UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE VETERINÁRIA
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA ANIMAL: EQUINOS

MUDANÇAS NO ÚTERO DA ÉGUA APÓS A INFUSÃO DE FRAGMENTOS DE CONCEPTOS

CESAR AUGUSTO CAMACHO ROZO

PORTO ALEGRE
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AUTOR: CESAR AUGUSTO CAMACHO ROZO

Tese apresentada ao Programa de Pós-Graduação em Medicina Animal: Equinos da Faculdade de Medicina Veterinária da UFRGS como requisito parcial para obtenção do grau de Doutor

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No es el conocimiento, sino el acto de aprender,
no es la posesión, sino el acto de llegar allí, que
concede el mayor placer.

Carl Friedrich Gauss Lee

RESUMO

A Comunicação materno embrionária equina ainda tem um pobre entendimento. Mudanças histológicas, vasculares e proteicas na prenhez inicial tem sido reportadas. O objetivo do presente trabalho foi determinar mudanças histológicas, vasculares e proteicas, comparando o endométrio e o liquido endometrial de éguas no sétimo dia após ovulação com éguas nas quais no quinto dia após ovulação foram infundidos fragmentos de conceptos de 13 dias. No presente estudo, foram utilizadas 10 éguas sadias e cíclicas, onde uma vez detectado o estro, as éguas foram examinadas diariamente até detectar a ovulação, sendo classificado como o dia 0. Após a ovulação, as éguas foram examinadas por palpação e ultrassonografia modo B e Doppler até o dia 7. Neste primeiro ciclo, liquido uterino e biopsias intrauterinas foram coletas no dia 7 após a ovulação, conformando o grupo Cíclico (n=10). No segundo ciclo, as mesmas éguas foram examinadas diariamente até detectar novamente a ovulação. Aos 5 dias pós-ovulação, fragmentos de conceptos de dia 13, que haviam sido previamente coletados, foram infundidos no útero de cada égua. Liquido uterino e biopsias endometriais foram coletadas no dia 7 pós-ovulação, compondo o grupo Fragmento (n=10). As éguas foram examinadas diariamente por ultrassonografia Power Doppler e Spectral Doppler desde a ovulação até o dia 7, nos dois ciclos. Biopsias foram divididas e armazenadas em solução de glutaraldeído a 2.5% para microscopia eletrônica de varredura e solução de paraformaldeído tamponado a 4% para estudos histológicos. Liquido uterino foi submetido a 2D-PAGE eletroforese, gerando géis que foram analisados e comparados. Os Spots com diferenças ($P < 0.01$) foram removidos, tripsinizados, liofilizados e submetidos a espectrometria de massa (LC-MS/MS e MALDI-TOF/TOF) e identificação.

Observou-se uma diminuição na porcentagem de células ciliadas e planas no grupo Fragmento em relação ao grupo Cíclico. No entanto, células ingurgitadas, secreção superficial e intraglandular, lúmen e diâmetro glandular, diâmetro dos vasos sanguíneos, vascularização endometrial e células imunes foram maiores no grupo Fragmento do que no grupo Cíclico. Mudanças na abundancia das proteínas

do líquido uterino foram identificadas por 2D-PAGE. 13 proteínas foram identificas por espectrometria de massa: Albumina, Apolipoproteina A1, Fator de complemento B, Fibrinogênio B, Fibrinogênio G, Hemopexin, IGL, IGHCp, IGHC1, Lipocalina 2, Serpin B1, Serotransferrin e Serotransferrin-semelhante. Mudanças histológicas e proteicas e suas interações sugerem novos achados para um melhor entendimento da comunicação materno-embrionária.
Palavras chave: biopsia, doppler, microscopia, eletroforese, proteína.

ABSTRACT

Equine maternal-embryonic communication still has poor understanding. Histological, vascular and protein changes in early pregnancy have been reported. The aim of the present study was to determine histomorphological, vascular and protein changes, comparing endometrium and uterine fluid of mares at 7th day after ovulation with mares in which at 5th day after ovulation were infused conceptus fragments of 13 days. In the present study, ten healthy and cyclic mares were used, where once estrus was detected, the mares were examined daily until ovulation was detected, being classified as day 0. After ovulation the mares were examined by palpation and ultrasonography mode B and Doppler since to day 7. In this first cycle, uterine fluid and intrauterine biopsies were collected on day 7 after ovulation, forming the Cyclic group (n = 10). In the second cycle, the same mares were examined daily until ovulation was detected again. At 5 days after ovulation, fragments of concepts from day 13, which had previously been collected, were infused into the uterus of each mare. Uterine fluid and endometrial biopsies were collected on day 7 post-ovulation, composing the Fragment group (n = 10). The mares were examined daily by Power Doppler ultrasonography and Spectral Doppler from ovulation to day 7, in both cycles. Biopsies were divided and stored in 2.5% glutaraldehyde solution for scanning electron microscopy and 4% buffered paraformaldehyde solution for histological studies. Uterine fluid was subjected to 2D-PAGE electrophoresis, generating gels that were analyzed and compared. Spots with differences ($P < 0.01$) were removed, trypsinized, lyophilized and subjected to mass spectrometry (LC-MS/MS and MALDI-TOF / TOF) and identification. A decrease in the percentage of ciliated and flattened cells was observed in Fragment group in relation to the Cyclic group. Nevertheless, protruded cells, superficial and intraglandular secretion, glandular lumen and diameter, blood vessel diameter, endometrial vascularization and immune cells were higher in Fragment group than in Cyclic group. Changes in uterine fluid protein abundance were identified by 2D-PAGE. 13 proteins were identified by mass spectrometry: Albumin, Apolipoprotein A1, Complement Factor B, Fibrinogen B, Fibrinogen G, Hemopexin, IGL, IGHC1, IGHCp, Lipocalin 2, Serpinb1

and Serotransferrin, Like-Serotransferrin. Histological and protein changes and their interactions suggest new findings for a better understanding of maternal-embryonic communication.
Key words: biopsy, doppler, microscopy, electrophoresis, protein.

