantification (target gene/endogenous control). Data were normalizedto a calibrator sample using the ΔΔCt method with correction for amplification efficiency (Pfaffl, 2001).

Table 1. Sequences of primers used for the quantitative PCR.

Gene (Número no GenBank)	**Oligonucleotídeo (5'-3')**	**Tamanho do amplicon (bp)**
ACP5	►AAGAGATTGCCAGGACAGTG	
(JN635352.1)	◄ACCTCTTGTCGTTGACATCC	123

CXCR4	► AGCAAGGGTGTGAGTTTGAG	
(NM_174301.3)	◄ AGGCATAGAGGATGGGATTC	99
DPP4	► ATACACCCACATGAGCCACT	
(NM_174039.2)	◄ TCCTGACAGTGCAGTTTTGA	124
GJB6	► AGTGTGTTTCAGGAGGTCCA	
(NM_001015546.1)	◄ GCTCGTTCATCTCGTTCTGT	92
IGFBP3	► TTCTTCCACATGGTGAACCT	
(BC149336.1)	◄ GGAAGCAACGAGAAAAATCA	79
INHBA	► CAAAGAAGGCAGTGACCTGT	
(NM_174363.2)	◄ GTTGAAAGAGACGGATGGTG	110
PTGS2	► TGCTGAGTTTAACACGCTCTACCA	
(NM_174445)	◄ TGAGACCATGTTCCAGTAAGACAGA	125
STC1	►AATGCTCCAAAACTCAGCAG	
(NM_176669.3)	◄ TTCCTGAGGCTCACAGAATC	93
THBS2	► ACCTCTTCAGCATCAGCAAC	106
(NM_176872.1)	◄ ATGTAGTCAAAGCGGACGAA	
TUBB	► CAGCAAGATCCGTGAAGAGT	123
(BT030522.1)	◄ACCAGCTGATGGACAGAGAG	

►= forward; ◄= reverse

Analysis of protein expression in uterine caruncles by Western blot

Uterine caruncles were subjected to immunoblotting to establish the protein expression PTGS2, THSB2 e IGFPB3. For this purpose, caruncular samples 30 (n = 3), 35 (n = 8) or 40 (n = 3) AI and 35 (n = 3) or 40 (n = 3) days IVF were homogenized in extraction buffer [0.1M potassium phosphate buffer (pH = 7.0), 1M Sucrose, 0.1M DTT, 0.5 M EDTA (pH = 8.0), 0.1M PMSF, 0.5M NaF, Fosfatase Inhibitor - 1:100] using aPolytron® (Tekmar Company, Mason, OH). Total protein concentration was determined by Bradford method (Protein Assay Kit, Bio-Rad, Hercules, CA, EUA) and50 µg/µl(PTGS2 and THSB2) or 30 µg/µl(IGFPB3)of total protein were denatured inLaemmlisample buffer (15% glycerol, 0.05M Tris, 0,05% bromphenol blue, 9% SDS, 6% 2-mercaptoethanol). Proteins were resolved by one-dimensional 8-10% SDS-PAGE minigel.Following migration, proteins were electrophoretically transblotted to PVDFmembranes (GE Healthcare Life Sciences,Pittsburgh, PA) using aSemi-Dry system (Bio-Rad, Hercules, CA). Membranes were blocked inTTBS [0.1% Tween 20 in Tris-HCL buffer (100 mM Tris, 0.9 % NaCl, pH 7.5)] containing 5% non-fat dried milk and incubated with primary antibodies anti PTGS2 (1:1000), THSB2 (1:500) and IGFPB3 (1:10000; Table 2) over nightat4ºC.Following addition of secondary antibodies,membranes were incubated with horseradish peroxidase anti-rabbit (GE Healthcare, NA934) or anti-goat (Merk Millipore, AP132P) IgG(1:10000) for 1.5 hours.The signal was detected by adding the peroxidase substrate (ECL Western Blotting Analysis System®, Amersham- GE Healthcare Life Sciences, Pittsburgh, PA) and exposure in the ChemiDoc MP System(Bio-Rad, Hercules, CA). Densities of protein bands were analyzed using Image Lab Software (Bio-Rad, Hercules, CA) and data were expressed relative to actin beta.

Table 2. Antibodies used for Western blot and immunohistochemistry analysis.

Antibody	Isotype	Immunogen	Supplier (order no.)
PTGS2	Rabbit polyclonal IgG	550 to the C-terminus of humanPTGS2	Abcam (AB102005)
IGFPB3	Goat Polyclonal IgG	Internal region of human IGFPB3	Abcam (AB77635)
THSB2	Rabbit polyclonal IgG	650 - 750 human THSB2	Abcam (AB84469)
Beta Actin	Rabbit polyclonal IgG	1 – 100 of human Beta Actin	Abcam (AB8227)

Cellular localization of proteins in uterine caruncles by immunohistochemistry

Fixed caruncular tissues were used to demonstrate cellular expression of PTGS2, THSB2 e IGFPB3. Samples 30 (n = 3), 35 (n = 8) or 40 (n = 3) AI and 35 (n = 3) or 40 (n = 3) days IVF were embedded in Paraplast® resin (Merck, Darmstadt, Alemanha). Sections (5 µm) were deparaffinized in xylene and rehydrated in a series of graded alcohols, andantigen retrieval was performed by boiling tissues in sodium citrate buffer (10 mM, pH 6.0). Slides were immersed in 1% hydrogen peroxide in methanol for quenching of any endogenous peroxidase activity and Protein Block (Dako North America, Inc., Carpinteria, CA) was added for blocking of non-specific binding. Sections were incubated for 20 h at 4 °C with primary antibodies anti- PTGS2, THSB2 e IGFPB3 (Table 2), diluted1:200 in 10% normal goat serum in phosphate-buffered saline/0.3% Triton X-100. Negative controls were set up with normal rabbit IgG or normal goat IgG(Santa Cruz Biotechnologies, Santa Cruz, CA).After washes in PBS (phosphate buffered solution, pH 7.2), the Universal LSAB™+ Kit, HRP (Dako North America, Inc., Carpinteria, CA) was employed according manufacturer's instructions. Briefly, sections were incubated with universal biotinylated horse anti-rabbit, anti-mouse and anti-goat second antibody and streptavidin conjugated to horseradish peroxidase. Reactions

were revealed by incubation with 3,3′-diaminobenzidine (DAB) chromogen (Sigma, St. Louis, MO) and sections were counterstained with hematoxylin.

Statistical analysis

Quantitative PCRand Western blot data are presented as mean ± S.E.M. Results were analyzed comparing relative expression between cotyledon-associated caruncles and caruncles from non-gravid horns at same gestational age and relative expression between natural and IVF gestations at same gestational age.Data were analyzed using unpaired bilateral t-test or ANOVA . Differences were considered statistically significant at $P<0.05$. Statistical analyses were conducted using GraphPad Prism Software (version 4.00 for Windows, GraphPad Software, San Diego, CA, USA).

RESULTS

Global transcriptome analysis

Transcriptome analysis was performed in caruncular samples obtained from AI pregnancies at 35 days. A library of 149 differentially expressed genes between cotyledon-associated caruncles and those from the non-gravid horn was generated. Among these transcripts, 114 were upregulated and 35 were downregulated in caruncles from the pregnant horn (±1.5-fold, $p<0.05$). The 10 most significantly upregulated genes were *SLPI*, *AQP11*, *Cyp2c44*, *RNASE1*, *TFRC*, *INHBA*, *ACP5*, *VSIG1*, *STC1* e *CYP4A11*, and 10 most significantly downregulated genes were *UPK1B*, *SLC27A2*, *CYP26A1*, *CDH17*, *AHSG*, *ATP6V0A4*, *MX2*, *GSTA3* e *PTGS2*. The complete list differentially expressed genes (up and downregulated) is demonstrated in Table S1.

Ontological classification of differentially expressed genes,based in the IPA software information, indicated that 44 functional groupswere modified. The molecular and cellular functions most significantly altered incotyledon-associatedcaruncles were cell movement, function and cell maintenance, antigen presentation, lipid metabolism and biochemistry of small molecules (Figure S1).Functional analysis demonstrated that many expressed genes were involved in cell motility (30.2%, 45 genes), cell morphology (29.5%, 44 genes) and cell differentiation (26.1%, 39 genes). Pathway interaction network analysis identified 10 networks of interrelationship involving differentially expressed genes (Table S2).