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1 INTRODUÇÃO

A fase inicial da gestação em éguas é um período crítico que depende da correta expressão de vários fatores associados à interação materna-embrionária, permitindo o apropriado desenvolvimento, implantação e manutenção da prenhez, e evitando a perda embrionária (KLEIN, 2016; WILSHER, 2017). A espécie equina apresenta características diferenciadas no desenvolvimento embrionário e fetal em comparação a outros animais domésticos (ALLEN, 2000; GINTHER, 1998).

O processo onde o embrião indica a sua presença, interrompendo o ciclo estral e mantendo a gestação, é conhecido como reconhecimento materno da prenhez (RMP) (SHORT, 1969). Porém ainda não foi identificada está sinalização química na espécie equina (KLEIN, 2016), mas acredita-se que é ativo em torno ao dia 13 após a ovulação (MCDOWELL et al., 1990).

Recentes estudos sobre a metabolômica, transcriptômica e proteômica da comunicação materno-embrionária ajudaram a um melhor entendimento dos eventos chave que podem influenciar o sucesso ou a falha no processo do reconhecimento materno (MERKL et al., 2010; KLEIN; TROEDSSON, 2011b; SWEGEN et al., 2017; SMITS et al., 2017, 2018; PILLAI et al., 2018; BASTOS et al., 2019). Proteínas detectadas no embrião, no útero e no fluido endometrial, tem sido associadas com processos de crescimento embrionário, nutrição, defesa uterino-embrionária, reestruturação endometrial e manutenção do corpo lúteo (SWEGEN et al., 2017; SMITS et al., 2018; BASTOS et al., 2019). Porém, devido à complexidade da comunicação materno-embrionária e as diferenças da espécie equina, muitos processos fisiológicos ainda precisam serem elucidados.

Tecidos embrionárias homogeneizados (ROWSON; MOOR, 1967) e células trofoblasticas (HEYMAN et al., 1984; BALL et al., 1990) foram infundidos depositados no útero de ovelhas, vacas e éguas como modelo experimental, tentando melhorar o entendimento do controle antiluteolitico, reconhecimento materno da prenhez, manutenção da prenhez e melhoramento das taxas de prenhez por transferência de embriões.

Hipotetiza-se que proteínas presentes em fragmentos de um concepto morto do 13º dia infundidos no útero equino 5 dias após a ovulação induzirão mudanças na estrutura, histologia, vascularização e do liquido uterino do endométrio equino, que podem ser detectadas no dia 7 após a ovulação. O objetivo do presente trabalho foi avaliar se fragmentos de conceptos de 13 dias infundidos no útero de éguas no quinto dia após a ovulação mudam o perfil proteico do fluido uterino, a estrutura do endométrio e a vascularização uterina no sétimo dia após a ovulação.

2 REVISÃO BIBLIOGRAFICA

A fisiologia da reprodução na espécie equina e em especial na prenhez inicial apresenta características diferentes com os outros animais domésticos. As interações entre o embrião e a tuba uterina, a chegada tardia no útero, uma amplo tempo de mobilidade embrionária, uma implantação tardia e acompanhado de padrões de hormonais diferenciados para a manutenção da gestação ainda não são totalmente compreendidos, assim como a sinalização química do reconhecimento materno da prenhez ainda não foi identificada (MERKL et al., 2010; KLEIN; TROEDSSON, 2011b; KLOHONATZ et al., 2015).

2.1 O Ciclo Estral e Endocrinologia da Égua

A égua é um animal poliestrico estacional cujo ciclo estral tem uma duração média de 21 dias. Está dividido em dois períodos: o estro de 7 dias de duração média, que é a fase da receptibilidade ao garanhão e é determinada pelo efeito do estrógeno com presença de folículos, e a fase lútea ou diestro de 14 dias aproximadamente, que é determinada pela progesterona liberada por um corpo lúteo (HUGHES; STABENFELDT; EVAN, 1977; GINTHER et al., 2003)

O Hormônio liberador de gonadotrofinas (GnRH) controla a liberação dos hormônios gonadotrópicos de origem adenohipofisária, o hormônio folículo estimulante (FSH) e o hormônio luteinizante (LH) (GINTHER, 1979). O FSH é responsável pelo recrutamento e crescimento folicular variando seus níveis pelo desenvolvimento do folículo dominante, chegando aos níveis menores de 1 a 2 dias antes da ovulação (GINTHER et al., 2005a). O LH tem ação em folículos antrais na seleção, maturação final do oócito, ovulação e aumentando gradualmente com o desenvolvimento folicular, logrando seus máximos níveis de 1 a 2 dias após a ovulação em presença de níveis altos de estradiol (E_2) (GINTHER et al., 2005b).