Analysis of differentially expressed genes in uterine caruncles

Genes potentially involved in cell differentiation and presenting some type of inter relationship (Figure S2) were chosen to validate microarray results

by quantitative PCR. Among them, *ACP5*, *DPP4*,*GJB6*,*IGFBP3*, *INHBA*, *STC1* and *THBS2* were upregulated and *CXCR4* and *PTGS2* were downregulated in cotyledon-associated caruncles.According IPA analysis, *ACP5, DPP4, CXCR4, IGFBP3, INHBA, PTGS2, STC1, THBS2*are related with cellular motility, *CXCR4, GJB6, IGFBP3, INHBA, PTGS2, STC1, THBS2*regulate number of cellsand*CXCR4, INHBA, PTGS2, STC1*are involved with epithelial cell differentiation (Figure S1).

Quantitative PCR demonstrated that expression ratios of selected genes were consistent with the microarray results. Transcript abundance of all studies genes were significantly different in cotyledon-associated caruncles ($p<0.05$) compared with caruncles from non-gravid horn at 35 days of AI gestations (Figure 1).

To further investigate the role of target genes in the endometrial remodeling during placentation, we examined their mRNA expression from days 30 to 40 in AI and FIV gestations.Real time PCR analysis of AI gestations demonstrated that*DPP4, ACP5 e STC1*mRNA levels were already increased at 30 days of gestation in cotyledon-associated caruncles. Besides having an enhanced expression at 30 and 35 days of gestation, INHBA message abundance remained increased at 40 days in caruncles from the gravid horn. The same pattern of expression was observed in relation to *PTGS2* mRNA, however the expression levels were decreased in cotyledon-associated caruncles from days 30 to 40 of gestation.*GJB6* transcript abundance was higher at 35 and 40 days of gestation in caruncles from the gravid horn. On the other hand, expression of *CXCR4, IGFBP3* e *THBS2* mRNAs was increased in cotyledon-associated caruncles only at day 35 in IA gestations. Considering gene expression in caruncles obtained from FIV gestations, we observed that only one transcript (*GJB6*) presented the same pattern of expression observed in AI gestations. The increase in mRNA levels of six genes (*ACP5, DPP4, INHBA, STC1, THBS2* and *PTGS2*) that was observed in caruncles from the gravid horn of AI gestations at 30 or 35 days, only occurred at 40 days in animals submitted to in vitro fertilization. Additionally, mRNA expression of two

genes (*CXCR4, IGFBP3*) did not vary between caruncles from gravid and non-gravid horns in FIV gestations (Figure 1).

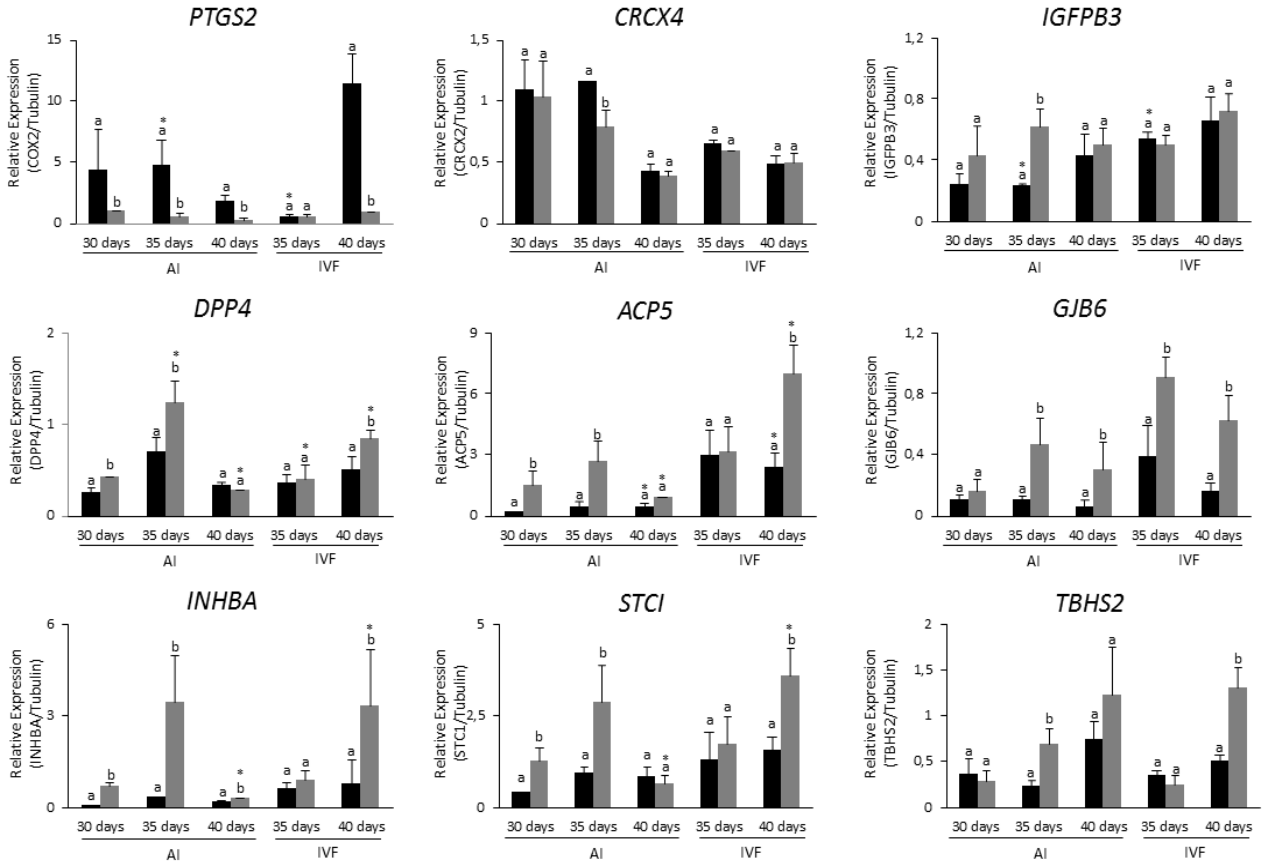

Figure 1. Quantitative real time PCR analysis of *PTGS2, CXCR4, IGFBP3, DPP4, ACP5, GJB6, INHBA, STC1* and *THBS2* transcripts in bovine caruncles from non-gravid horn (black bars) and in cotyledon-associated caruncles (gray bars). All genes were up or down regulated ($p<0.05$) in caruncles from gravid horns at 35 days of gestation. In AI gestations, *DPP4, ACP5, STC1* and *INHBA* transcripts abundance increased in cotyledon-associated caruncles at day 30 and remained higher until 35 (*DPP4, ACP5, STC1*) or 40 days of gestation (*INHBA)*. Caruncles from gravid horn showed a higher expression of GJB6 mRNA at 35 and 40 days of pregnancy. *CXCR4, IGFBP3* and *THBS2* transcript abundance increased in cotyledon-associated caruncles only at 35 days of gestation. *PTGS2* mRNA expression was decreased in caruncles from pregnant horn at 30, 35 and 40 days of pregnancy. In IVF animals, only GJB6 gene expression showed the same pattern observed in AI pregnancies. Up-regulation of six transcripts (*DPP4, ACP5, INHBA, STC1, THBS2* and *PTGS2*) occurred at 40 days of gestation in gravid caruncles and expression of two genes (*CXCR4, IGFBP3*) did not vary between caruncles from gravid and non-gravid hors at 35 and 40 days. Different superscripts represent significant differences in means between cotyledon-associated caruncles and caruncles from non-gravid horn ($P < 0.05$) at same gestational age. Asterisks represent significant differences in means between IA and IVF gestations ($P < 0.05$) at same gestational age. IA: artificial insemination; FIV: in vitro fertilization.

Analysis of protein expression in uterine caruncles

Western blot analysis was performed to investigate PTGS2, THSB2 e IGFPB3 protein content in uterine caruncles from IA and FIV gestations. Immunoblots showed specific bands of 69, 32 and 129 kDa to PTGS2, IGFPB3 e THSB2 proteins, respectively (Figure 2). Densitometric evaluation indicated an increase in protein content of PTGS2 at 40 days (Figure 2A) and IGFPB3 and THSB2 at 35 and 40 days (Figures 2B and 2C) in cotyledon-associated caruncles obtained from IA gestations.In FIV gestations, PTGS2 protein abundance was significantly higher at 35 days in caruncles from the gravid horn (Figure 2A). On the other hand,the pattern of IGFPB3 e THSB2 protein expression in FIV gestations was similar to that observed in IA gestations. In these cases, an increase in protein levels was observed at 35 and 40 days of gestation in cotyledon-associated caruncles (Figures 2B and 2C).

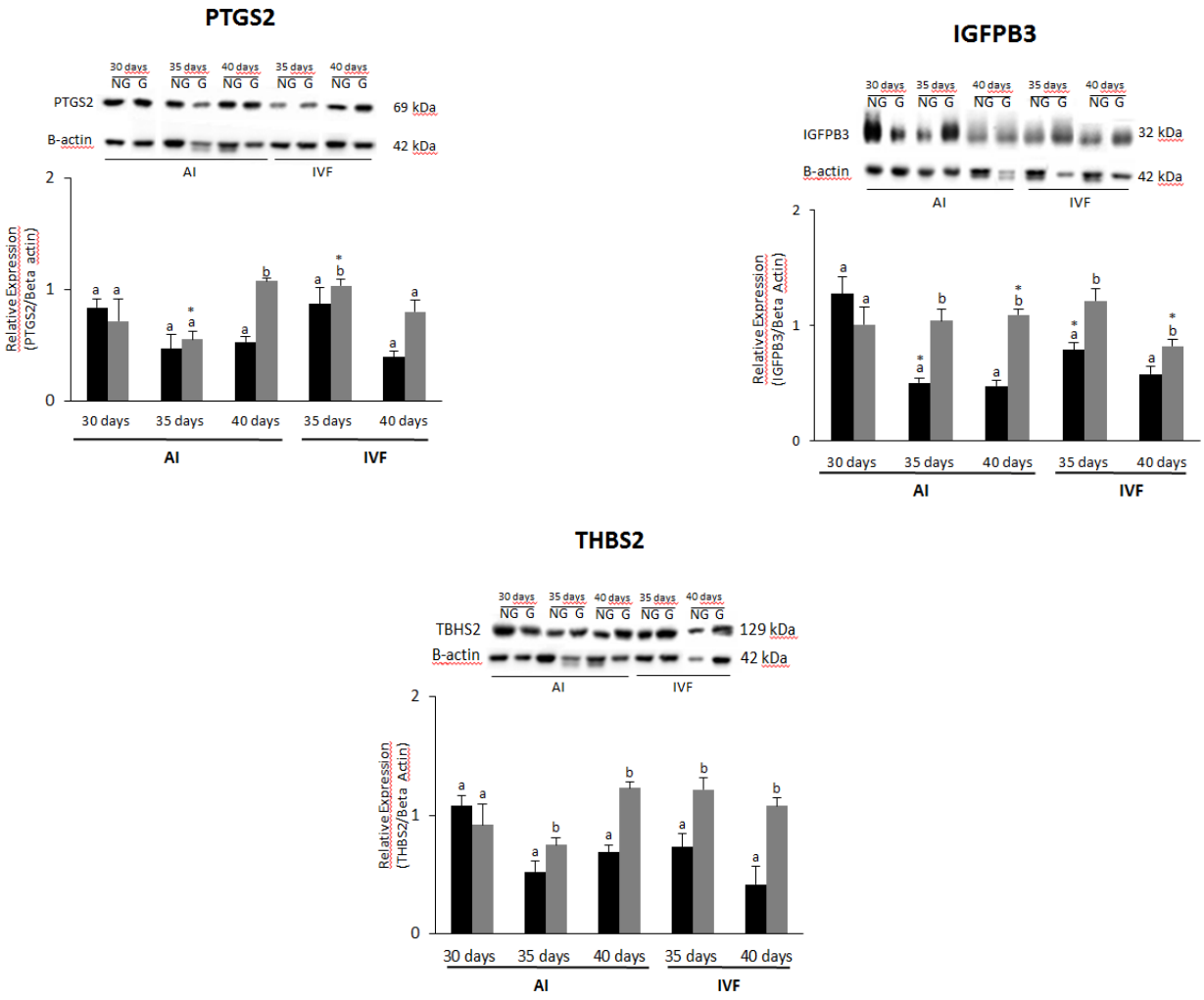

Figure 2. Western blot analysis of PTGS2, IGFBP3 and THBS2 proteins in bovine caruncles from non-gravid horn (black bars) and in cotyledon-associated caruncles (gray bars). In AI gestations, PTGS2 showed a higher protein abundance in gravid caruncles at 40 days of gestation. IGFPB3 and THSB2 proteins were increased in cotyledon-associated caruncles at 35 and 40 days of pregnancy. In FIV animals, PTGS2 was more abundant in gravid caruncles at 35 days of gestation. The patter of IGFPB3 and THSB2 protein expression in gravid caruncles from FIV gestations were similar from that observed in AI gestations. Different superscripts represent significant differences in means between cotyledon-associated caruncles and caruncles from non-gravid horn ($P < 0.05$) at same gestational age. Asterisks represent significant differences in means between AI and IVF gestations ($P < 0.05$) at same gestational age. IA: artificial insemination; FIV: in vitro fertilization; NG: non-gravid horn; G: gravid horn.

Protein immunolocalization in uterine caruncles

Protein immunostaining was observed in the cytoplasm of epithelial and stromal uterine cells, glandular uterine cells and endothelial uterine cells. In addition, we observed that staining intensity was associated to western blot data, with cells from cotyledon-associated caruncle showing more intensity of staining at 35 and 40 days in both AI and FIV gestations. All cell types showed PTGS2 positive staining in caruncules from gravid and non-gravid horns from AI and FIV gestations (Figure 3A). Signal was less intense in endothelial cells when compared with epithelial, stromal and glandular uterine cells (Figure3B). At 35 days of IA gestation, IGFPB3 staining was observed in all cell types, but predominantly in the glandular and epithelial cells of caruncles from both uterine horns. IGFBP3 positive signal was observed only in epithelial and glandular cells of non-gravid horn at 30 and 40 days in IA gestation. In FIV gestations, we also observed a predominant staining of the protein in uterine epithelial and glandular cells, however, all cell types present positive signal in both uterine horns (Figure 3C).THSB2 immunostaining was also detected in all caruncular cell types in both uterine horns from IA and FIV gestations. Stromal uterine cellsshowed lesserintensity of staining when compared with epithelial, glandular and endothelial cells in both uterine horns from AI and FIV gestations at 35 and 40 days of pregnancy (Figure 3D).