A ovulação na égua acontece 24 a 48 horas antes do fim dos sinais do estro (HUGHES; STABENFELDT; EVAN, 1977), com um aumento importante nos níveis

de prostaglandina $F_{2\alpha}$ ($PGF_{2\alpha}$) e prostaglandina E_2 (PGE_2) no fluido folicular do folículo dominante perto da ovulação (SIROIS; DORÉ, 1997).

Depois de 24 a 48 horas da ovulação se observa a formação do corpo lúteo, estrutura encarregada da produção de Progesterona (P_4). Esse corpo lúteo mantém-se funcional na égua não gestante por um tempo médio de 14 dias, que coincide com a fase de diestro. A ovulação é considerada como o início da fase lútea, que culmina com a luteólise, apresentando níveis plasmáticos de progesterona inferiores a 1 ng/mL, pela ação do $PGF_{2\alpha}$ de origem endometrial (GINTHER et al., 2005b).

2.2 Endométrio da égua e sua avaliação

Avaliação por histologia convencional e microscopia eletrônica de varredura têm sido usadas amplamente na determinação dos padrões celulares e de atividades secretórias nos diferentes ciclos no útero da égua (RICKETTS et al., 1978), sendo semelhantes a outras espécies (AURICH; MARIC; AURICH, 2011). A histologia convencional fornece uma avaliação da parede do endométrio (KENNEY, 1978) e a microscopia eletrônica de varredura (MEV) proporciona uma representação de aspecto tri-dimensional da superfície do tecido, exibindo o epitélio luminal (SAMUEL et al., 1979).

O útero da égua é composto por 3 camadas. A lâmina própria é a mucosa ou endométrio, que não é decíduo; a segunda camada ou intermediária é o miométrio e a terceira camada externa é o perimétrio. Uma biópsia endometrial usualmente consiste exclusivamente da porção de prega endometrial com ausência de musculo, porém eventualmente pode conter uma porção da camada circular do miométrio (KENNEY, 1978) (Fig. 1).

O endométrio é formado por uma camada de epitélio luminal com sua lâmina basal e lâmina própria. O epitélio luminal é composto de células cubóides a colunares altas que residem na membrana basal, que varia na altura de acordo com a fase do ciclo estral. Menos da metade das células são ciliadas (KENNEY, 1978).

A lâmina própria é composta de duas camadas que se mesclam imperceptivelmente entre elas, e divididas para melhor descrição baseada na população de células do estroma. O estrato compacto (EC) que sustenta o epitélio luminal, tem cerca 0,75 à 1 mm de espessura e é caracterizado por uma relativa alta concentração de células do estroma e numerosos capilares juntos à membrana basal. As células do estroma são estreladas, irregulares, com núcleo oval a redondo e escasso citoplasma. As células formam uma rede de fibras reticulares, onde normalmente não tem produção de colágeno extracelular. O estrato esponjoso (EE) tem menor densidade celular de estroma formando uma rede de fibras entre as células, conferindo a aparência esponjosa. O espaço entre as áreas esponjosas normalmente está preenchido com fluído tissular. Os vasos no EE são capilares, arteríolas, vênulas e ocasionalmente pequenas artérias musculares, além de numerosos vasos linfáticos (KENNEY, 1978).

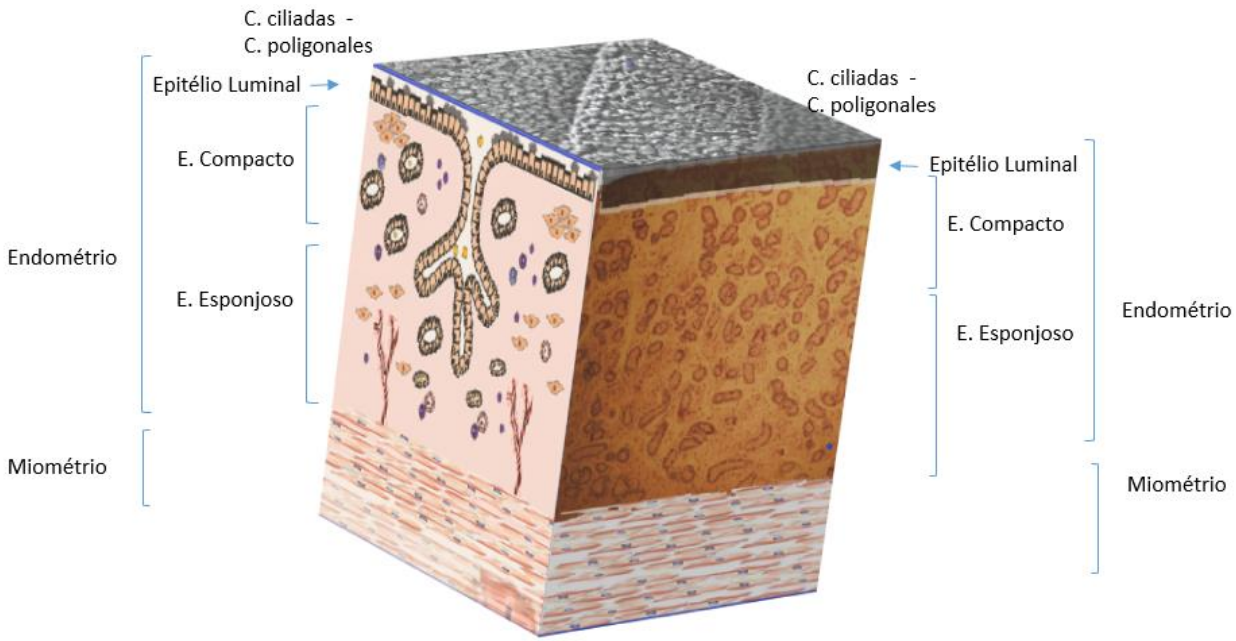

Fig. 1. Representação esquemática de vista de corte seccional de endométrio equino. A relativa espessura das camadas foram diminuídas para a proposta ilustrativa. Fonte:Autor.