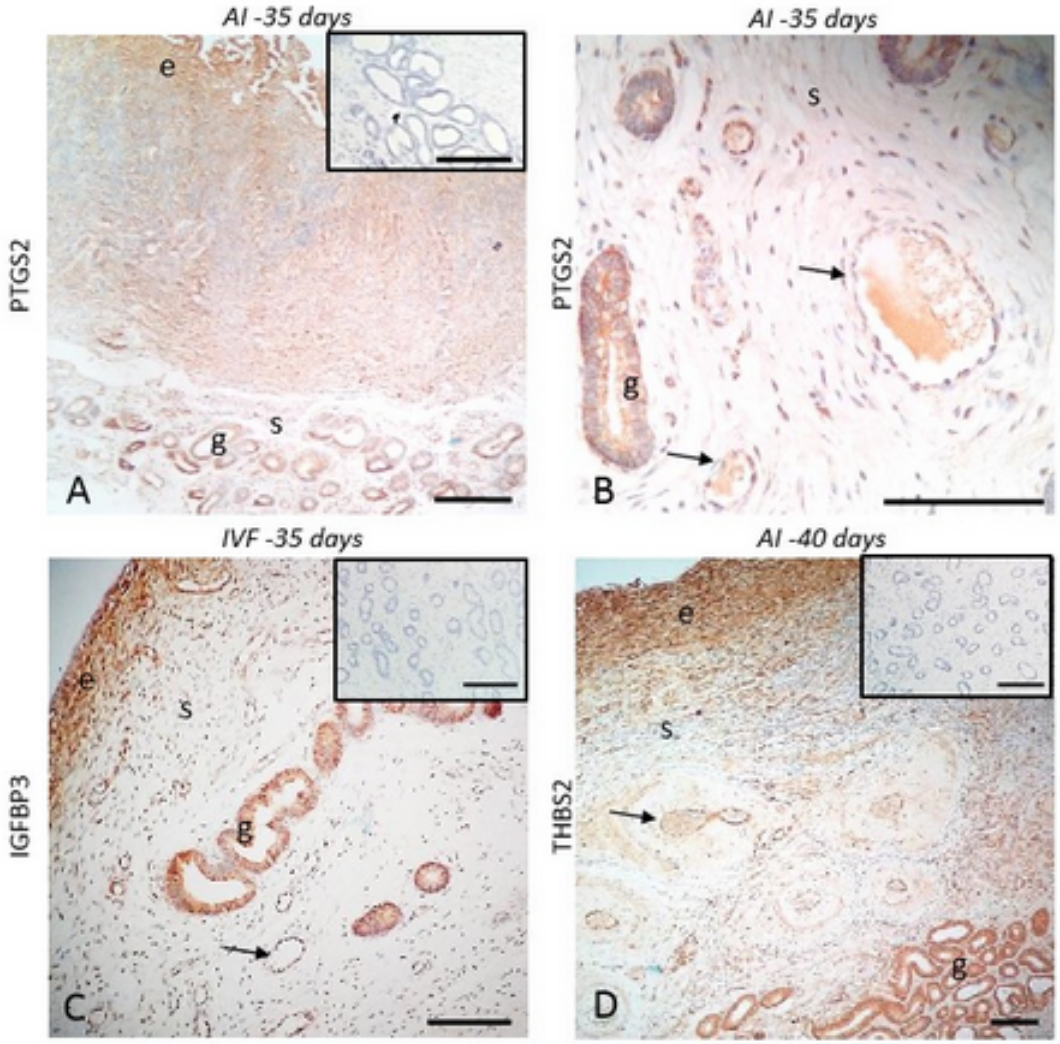

Figure 3. Immunohistochemical characterization of PTGS2, IGFPB3 and THSB2 protein in uterine tissue. PTGS2 (A and B), IGFPB3 (C) and THSB2 (D) localize in uterine epithelial (e), stromal (s), glandular (g) and endothelial cells (black arrow). A) PTGS2 protein immunolocalization in IA gestation at 35 days showing positive signal in all analyzed uterine cells. B) Positive signal of PTGS2 protein was less intense in endothelial cells when compared with other cell types in IA gestations at day 35. C) Localization of IGFPB3 protein in IVF gestation at 35 days demonstrating positive staining in uterine epithelial, stromal, glandular and endothelial cells. D) THSB2 protein localization in AI gestation at 40 days showing that stromal cells present a less intense signal when compared to other uterine cells. Inserts represent negative controls. AI: artificial insemination; FIV: in vitro fertilization. Bars = A, C, D and inserts: 100 μm, B: 200 μm.

DISCUSSION

Placentation process requires the production of a plethora of growth factors, cell-adhesion molecules, extracellular matrix proteins, hormones and transcription factors(Regnault et al., 2002). Several studies have demonstrated that the embryo can play an important role in controlling endometrial gene expression during placentation (Barnea et al., 2012; Kashiwagi et al., 2007; Kikuchi et al., 2011; Thatcher et al., 2001). Here in, we identified a list of genes that are differentially expressed in cotyledon-associated caruncles compared with caruncles form non-gravid horn during placentation.Ontological classification indicated that a high percentage these genesare involved in functions related to tissue remodeling during placentation, such as cell motility, cellmorphology and cell differentiation. Placentation process involves a coordinate maternal-fetal cross talk, and, in bovines, most of gestational losses occur during this period, especially in the case embryos manipulated in laboratory where most gestational losses occur by deficiencies in placental formation(Bertolini et al., 2007; Thatcher et al., 2001). According gene interrelationship analysis, many genes here investigated had their expression associated to progesterone, the predominant hormone during pregnancy. It has been reported, for example, an increase in expression of IGFBP3 in sheep endometrium after progesterone stimulation(Satterfield et al., 2008). However, in the animal model used in this study, caruncles from both uterine horns were under influence of circulating progesterone provided by the ovary, indicating that bovine embryos can regulate specific genes in their microenvironment in order to ensure the success of placental development process.

In this study, we observed a distinct pattern of gene and proteinexpression between caruncles from AI and FIV gestations. Only *GJB6* mRNA expression and IGFBP3 e TBHS2 protein abundance showed a similar pattern of expression. The higher expression of *ACP5*in cotyledon-associated

caruncles from FIV gestations only was observed at 40 days, when expression levels similar to those observed at 35 days in AI pregnancies were achieved. This pattern of expression was repeated for other genes, including*DPP4*, *INHBA* and *SCT1*. In the case of *CXCR4*mRNA expression, the difference in gene expression observed at 35 days in AI gestations was not observed at 35 or 40 days in FIV gestations. Together, these data suggest that the process of in vitro fertilization, although allow placental development, seems to alter its mechanisms, including delaying the events that occur during the process.All genes here investigated are somehow related with the caruncular tissue remodeling required for placentation, however, it is not known if the changes (or what changes) in uterine gene expression profile here observed would cause early or late pregnancy loss or otherwise cause damage to the fetus determining his death after birth. However, our data suggest that embryos manipulation can change their ability to properly control the uterine microenvironment, and this, at least in part, can be one of the factors contributing forlow pregnancy rates described for in vitro produced embryos(Sartori & Dode, 2008).

Analysis of genes interrelationship demonstrated that a number of differentially expressed genes had direct or indirect association with hCG (*PTGS2*, *STC1*, *GJB6*, *CXCR4*, *INHBA*, *IGFBP3*). hCG is a glycoprotein produced by the human fetus with action similar to LH and, besides ensuring the production of ovarian progesterone, plays a crucial role in hemochorial placentation, modulating trophoblast invasion and implantation, cytotrophoblast cell growth and development of placental villi (Cole, 2012). Although hCG is not secreted by bovine embryo, the relationship of differentially expressed genes with an embryo-secreted molecule reinforces its importance as an active agent in the coordination of events occurred in the endometrium during placentation process.

Most reports in literature describe a positive correlation between embryonic factors, including hCG, and PTGS2 expression (Han et al., 1996; Zhou et al., 1999). In this study, we observed a decrease of *PTGS2* mRNA abundance incotyledon-associatedcaruncles obtained from AI and IVF

pregnancies, however protein analysis showed that PTGS2 is upregulated in the gravid horn. This contrast between gene and protein expression could be explained by an increase in mRNA translation or a greater protein stability in cotyledon-associated caruncles. Expression of PTGS2 is deemed critical at all reproduction stages, since ovulation and implantation until placentation and delivery (Chan, 2004). However, it is worth mentioning that lower *PTGS2*gene expression may be involved in regulation of other important genes in this particular phase. Inhibition of PTGS2 expression in tumor cells was related to attenuation ofCXCR4 expression (Katoh et al., 2010), and this pattern of expression was also observed in our model; a decrease in expression of *PTGS2* and *CXCR4*was observed in caruncles from gravid horn. CXCR4 is a chemokine receptor that, unlike other receptors, has CXCL12 as its only one ligand(Murdoch, 2000). CXCL12/CXCR4 system is involved in the endometrial-trophoblast cross talk, recruitment of lymphocytes to the pregnant uterus and placental vascularization (Dominguez et al., 2003; Kumar et al., 2004; Murdoch, 2000; Valles & Dominguez, 2006).In this study,we observed that *CXCR2* mRNA expression is decreased at 35 days of gestation in cotyledon-associated caruncles and, at the same gestational period, *DPP4* transcript abundance was increased in these caruncles. DPP4, also known as CD26, directly regulates CXCL12 activity (Lambeir et al., 2001). Thus, data suggest that the increase in *DDP4* mRNA abundance could be related to the decrease of *CXCR2*. In this case, an increase in the availability of its unique ligand could support the system function during placentation.

Other differentially expressed molecules here investigated have been described as important for placental tissue remodeling in several species. INHBA or activin, which was originally identified as a peptide growth factor obtained from ovarian follicular fluid (Ling et al., 1986), is a dimeric glycoprotein consisting of two βA subunits (Shimasaki et al., 2004). INHBA role during implantation and placentation has been described in mice and humans (Jones et al., 2006; Stoikos et al., 2008). Moreover, a study has suggested that INHBA coordinates endometrial remodeling during placentation in cattle (Sugawara et al., 2010). Additionally, our data suggest that modulation of caruncular *INHBA*