A característica proeminente da lâmina própria é a presença de numerosas glândulas uterinas. As glândulas procedem e conectam-se do epitélio luminal por ductos. As glândulas em sua porção meia e basal estão conformadas por mais de 10 ramos primários e alguns poucos secundários. Além disto, a superfície endometrial tem pregas que formam um espaço capilar. O espaço capilar serve como coletor de deposito de células migratórias e tecidos (KENNEY, 1978). A estrutura endometrial modifica-se conforme o status reprodutivo das éguas (fase cíclica, prenhe ou anestro), assim como dentro de sua fase cíclica (estro ou diestro) (KENNEY, 1978).

No estro por histologia convencional, o epitélio luminal varia entre colunar alto ou pseudoestratificado, resultado da atividade mitótica (RICKETTS et al., 1978); a altura do epitélio luminal varia de 20 a 30 μm, podendo alcançar até 50 μm na fase inicial (BARROS; MASUDA, 2013). Vacuolização citoplasmática é comum, especialmente no terço basal das células. No estro, há tendência à presença de neutrófilos polimorfonucleares. A densidade glandular por unidade de área é menor no estro do que em outras fases como resultado do edema endometrial, sendo as glândulas menos tortuosas que durante o diestro (KENNEY, 1978). Na visualização por MEV, o epitélio luminal presenta células microvilosas poligonais, células ciliadas, células pró-ciliadas, bolhas apicais de células secretórias e nos cumes das dobraduras endometriais (SAMUEL et al., 1979).

No diestro inicial, 7 dias após do estro por histologia convencional, o epitélio pode ser de 15 a 20 μm, ocasionalmente 10 μm, dependendo se as células expulsarão seu conteúdo durante o estro. Depois do dia 7, a altura do epitélio incrementa a 20 μm. No diestro a densidade glandular aumenta pela diminuição do edema e pelo incremento da tortuosidade (KENNEY, 1978). Com a MEV, as células secretórias diminuem em proporção aumentando o número das células ciliadas para a metade do diestro (SAMUEL et al., 1979).

2.3 Fisiologia da Prenhez inicial na égua

Atualmente, múltiplos estudos tem identificado proteínas e transcritos da prenhez inicial em tecidos embrionários, endometriais e em fluido intrauterinos, determinando uma complexa rede ainda pobremente compreendida (SWEGEN et al., 2017; SMITS et al., 2018; PILLAI et al., 2018; BASTOS et al., 2019). Proteínas secretadas pelo concepto, endométrio e ovários maternos desempenham importantes papel no controle hormonal, orquestrando mudanças para a manutenção de prenhez (KLEIN; TROEDSSON, 2011b; WILSHER; LEFRANC; ALLEN, 2011; LEISINGER et al., 2017; WILSHER, 2017). Devido à complexidade da interações materno-embrionárias e a grande número de informações geradas nos últimos anos (Fig. 2), é necessário desenhar novas abordagens que resumam e foquem nossos resultados.

2.3.1 Desenvolvimento do embrião equino na oviduto

Após a ovulação, o oócito migra até a ampola, ainda protegido pelas células do cumulus. O complexo cumulus oócito (CCO) atravessa a ampola até a junção ampola-istmo, onde ocorre a fecundação pelo espermatozoide, após o acasalamento ou inseminação (GINTHER, 1979). Quando o espermatozoide penetra o oócito, este inicia a segunda divisão meiótica com a reorganização de cromossomos produzindo a singamia e formando o zigoto (GINTHER, 1979). Dois dias após a fecundação, com 6 ou 8 células, o genoma embrionário é ativado. O embrião continua seu crescimento e no dia 5 no estado de mórula compacta começar a sintetizar PGE_2, que induz o relaxamento e a contração da união istmo-ampola, para que o embrião entre no lúmen do útero ao dia 6 no estádio de mórula tardia ou blastocisto inicial (BETTERIDGE et al., 1982; WEBER; VANDERWALL, 1991; WEBER et al., 1992; WEBER; WOODS; LICHTENWALNER, 1995). Os oócitos não fecundados permanecem nas pregas da mucosa do oviduto até a ocorrência de um processo de degeneração lenta (FLOOD; JONG; BETTERIDGE, 1979).

A proliferação endometrial equina é dividida em duas fases: a proliferação de estroma no estro e a proliferação de epitélio no diestro (AUPPERLE et al., 2000).