mRNA expression by bovine embryo is important in the early stages of placental development, since an increase in the *INHBA* transcript abundance was already observed at day 30 of gestation.IGFBP3 is a protein with high binding affinity for IGF, binding 70 to 80% of serum IGF-I and IGF-II (Holmes et al., 1999). IGFs have mitogenic and anabolic functions (Sara & Hall, 1990). Binding acts as a method of IGF reserve, increasing its half-life of minutes to hours(Guler et al., 1989). Here in, we observed an increase in*IGFBP3* mRNA expression in caruncles from gravid horn, which could be related to the maintenance of higher tissue levels of IGFs during placentation. Furthermore, increased *IGFBP3* transcript levels in cotyledon-associated caruncles at 35 days of gestation may be related to the increase in*THBS2* mRNA expression also observed at this stage, since positive relationship between the two genes has been described in hepatic tumor cells (Lin et al., 2011).THBS2 is an anti-angiogenic factor that controls the tissues level of vascularization and participates in connective tissue organization(Bornstein et al., 2000; Czekierdowski et al., 2008), and its role during placentation can be precisely related to these functions, providing a fine control of vascular and tissue development.*ACP5* was the seventh most expressed gene in cotyledon-associated caruncles at 35 days of gestation, and despite being related to hCG, IPA analysis showed its relationship to FSH and LH, reinforcing its importance in reproductive events. ACP5 is a metalloprotein present in the pregnant uterus of mammals, however, does not have its particular function determined (Padua et al., 2012). The increase in ACP5 expression is related to the more aggressive breast and ovarian tumors (Adams et al., 2007; Honig et al., 2006) and the molecule is involved in the process of melanoma metastasis (Scott et al., 2011). This information, taken together, suggest that ACP5 could be one of the important molecules controlling placental tissue remodeling in mammals (Padua et al., 2012).STC1 is a glycoprotein which main function is to regulate the concentration of blood calcium. However, the protein is expressed in several tissues, including heart, lung, liver, adrenal glands, prostate and ovary, suggesting that the molecule may have other functions (Chang et al., 1995; Olsen et al., 1996; Varghese et al., 1998). Recently, expression of STC1 was described in specific sites of the endometrium of pregnant mares, indicating that

embryo stimulates the expression of this molecule and that STC1 is involved in placental development (Kikuchi et al., 2011). The importance of GJB6, also known as connexin 30, during implantation and placentation has not been described yet;however, it is known that various connexins are crucial during these events. Connexins junctions play an important role in cellular function allowing the exchange of small molecules and second messengers and facilitating cell growth and homeostasis (Bruzzone et al., 1996).

Based on our results, we can conclude that bovine embryo can influences a cluster of endometrial genes, including genes involved in cell motility, differentiation and morphology, crucial events during placentation process. Furthermore, our data provide new insight into the mechanisms by which embryos control uterine development, suggesting that in vitro manipulation may alter the embryo's capability to modulate the uterine microenvironment in a timely manner. These findings indicate that embryo influences, or lack thereof, may contribute to high levels of loss of IVF gestations.

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Table S1: Probe name, gene symbol, entrez gene name and fold change (Log) of differentially expressed genes (≥1.5 fold, $p \leq 0.05$) in cotyledon-associated caruncles compared to the caruncles from non-gravid horn.

Probe Name	GeneSymbol	Entrez Gene Name	Fold change (Log)
Bt.15484.2.A1_at	SLPI	secretory leukocyte peptidase inhibitor	1,144
Bt.20574.1.S1_at	AQP11	aquaporin 11	1,006
Bt.19501.1.A1_at	Cyp2c44	cytochrome P450, family 2, subfamily c, polypeptide 44	0,916
Bt.4630.1.S1_at	RNASE1	ribonuclease, RNase A family, 1 (pancreatic)	0,886
Bt.19482.1.A1_at	TFRC	transferrin receptor (p90, CD71)	0,885
Bt.12760.1.S1_at	INHBA	inhibin, beta A	0,793
Bt.5193.1.S1_at	ACP5	acid phosphatase 5, tartrate resistant	0,787
Bt.28172.2.A1_at	VSIG1	V-set and immunoglobulin domain containing 1	0,736
Bt.10272.1.S1_at	STC1	stanniocalcin 1	0,724
Bt.4126.2.S1_at	CYP4A11	cytochrome P450, family 4, subfamily A, polypeptide 11	0,653
Bt.8624.1.S1_at	ARG2 (includes EG:11847)	arginase, type II	0,593
Bt.10013.1.S1_at	ASL	argininosuccinate lyase	0,574
Bt.18557.1.S1_at	C7	complement component 7	0,555
Bt.6349.1.A1_at	SLC25A48	solute carrier family 25, member 48	0,545
Bt.25989.1.A1_at	GJB6	gap junction protein, beta 6, 30kDa	0,541
Bt.4102.2.S1_a_at	NPPC	natriuretic peptide C	0,522
Bt.6521.1.A1_at	PARD6B	par-6 partitioning defective 6 homolog beta (C. elegans)	0,520
Bt.1525.1.S2_at	AQP1	aquaporin 1 (Colton blood group)	0,512

Bt.1616.1.A1_at	SOD3	superoxide dismutase 3, extracellular	0,510
Bt.12744.1.S1_at	ATP2C2	ATPase, Ca++ transporting, type 2C, member 2	0,510
Bt.28174.1.S1_a_at	ANXA10	annexin A10	0,508
Bt.5515.1.S1_at	NT5E	5'-nucleotidase, ecto (CD73)	0,505
Bt.17910.1.A1_at	CORO2A	coronin, actin binding protein, 2A	0,502
Bt.6375.1.S1_at	PAGE4	P antigen family, member 4 (prostate associated)	0,495
Bt.7156.1.S1_at	SLC4A4	solute carrier family 4, sodium bicarbonate cotransporter, member 4	0,482
Bt.26655.1.S1_at	CCL19	chemokine (C-C motif) ligand 19	0,474
Bt.19901.1.A1_at	C4orf34	chromosome 4 open reading frame 34	0,469
Bt.25070.1.S1_at	C4orf49	chromosome 4 open reading frame 49	0,469
Bt.8282.1.S1_at	DPP4	dipeptidyl-peptidase 4	0,460
Bt.20397.1.S1_at	CXCL14	chemokine (C-X-C motif) ligand 14	0,456
Bt.2712.1.S1_at	SERPINA5	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 5	0,454
Bt.29198.1.S1_a_at	MEP1B	meprin A, beta	0,416
Bt.13434.2.A1_at	Mkrn1	makorin ring finger protein 1	0,409
Bt.21180.1.S1_at	CSDC2	cold shock domain containing C2, RNA binding	0,403
Bt.23998.1.A1_a_at	CUX2	cut-like homeobox 2	0,396
Bt.28906.1.A1_at	C2CD4B	C2 calcium-dependent domain containing 4B	0,391
Bt.24185.1.A1_at	RAD21L1	RAD21-like 1 (S. pombe)	0,387
Bt.126.1.S2_at	KCNJ2	potassium inwardly-rectifying channel, subfamily J, member 2	0,386
Bt.1739.1.S1_at	FZD4	frizzled family receptor 4	0,380
Bt.487.1.S1_at	EDNRB	endothelin receptor type B	0,373
Bt.137.1.S1_at	ANGPT2	angiopoietin 2	0,351

Bt.16370.1.S1_at	FAM107B	family with sequence similarity 107, member B	0,348
Bt.20253.1.S1_at	SIKE1	suppressor of IKBKE 1	0,337
Bt.18887.3.A1_at	FOXO1	forkhead box O1	0,331
Bt.300.1.A1_at	SULT1E1	sulfotransferase family 1E, estrogen-preferring, member 1	0,328
Bt.22749.1.A1_at	PTGFRN	prostaglandin F2 receptor negative regulator	0,325
Bt.6520.1.A1_at	FRAT2	frequently rearranged in advanced T-cell lymphomas 2	0,322
Bt.25599.1.A1_at	CRHBP	corticotropin releasing hormone binding protein	0,321
Bt.16235.1.A1_at	MOB1B	MOB kinase activator 1B	0,318
Bt.27077.1.S1_at	DARC	Duffy blood group, chemokine receptor	0,317
Bt.20173.1.A1_at	DPT	dermatopontin	0,312
Bt.20862.1.A1_at	MTHFD2	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2, methenyltetrahydrofolate cyclohydrolase	0,310
Bt.13387.1.S1_at	CFI	complement factor I	0,310
Bt.25462.1.S1_at	CDK6	cyclin-dependent kinase 6	0,309
Bt.25668.1.S1_at	PYROXD2	pyridine nucleotide-disulphide oxidoreductase domain 2	0,304
Bt.3435.1.A1_at	FIBIN	fin bud initiation factor homolog (zebrafish)	0,296
Bt.13278.2.S1_a_at	STEAP3	STEAP family member 3, metalloreductase	0,292
Bt.10949.1.S1_at	SLC7A1	solute carrier family 7 (cationic amino acid transporter, y+ system), member 1	0,283
Bt.1565.1.S1_at	ELL3	elongation factor RNA polymerase II-like 3	0,282
Bt.20012.1.S1_at	TNFRSF18	tumor necrosis factor receptor superfamily, member 18	0,279
Bt.22869.1.S1_at	FABP5	fatty acid binding protein 5 (psoriasis-associated)	0,275
Bt.2421.1.A1_at	PLEKHF1	pleckstrin homology domain containing, family F (with FYVE domain) member 1	0,269
Bt.12927.2.S1_at	RCC2	regulator of chromosome condensation 2	0,268