Diferentes grupos sugeriram que o transporte lento do embrião na tuba uterina equina está vinculada ao padrão de proliferação endometrial, de maneira que a presença do embrião no útero no 6º dia estimula o crescimento do epitélio glandular e a secreção do histotrofo (GERSTENBERG; ALLEN; STEWART, 1998), pelos altos níveis de progesterona e baixos níveis de estrógenos (BRUNCKHORST et al., 1991; KALPOKAS et al., 2010). Esta estimulação hormonal foi associada com uma alta expressão dos receptores esteroides nas células epiteliais, especificamente no epitélio glandular (AUPPERLE et al., 2000; KALPOKAS et al., 2010) e com uma elevada expressão do marcador de proliferação em células epiteliais, o antígeno Ki 67 (AUPPERLE et al., 2000).

2.3.2 Períodos de prefixação e cápsula embrionária

O período de prefixação do embrião na gestação tem muitas particularidades em comparação com outras espécies de animais domésticos, entre elas a forma esférica, alta mobilidade do embrião e a presença de uma cápsula acelular glicoproteica de caraterística anti-adesiva (ALLEN, 2001, 2005)

O deslocamento do embrião, no útero, pode ocorrer de um corno a outro 20 vezes por dia, decorrente das fortes contrações miometriais, sem elongação do trofoblasto, mantendo sua forma esférica. A movimentação e sinalização embrionária bloqueiam a secreção de pulsos luteolíticos de $PGF_{2\alpha}$ (GINTHER, 1983, 1995). A aplicação de inibidores de ciclodexinas como o flunixin meglumine diminuem os movimentos do embrião, sugerindo que as prostaglandinas são o estimulo primário da contração uterina na prenhez inicial (STOUT; ALLEN, 2001a). O embrião é capaz de produzir as prostaglandinas em condições in vitro, sugerindo que o concepto provavelmente tem a capacidade de estimular a contração e relaxamento do miométrio (STOUT; ALLEN, 2002).

A cápsula é detectada no dia 6.5 após a ovulação entre o trofectoderma e a zona pelúcida, no momento da blastulação (BETTERIDGE et al., 1982). Esta cápsula é inicialmente produzida pelo trofectodema até que endureça em uma

membrana elástica fina que cobre completamente o embrião esférico até o dia 23. O peso da cápsula aumenta gradualmente desde o dia 12 até o dia 18; além disto, ocorre uma maior movimentação do embrião nos dias 11 a 14 (GINTHER, 1985; ALLEN, 2001, 2005; STOUT; MEADOWS; ALLEN, 2005). A presença da cápsula e o deslocamento embrionário é importante para o desenvolvimento da prenhez inicial para evitar a luteólise, pois vesículas embrionária equinas foram fixadas cirurgicamente no endométrio, resultando na luteólise total ou parcial (MCDOWELL et al., 1988). Em outro experimento foram retiradas as cápsulas de 15 embriões, resultando na não manutenção da prenhez (STOUT; MEADOWS; ALLEN, 2005).

A cápsula é composta por ácido siálico e glicoproteínas tipo mucina (MUC1) secretadas pelo trofoblasto, sendo fundamental na proteção, movimentação e comunicação materno-embrionária. A composição e a carga da cápsula é alterada devido à glicosilação de N-acetil-galactosamina associada à MUC1, a mudança na porcentagem de ácido siálico e carboidratos sobre o dia 16, quando o embrião se fixa, sugere um mecanismo de regulação da movimentação e dos efeitos anti-adesivos do útero na prevenção da implantação prematura do embrião (ORIOL et al., 1993; ARAR et al., 2007). Isto também é coincidente com o aumento do diâmetro do concepto (STOUT; ALLEN, 2001a). A cápsula, por sua carga eletroestática negativa do lado externo torna-se "adesiva", pela configuração do glicocálice, atraindo proteínas e acumulando uma série de componentes das secreções das glândulas endometriais em sua superfície, que servem na nutrição embrionária, à medida que o concepto se movimenta pelo interior do útero (ORIOL et al., 1993; ALLEN, 2001).

A expressão de MUC1 parece ser regulada pela P_4 e E_2. O mRNA da MUC1 foi detectado no endométrio tanto de éguas prenhes como não prenhes nos dias 11 e 18 da prenhez/ciclo estral respectivamente, assim como no trofoblasto da maioria dos embriões obtidos nesse período (GILLIES et al., 1999). Por outro lado, através de imunofluorescência se determinou uma expressão da proteína MUC1 uniformemente nas superfícies apicais do epitélio luminal e glandular, sendo a intensidade de coloração de MUC1 superior no corno uterino gravídico que no não

gravídico nos dias de obtenção das amostras (dias 14, 17, 21, 27, 37 da prenhez) (AL-RAMADAN et al., 2002).

No entanto, a cápsula embrionária também contém uma proteína de ligação o fator de crescimento semelhante à insulina tipo 3 (IGFBP3), que pode concentrar fatores de crescimento do tipo insulínico (IGF) na cápsula e, eventualmente, libera-los de uma forma controlada, portanto, regulando a influência do IGF materno no concepto (HERRLER et al., 2000).

Merkl et al., (2010) e Klein e Troedsson (2011a) demonstraram que o endométrio e o embrião expressam uma ampla gama de proteínas transportadoras de soluto (genes SLC) e nutrientes, tais como ácidos e íons amino, de modo que o embrião expressa várias apolipoproteínas que facilitam o transporte dos lipídios.

2.3.3 Reconhecimento materno da prenhez (RMP)

O corpo lúteo é formado após a ovulação, sendo fonte de progesterona. Em éguas não prenhes ocorre a luteólise entre os dias 13 a 16 pós ovulação pela ação da $PGF_{2\alpha}$ liberada pelo endométrio. Assim, a presença do embrião inibe a liberação de $PGF_{2\alpha}$ e mantém o corpo lúteo (ALLEN, 2001), sinalizando sua presença interrompendo o ciclo estral e mantendo a gestação no processo conhecido como “Reconhecimento materno da prenhez" (RMP) (SHORT, 1969). O sinal do RMP para os equinos ainda não foi identificado (KLEIN et al., 2010). O concepto tem uma complexa interação com o útero da égua, mediante liberação de hormônios até sua implantação no dia 35 para manter a gestação e inibir a luteólise (KLEIN et al., 2010; BAUERSACHS; WOLF, 2012). Acredita-se que o sinal do RMP equino tenha em torno de 3000 e 10000 Dalton, sendo resistente à proteinase k, mas pode ser absorvido por carvão revestido com dextrano (ABABNEH et al., 2000).

2.3.3.1 Histologia e ultraestrutura da comunicação útero embrionária

Eventos comuns aos encontrados durante o diestro e na gestação inicial em equinos tem sido documentados. Presença de epitélio cubóide e colunar simples ou pseudoestratificado, vacúolos celulares, presença de leucócitos no estroma, adensamento de glândulas foram descritos no diestro e na gestação até os 21 dias. Entretanto, apenas nas éguas gestantes foi relatada a presença de pleomorfismo celular do epitélio superficial (KEENAN et al., 1987). Mudanças histomorfométricas têm sido relatadas no endométrio usando analisadores de imagem semiautomáticos (MANSOUR et al., 2004).

Na avaliação histológica e ultraestrutural do endométrio no dia cinco após a inseminação, determinou-se diferenças no diâmetro glandular, diminuição na proporção de células ciliadas por campo e aumento no número de linfócitos em éguas inseminadas, comparadas com éguas cíclicas, sugerindo uma mudança da estrutura endometrial previa à entrada do embrião (CABALLEROS et al., 2019). Adicionalmente, no dia 7 após ovulação foram detectadas mudanças histomorfométricas e aumento de secreções histotróficas no endométrio de éguas prenhas em comparação com éguas cíclicas, sendo associadas à comunicação materno-embrionária (CAMOZZATO et al., 2019), além de um aumento no número de leucócitos por campo (MARTÍNEZ PEREIRA, 2016).

2.3.3.2 Hormônios envolvidos no RMP

A prostaglandina $F_{2\alpha}$ é metabolizada através do ácido araquidônico (AA), ácido graxo que constitui a membrana fosfolipídica. O AA é hidrolisado e liberado pela ativação da fosfolipase A2 (PLA2). O AA é metabolizado pelas enzimas prostaglandina-endoperóxido sintase 1 e 2 (PTGS1 e PTGS2), anteriormente conhecida como ciclo-oxigenase 1 e 2 (COX 1 e 2) (NEEDLEMAN et al., 1986) produzindo PGFH1 e PGFH2 (BOERBOOM et al., 2004; CROFFORD, 2001). O PTGS1 é uma isoforma que não altera seus níveis independentemente do momento da prenhez e é essencial. No tanto, a expressão de PTGS2 aumenta na luteólise, mas não apresenta um aumento considerável na égua prenhe (NEEDLEMAN et al., 1986; BOERBOOM et al., 2004). Finalmente, a prostaglandina H2 é convertida em

13,14-dihydro-15-keto $PGF_{2\alpha}$ (PGFM) comumente conhecida como prostaglandina $F_{2\alpha}$, pela ação da PGF sintetase ou em Prostaglandina E_2 pela ação da PGE sintetase (PGES2). A PGE_2 pode ser transformada em $PGF_{2\alpha}$ pela ação da 9-ceto-PGE_2 redutase (9k-PGR) (NEEDLEMAN et al., 1986).

As prostaglandinas são liberadas do citosol pela proteína transportadora de prostaglandinas (PGT) (CHAN et al., 1998; AROSH et al., 2004), pertencente à família das SLC mencionadas anteriormente, membro 2A1 (HAGENBUCH; MEIER, 2003). A PGT, em conjunto com as prostaglandinas, exercem suas funções em seu influxo e inativação (SCHUSTER, 1998) sem alterar suas concentrações no endométrio equino durante o estro, diestro ou prenhez inicial (NASCIMENTO, 2011). Finalmente, a PGF2α entra na circulação periférica e alcança os ovários resultando na luteólise e na continuação do ciclo estral (ALLEN; STEWART, 2001).

Concentrações do PGFM no plasma de amostras coletadas diretamente da veia uterina da égua nos dias 10 e 14 de gestação são menores que aos mesmos dias correspondentes do ciclo estral (DOUGLAS; GINTHER, 1976), porém lavados uterinos de éguas prenhes no dia 18 contém concentrações similares às observadas durante o momento da luteólise em éguas cíclicas (STOUT; ALLEN, 2002). Assim, Klein e Troedsson (2011b) concluíram que o embrião retarda mais do que impede a liberação de $PGF_{2\alpha}$ uterina durante o RMP. O sinal produzido pelo embrião equino é, portanto, mais de natureza antiluteolítica do que luteoprotetiva (inibe a ação da $PGF_{2\alpha}$) ou luteotrópica (apoia a função do corpo lúteo) (KLEIN et al., 2010). Em éguas cíclicas existe um aumento do RNAm da PTGS2 e proteínas coincidindo com a luteólise, e em éguas prenhes, tal aumento está ausente (ATLI et al., 2010). Portanto, a transcrição reprimida de PGTS2 se deve a um produto secretado pelo concepto (EALY; EROH; SHARP, 2010; EISELE; DESVOUSGES; SHARP, 2002). Além disto, a PLA2 também foi avaliada no endométrio equino, concluindo que os mecanismos de regulação uterina da enzima não são influenciados pela prenhez, e podem estar associados a modificações pós-transducionais (ABABNEH; ABABNEH; SHIDAIFAT, 2011).