Bt.28130.1.S1_at	ALDH3A2	aldehyde dehydrogenase 3 family, member A2	0,266
Bt.27339.1.A1_at	MME	membrane metallo-endopeptidase	0,265
Bt.15723.1.S1_at	WWC2	WW and C2 domain containing 2	0,263
Bt.20261.1.S1_at	PTPN3	protein tyrosine phosphatase, non-receptor type 3	0,261
Bt.12297.1.S1_at	LOXL4	lysyl oxidase-like 4	0,259
Bt.21867.1.S1_at	STAB1	stabilin 1	0,256
Bt.29716.1.S1_at	GJB2	gap junction protein, beta 2, 26kDa	0,256
Bt.10001.1.A1_at	SLC39A14	solute carrier family 39 (zinc transporter), member 14	0,254
Bt.12645.1.S1_at	RAB15	RAB15, member RAS oncogene family	0,253
Bt.20649.2.S1_at	CELF4	CUGBP, Elav-like family member 4	0,250
Bt.4394.1.S1_at	GUCY1B3	guanylate cyclase 1, soluble, beta 3	0,246
Bt.22472.1.S1_at	CREM	cAMP responsive element modulator	0,246
Bt.422.1.S1_at	IGFBP3	insulin-like growth factor binding protein 3	0,243
Bt.26774.1.S1_at	IGSF10	immunoglobulin superfamily, member 10	0,242
Bt.20793.1.A1_s_at	LAMC1	laminin, gamma 1 (formerly LAMB2)	0,242
Bt.1052.1.A1_at	CH25H	cholesterol 25-hydroxylase	0,242
Bt.3806.1.A1_at	CADM1	cell adhesion molecule 1	0,240
Bt.22552.1.S1_at	CLSTN1	calsyntenin 1	0,237
Bt.26619.1.S1_at	GPD1L	glycerol-3-phosphate dehydrogenase 1-like	0,236
Bt.5522.1.S1_at	THBS2	thrombospondin 2	0,234
Bt.25436.1.S1_a_at	RNF2	ring finger protein 2	0,222
Bt.22480.1.A1_at	SLC35E3	solute carrier family 35, member E3	0,221
Bt.4614.1.S1_at	SLC37A1	solute carrier family 37 (glycerol-3-phosphate transporter), member 1	0,220

Bt.2990.1.S1_at	SLCO2A1	solute carrier organic anion transporter family, member 2A1	0,220
Bt.19690.1.A1_at	PON1	paraoxonase 1	0,219
Bt.12603.1.S1_at	ARHGAP29	Rho GTPase activating protein 29	0,215
Bt.24694.1.A1_at	HEG1	HEG homolog 1 (zebrafish)	0,213
Bt.18751.1.S1_at	PIP4K2A	phosphatidylinositol-5-phosphate 4-kinase, type II, alpha	0,211
Bt.12610.1.A1_at	CP	ceruloplasmin (ferroxidase)	0,211
Bt.9226.1.S1_at	HPCAL1	hippocalcin-like 1	0,208
Bt.3964.1.S1_at	SLC2A3	solute carrier family 2 (facilitated glucose transporter), member 3	0,208
Bt.12848.1.S1_at	PTHLH	parathyroid hormone-like hormone	0,206
Bt.20330.1.S1_at	PRSS23	protease, serine, 23	0,205
Bt.9406.1.S1_at	RAB26	RAB26, member RAS oncogene family	0,205
Bt.24513.1.S1_at	USP12	ubiquitin specific peptidase 12	0,204
Bt.612.1.S1_at	GSR	glutathione reductase	0,203
Bt.18503.1.A1_at	PPIH	peptidylprolyl isomerase H (cyclophilin H)	0,199
Bt.11917.1.S1_at	C17orf28	chromosome 17 open reading frame 28	0,198
Bt.23449.1.S1_at	FAR2	fatty acyl CoA reductase 2	0,198
Bt.5083.1.S1_at	SLC27A4	solute carrier family 27 (fatty acid transporter), member 4	0,197
Bt.18275.2.A1_at	TET3	tet methylcytosine dioxygenase 3	0,193
Bt.6542.1.S1_at	SLC22A5	solute carrier family 22 (organic cation/carnitine transporter), member 5	0,192
Bt.16427.1.S1_at	CCBP2	chemokine binding protein 2	0,192
Bt.19717.1.S1_at	FAM109A	family with sequence similarity 109, member A	0,190
Bt.5861.1.S1_at	CD200	CD200 molecule	0,189
Bt.26328.1.A1_at	SGIP1	SH3-domain GRB2-like (endophilin) interacting protein 1	0,188

Bt.13476.1.S1_at	KIF23	kinesin family member 23	0,188
Bt.27689.1.A1_at	WISP2	WNT1 inducible signaling pathway protein 2	0,187
Bt.22740.1.A1_at	C1GALT1	core 1 synthase, glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase, 1	0,186
Bt.276.1.S1_at	SLC6A6	solute carrier family 6 (neurotransmitter transporter, taurine), member 6	0,182
Bt.17955.1.A1_at	SOBP	sine oculis binding protein homolog (Drosophila)	0,181
Bt.18817.2.A1_at	SMG7	smg-7 homolog, nonsense mediated mRNA decay factor (C. elegans)	0,180
Bt.24969.1.S1_at	FGGY	FGGY carbohydrate kinase domain containing	0,179
Bt.1364.1.S1_a_at	SERPINB9	serpin peptidase inhibitor, clade B (ovalbumin), member 9	0,179
Bt.12048.1.S1_at	FAM213A	family with sequence similarity 213, member A	-0,180
Bt.21547.2.S1_at	PKP2 (includes EG:287925)	plakophilin 2	-0,182
Bt.8957.1.S1_at	CXCR4	chemokine (C-X-C motif) receptor 4	-0,183
Bt.1330.1.S1_at	AKR1B1	aldo-keto reductase family 1, member B1 (aldose reductase)	-0,201
Bt.12755.1.S1_a_at	CHGA	chromogranin A (parathyroid secretory protein 1)	-0,202
Bt.22726.1.A1_at	CA8	carbonic anhydrase VIII	-0,239
Bt.452.1.S1_at	GPLD1	glycosylphosphatidylinositol specific phospholipase D1	-0,206
Bt.8544.1.S1_at	CD69	CD69 molecule	-0,217
Bt.14100.1.S1_at	OLFM4	olfactomedin 4	-0,217
Bt.22253.1.A1_a_at	AFP	alpha-fetoprotein	-0,235
Bt.2346.1.S1_at	LY6E	lymphocyte antigen 6 complex, locus E	-0,240
Bt.14395.2.S1_at	SPTSSB	serine palmitoyltransferase, small subunit B	-0,240
Bt.6963.1.A1_at	EVI2B	ecotropic viral integration site 2B	-0,245
Bt.5050.1.A1_at	CDH16	cadherin 16, KSP-cadherin	-0,248
Bt.7393.1.S1_at	NPNT	nephronectin	-0,252