Assim mesmo, a Ocitocina (Ox) é um estimulador da liberação de PGF do endométrio, pela união ao receptor endometrial (BURNS; GRAF; HAYES, 1997). Estudos demonstraram que a PGF2 é Ox dependente, apresentando um maior número de receptores para Ox em biopsias endometriais no dia 14 após a ovulação (STARBUCK et al., 1998), e um aumento de metabólitos de $PGF_{2\alpha}$ após a aplicação exógena de Ox, além de uma expressão diferenciada em éguas prenhes (GOFF; PONTBRIAND; SIROIS, 1987). Redução da resposta à ocitocina coincide com uma diminuição da capacidade de união com o receptor endometrial (STARBUCK et al., 1998).

A estimulação do receptor de Ox (ROx) gera uma cascata de eventos que culminam com a ativação da fosfolipase A (PLA), resultando na liberação hidrolítica do ácido araquidônico dos fosfolipídios de membrana. Na égua, a produção da Ox é de origem endometrial, enquanto que o ovário está essencialmente desprovido de Ox. A expressão de RNAm de Ox tende a ser maior durante o estro e final do diestro que em outras etapas do ciclo (BEHRENDT–ADAM et al., 1999). Propõe-se que a $PGF_{2\alpha}$ uterina seja o gatilho da secreção de Ox da hipófise durante o início da luteólise na égua (VANDERWALL; SILVIA; FITZGERALD, 1998), mas o mecanismo que regula a liberação inicial da $PGF_{2\alpha}$ uterina segue desconhecido. Dando continuidade ao processo, a Ox da pituitária estimula a secreção de $PGF_{2\alpha}$ endometrial através da união a seu receptor no endométrio, o que estabelece uma retroalimentação positiva (KLEIN; TROEDSSON, 2011a).

Por outro lado, Klein e Troedsson (2011b) encontraram genes envolvidos no transporte e síntese do colesterol. A proteína reguladora da esteroidogênese aguda (StAR) é uma proteína de transporte que regula a transferência do colesterol na mitocôndria, o que é um ponto limitante na produção de hormônios esteroides. A proteína StAR demonstrou maior expressão nos embriões dos dias 12 e 14 em comparação com os do dia 8. A enzima de clivagem da cadeia de colesterol-citocromo P450 (P450SCC) é uma enzima mitocondrial associada à conversão de colesterol em pregnenolona e teve um padrão similar de expressão da StAR, assim como a HSD3B1, uma enzima que desempenha um papel fundamental na biossíntese de todos os hormônios esteroides (KLEIN; TROEDSSON, 2011b), A

expressão do RNAm de CYP19A1, comumente conhecida como aromatase, começou a aumentar nos embriões do dia 12 (WALTERS et al., 2000), da mesma forma que a CYP17A1, uma enzima citocromo P450 atua sobre a pregnenolona e a progesterona (KLEIN; TROEDSSON, 2011a). No dia 12 o endométrio de éguas prenhes apresenta um aumento na transcrição do gene FGF9 (MERKL et al., 2010), um importante mediador da sinalização do estradiol (E_2), descrito como um fator de crescimento endometrial do estroma autócrino, induzido pelo E_2 em humanos (TSAI et al., 2002).

O E_2 e a progesterona (P_4) são hormônios derivados do colesterol que mediam mudanças nos tecidos reprodutivos da égua durante o ciclo estral e a prenhez inicial (GINTHER, 1979). As ações destes hormônios estão mediadas por receptores situados nos núcleos celulares (CLARK et al., 1987). Os hormônios esteroides atravessam o citoplasma, unem-se a seus receptores (receptor de E_2-RE- e receptor de P_4-RP-), e estes complexos ligante-receptor servem como fatores de transcrição que interatuam com o ácido desoxirribonucleico (DNA) diretamente para regular a expressão gênica, regulando o crescimento e a diferenciação endometrial (modelo "clássico" de ativação/ via genômica (CLARK; PECK; MARKAVERICH, 1987; AUPPERLE et al., 2000; HARTT et al., 2005).

As concentrações de E_2 aumentam 14 a 16 dias após a ovulação, no fim do diestro e início do estro, apresentando seus níveis máximos 1 a 2 dias antes da ovulação (GINTHER, 1979). No ciclo estral, o E_2 é responsável por mudanças físicas, endócrinas e comportamentais que colaboram no relaxamento da cérvice, formação do edema uterino, estimulação da secreção de LH e sinais externos de cío (MCKINNON et al., 2011).

Os estrógenos são produzidos pelo embrião antes da implantação sobre o dia 10 (ZAVY et al., 1978; FLOOD; JONG; BETTERIDGE, 1979). Os estrógenos estão envolvidos na migração do embrião pelo útero, mudanças na tonicidade uterina, vascularização e atividade secretora endometrial antes da fixação do embrião (MERKL et al., 2010; STOUT; ALLEN, 2001a). Além disto, estão relacionadas com a produção de proteínas glandulares da prenhez (WILSHER; KÖLLING; ALLEN, 2006).