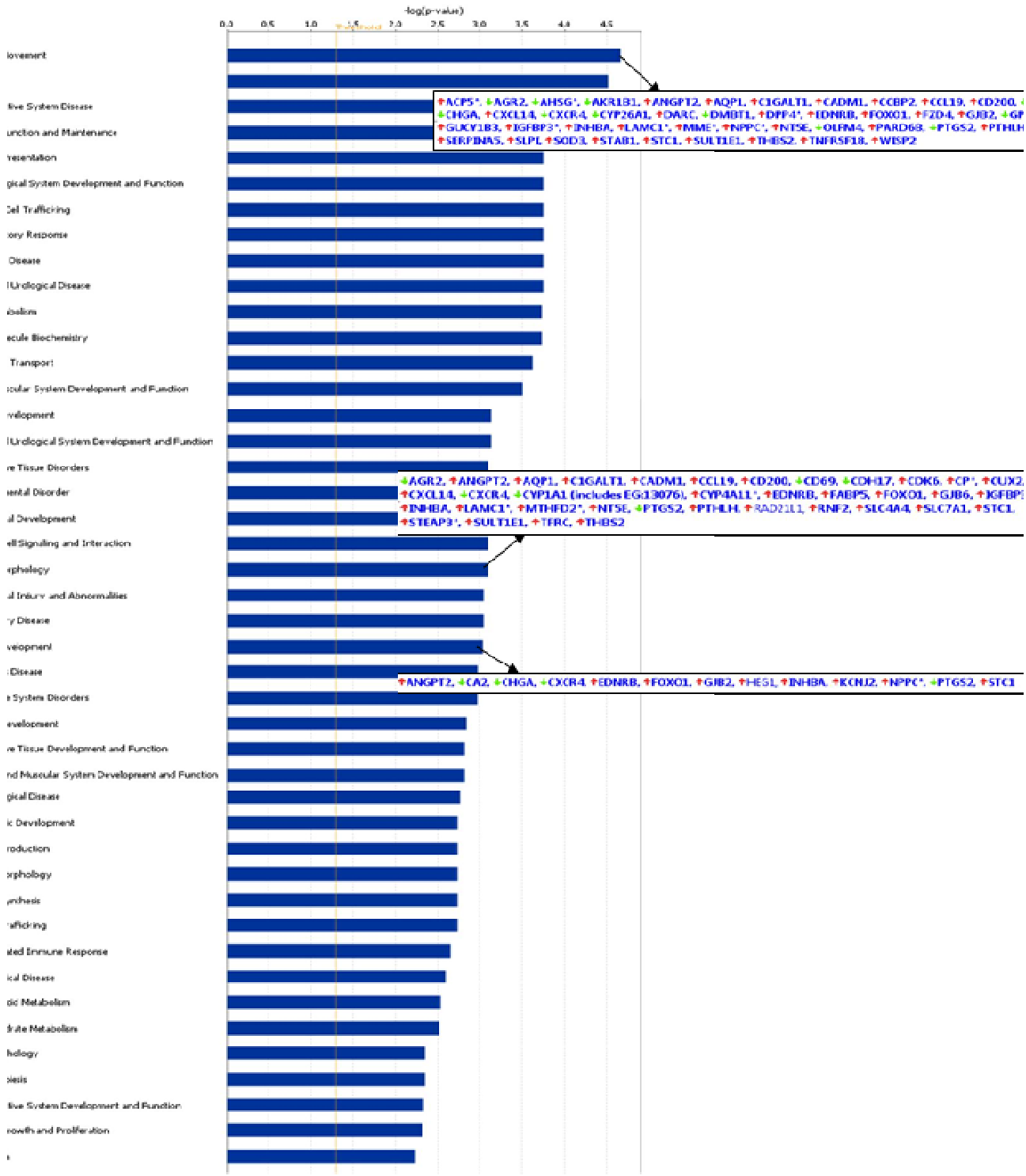
Bt.13772.1.A1_at	CYP1A1 (includes EG:13076)	cytochrome P450, family 1, subfamily A, polypeptide 1	-0,255
Bt.22763.1.S1_at	HMGCS1	3-hydroxy-3-methylglutaryl-CoA synthase 1 (soluble)	-0,259
Bt.9632.2.S1_at	DMBT1	deleted in malignant brain tumors 1	-0,279
Bt.227.1.A1_at	GSTA1	glutathione S-transferase alpha 1	-0,304
Bt.19805.1.A1_at	SLC2A5	solute carrier family 2 (facilitated glucose/fructose transporter), member 5	-0,324
Bt.22854.1.S1_at	CA2	carbonic anhydrase II	-0,356
Bt.23250.2.S1_at	GATM	glycine amidinotransferase (L-arginine:glycine amidinotransferase)	-0,357
Bt.29129.1.S1_at	AGR2	anterior gradient 2 homolog (Xenopus laevis)	-0,365
Bt.15758.1.S1_at	PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	-0,371
Bt.227.2.A1_at	GSTA3	glutathione S-transferase alpha 3	-0,377
Bt.8143.1.S1_at	MX2	myxovirus (influenza virus) resistance 2 (mouse)	-0,385
Bt.22301.1.S1_at	ATP6V0A4	ATPase, H+ transporting, lysosomal V0 subunit a4	-0,432
Bt.23250.1.A1_x_at	AHSG	alpha-2-HS-glycoprotein	-0,454
Bt.28258.1.S1_at	CDH17	cadherin 17, LI cadherin (liver-intestine)	-0,486
Bt.9699.1.S1_at	CYP26A1	cytochrome P450, family 26, subfamily A, polypeptide 1	-0,593
Bt.28620.1.S1_at	SLC27A2	solute carrier family 27 (fatty acid transporter), member 2	-0,673
Bt.4089.1.S1_at	UPK1B	uroplakin 1B	-0,779

Table S2: Pathway interaction network analysis in IPA. Analysis demonstrated 10 networks involving differentially expressed genes. Genes potentially involved in cell differentiation were chosen for PCR analysis (network highlighted in gray). The color indicates upregulated (red) or douwnregulated (green) genes in cotyledon-associated caruncles compared with the caruncles from non-gravid horn.

Top Functions	Genes	Changed genes
Cancer, Reproductive System Disease, Nucleic Acid Metabolism	**AKR1B1**, **ALDH3A2**, **AQP1**, C/ebp, **CCL19**, **CLSTN1**, **CXCL14**, **DMBT1**, **FABP5**, Growth hormone, **GSR**, **GSTA1**, **GSTA3**, **GUCY1B3**, Ifn, IFN alpha/beta, IL12 (complex), **LY6E**, **MEP1B**, **MME**, N-cor, NFkB (complex), Notch, **NPPC**, **OLFM4**, **PARD6B**, PI3K (family), PXR ligand-PXR-Retinoic acid-RXRα, **SERPINB9**, **SLC2A5**, **SLC7A1**, **SLPI**, **TFRC**, thyroid hormone receptor, **TNFRSF18**	24
Cardiovascular System Development and Function, Organismal Development, Cellular Movement	**ACP5**, BCR (complex), **CCBP2**, **CD69**, **CDH16**, **CDK6**, **CH25H**, Collagen Alpha1, **CREM**, **CXCR4**, Cyclin A, **CYP26A1**, **DPP4**, ERK1/2, FSH, **GJB2**, **GJB6**, hCG, Iga, **IGFBP3**, Igg3, IgG2a, Igm, Immunoglobulin, **INHBA**, Lh, MAP2K1/2, **PPIH**, **PTGS2**, **SLC4A4**, **STAB1**, **STC1**, **SULT1E1**, Tgf beta, **THBS2**	21
Developmental Disorder, Renal and Urological Disease, Cell Morphology	Akt, Alp, **ANGPT2**, Ap1, **ARG2 (includes EG:11847)**, **CADM1**, **CHGA**, Collagen type I, Collagen type IV, Collagen(s), **CP**, Creb, Fibrinogen, HDL, **HMGCS1**, IL1, **LAMC1**, Laminin, LDL, Mek, Nos, **NPNT**, **NT5E**, Pdgf (complex), PDGF BB, PP2A, Proinsulin, **PTPN3**, **SERPINA5**, **SLC22A5**, **SLC2A3**, **SLC6A6**, **SMG7**, **SOD3**, STAT5a/b	16
Lipid Metabolism, Small Molecule Biochemistry, Vitamin and Mineral Metabolism	AGT, aldosterone, **ATP6V0A4**, beta-estradiol, **C2CD4B**, Ca2+, CALB1, **CELF4**, **CORO2A**, **CRHBP**, CYP11B1, **Cyp2c44**, Cyp2c, D-glucose, DNAJC3, **ELL3**, ESR1, **FRAT2**, GC-GCR dimer, **HPCAL1**, INHIBIN B, Ivl, **KCNJ2**, MMP2, NR3C1, **PKP2 (includes EG:287925)**, POMC, PPY, Rcan1, **SLC27A2**, **SLC4A4**, **STC1**, **USP12**, **WWC2**, YWHAZ	16
Molecular Transport, Lipid Metabolism, Small Molecule Biochemistry	**ASL**, **ATP2C2**, **CA8**, Ck2, CSNK2B, EGFL7, FGFR1OP2, GAMT, HP1BP3, IREB2, **KIF23**, L3MBTL3, **LOXL4**, MYL3, NOP2, NUCKS1, ODF2L, PCGF3, PCGF5, PDDC1, PHF23, **PIP4K2A**, **PRSS23**, **PYROXD2**, **RCC2**, **RNF2**, **SIKE1**, **SLC27A2**, **SLC27A4**, **SLC39A14**, THOC6, TMEM222, UBC, Ubiquitin, ZNF687	14
Lipid Metabolism, Small Molecule Biochemistry, Molecular Transport	**ANXA10**, **BCL11A**, **C17orf28**, **C1GALT1**, C1GALT1C1, **C4orf34**, CTBP1, DSG2, EDC3, EXOC6, **FAM213A**, **FAR2**, FGFR1, **GPD1L**, HSPA2, KIAA1279, LATS1, LATS2, LSM4 (includes EG:25804), **MOB1B**, **PLEKHF1**,	

	RAB15, RAB26, RABIF, RCN3, RPH3A, SMAD6, SOBP, SPTLC1, SPTLC2, SPTLC3, SPTSSB, STK38L, STX12, UBC	13
Carbohydrate Metabolism, Lipid Metabolism, Small Molecule Biochemistry	AFP, AHSG, AMPK, CA2, Calcineurin protein(s), CD3, CD200, CDH17, CSDC2, CYP1A1 (includes EG:13076), estrogen receptor, Focal adhesion kinase, FOXO1, GOT, hemoglobin, Hsp70, IgG, Insulin, Interferon alpha, Jnk, MTHFD2, MX2, Nfat (family), P38 MAPK, p85 (pik3r),PI3K (complex), Pkc(s), PON1, RNA polymerase II, SLC27A2, Sos, SRC (family), STEAP3, TCR, Tnf	13
Cellular Movement, Hematological System Development and Function, Immune Cell Trafficking	8,9-epoxyeicosatrienoic acid, ACSL4, AGR2, AQP11, C4orf49, CCDC88A, CCL1, CCL5, CCL23, CCL24, CCRL2, CFI, CXCL5, DPT, EDN2, EGF (includes EG:13645), EGFR, ERBB2, FSTL1, FSTL3, IL13, MAPK1, MGAT3, NDST1, PROK1, prostaglandin E2, PTGFRN, RNASE1, RNase A, singlet oxygen, SLC37A1, SLCO2A1, SLPI, VPS4B, WISP2	11
Antigen Presentation, Humoral Immune Response, Inflammatory Response	AK2, AMT, ANKZF1, AP3M1, C6, C7, C8, C9, C5-C6-C7-C8, C5-C6-C7-C8-C9, C8A, C8B, C8G, CUX2, FAM107B, GATM, GJB1, GPLD1, GRHPR, HAL, HLA-G, HNF1A, HNF4A, JMJD5, membrane attack, ONECUT1, PIH1D1, PRELP, RBM23, RPAIN, SGIP1, SLC2A2, SLPI, TDO2, WDFY2	7
Cellular Movement, Carbohydrate Metabolism, Molecular Transport	ARHGAP29, AVPR2, Beta Arrestin, caspase, CCR10, CCRL1, CCRL2, chemokine, CYP4A11, DARC, EDNRB, ERK, FZD4, G protein alphai, GNRH, Gpcr, GPR68, Gαq, HCRTR2, Hdac, Histone h3, Histone h4, LPAR2, LPAR5, Mapk, Mmp, PIK3R5, Pka, PTGDR, PTHLH, Rac, Ras homolog, Relaxin, TBC1D8, Vegf	6

Figure S1: Functional grouping of differentially expressed genes in bovine cotyledon-associated caruncles at 35 days of gestation. Most significant functional groups are shown (p <0.05). The bars represent the P-value on a logarithmic scale for each functional group. Up- (red) and downregulated (green) genes involved in cell motility (46 genes), number of cells (36 genes) and epithelial cell differentiation (13 genes) are showed in the boxes.



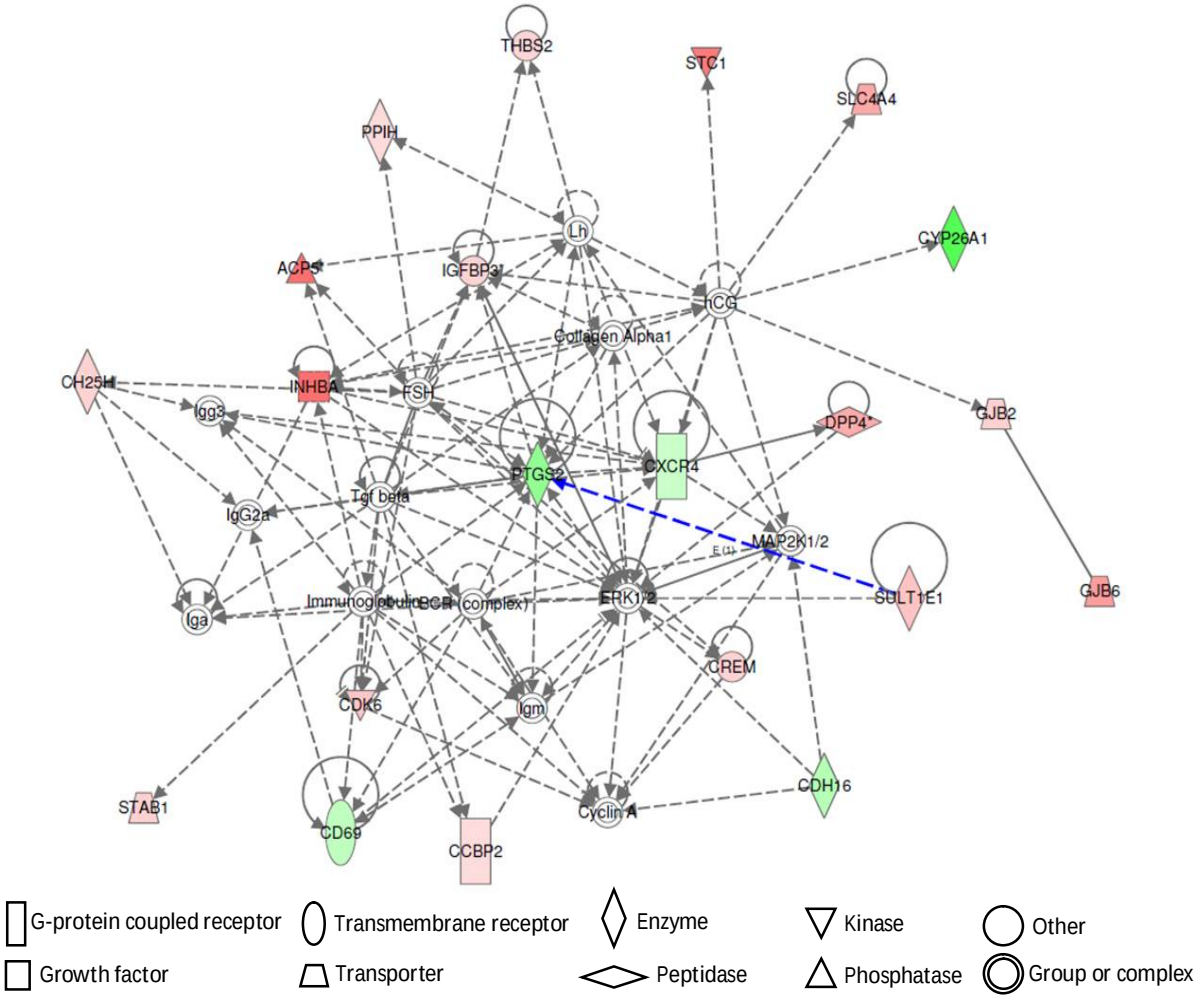


Figure S2: Ingenuity pathway interaction network analysis. Genes involved in cardiovascular system development and function, organismal development, cellular movement, that are differentially expressed in bovine cotyledon-associated caruncles compared to non-gravid caruncles. The colour intensity of the nodes indicates the fold-change increase (red) or decrease (green) associated with a particular gene in caruncles from pregnant horn compared with caruncles form non-pregnant horn. Direct or indirect relationships between molecules are indicated by solid or dashed connecting lines, respectively.

CONSIDERAÇÕES FINAIS

Neste trabalho podemos observar a existência de diferença do padrão da expressão gênica e proteica durante os primeiros dias de gestação do embrião bovino no endométrio. Pois com o passar dos dias, a expressão da maioria dos genes no corno gestante aumenta, demonstrando que a gestação não progride apenas por influência hormonal, mas que a presenças de um embrião viável tem forte participação durante este evento. Mesmo as técnicas de reprodução serem bastante utilizadas e avançadas, percebemos a importância da produção de um embrião viável e com boa qualidade, para que ocorra o estabelecimento da gestação.

Tais mecanismos apresentados poderão auxiliar na compreensão das perdas gestacionais, comuns principalmente nos embriões manipulados como FIV e clones, que devido às várias manipulações necessárias para sua obtenção acabam por alterar a morfologia embrionária, expressão gênica fetal e o desenvolvimento feto/placentária. E com isso ser possível a elaboração de técnicas que otimizem os resultados de prenhes oriundos de biotecnias, e estas, sejam ainda mais difundidas. Pois só melhoram os resultados desejados.

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