O E_2 também promove a angiogênese uterina pelo estímulo e migração de fatores angiogênicos e das células endoteliais (LOSORDO; ISNER, 2001; ALBRECHT et al., 2003), de modo que a produção de estradiol, pelos embriões equinos, pode estar relacionada com o aumento da vascularização uterina na prenhez inicial (KLEIN, 2016). Acredita-se que o estradiol possa estar envolvido com o sinal do reconhecimento materno (ALLEN, 2000; WILSHER; GOWER; ALLEN, 2011). A administração sistêmica ou intrauterina de estrógenos na égua induz uma alteração na vida do corpo lúteo, pela estimulação de ocitocina (GOFF et al., 1993; GOFF; SIROIS; PONTBRIAND, 1993; WILSHER; GOWER; ALLEN, 2011).

Aparentemente o E_2 pode estar envolvido no estimulo da liberação de $PGF_{2\alpha}$ (STOUT; ALLEN, 2001b, 2002). Assim, foram analisadas amostras endometriais de éguas prenhes com menores níveis de RNAm para o RER1 nos dias do reconhecimento materno, que em éguas cíclicas (KLEIN; TROEDSSON, 2011b) sugerindo que essa diminuição do RNAm de receptores de $E_{2\text{-}\alpha}$ é feita pelos estrógenos embrionários. O E_2 também exerce efeito direto sobre o embrião, já que o RNAm do receptor de estrógeno beta foi detectado nos embriões nos dias 10, 12 e 14, bem como neste último dia foi localizado o receptor no trofectoderma, sugerindo a possibilidade de que estes ajudem no desenvolvimento embrionário de forma autócrina - parácrina (RAMBAGS et al., 2008).

A P_4 é sintetizada pelo corpo lúteo, e é o principal hormônio da gestação, responsável por uma série de mudanças no endométrio, necessárias para o desenvolvimento e sobrevivência do embrião (ASHWORTH, 1995; AURICH; BUDIK, 2015). A P_4 tem efeitos indiretos sobre o embrião mediante a produção de histotrofo e diretos pela presença de receptores de P_4. Rambags (2008) determinou a expressão de RNAm de receptores de membrana (progesterone membrane receptor component 1 -PGRMC1 e mPR) e de receptores nucleares nos dias 7, 10 e 14, permitindo ao hormônio esteróide atuar por vias rápidas, diferentes à clássica genômica (FALKENSTEIN; NORMAN; WEHLING, 2000; RAMBAGS et al., 2008). Os níveis máximos do RNAm de PGRMC1 também foram encontrados no endométrio no 8º dia do ciclo (GEBHARDT et al., 2012) e receptores intranucleares diminuíam ao longo dos dias (RAMBAGS et al., 2008). O receptor de membrana de

progestina (PAQR5) é outro dos receptores que mediam efeitos não genômicos da P_4 e ainda é capaz de se unir à 17-alfa-OH-progesterona, um dos metabólitos esteroides produzidos pelo embrião equino no dia 12 (SMITH et al., 2008; MERKL et al., 2010).

A progesterona induz um aumento na densidade glandular, mudanças no epitélio luminal, nas secreções intra-glandulares (KEENAN et al., 1991). Além disto, a P_4 diminui a migração de polimorfonucleares e mononucleares no dia 7 após a ovulação (ACUÑA; FUMOSO, 2013), mesmo efeito observado em éguas ovariectomizadas tratadas com P_4 (WATSON; STOKES; BOURNE, 1987). Por outro lado, a progesterona regula a expressão de diversas proteínas do fluido uterino, uma delas é a uterocalina, uteroglobina e uteroferrina (BEIER-HELLWIG et al., 1995; CROSSETT et al., 1998; HOFFMANN et al., 2009).

2.3.3.3 Fluído uterino e histotrofo

O endométrio e o embrião expressam uma ampla gama de transportadores de soluto (genes SLC) transportadores de nutrientes, tais como ácidos e íons amino. (MERKL et al., 2010; KLEIN; TROEDSSON, 2011b). O embrião expressa várias apolipoproteínas que facilitam o transporte dos lipídios. Antes da implantação, o concepto é mantido unicamente pelas secreções que se acumulam no lúmen uterino, um fenômeno conhecido como nutrição histotrófica (ASHWORTH, 1995). Na égua, a implantação ocorre sobre o dia 36 (GINTHER, 1998), muito mais tarde que em outras espécies (BAZER et al., 2009).

No fluido uterino de éguas cíclicas e prenhas nos dias 7, 10 e 13 foram observadas diferenças de abundância em 19 proteínas. As proteínas identificadas foram associadas com a nutrição histotrofica (uterocalina e albumina), metabolismo e produção de energia (isocitrato desidrogenase e enolase 1), estimulo de E_2 (creatinina kinase tipo-B, proteína de união a vitamina D), imuno-tolerância materna e metabolismo COX (haptoglobina e fibrinogênio B), vascularização e angiogênese (haptoglobina e proteína de canais intracelulares de cloro), estrutura e movimentação (actina citoplasmática 1 e proteína protetora de actina X),

crescimento, desenvolvimento e apoptose (HSPA8 e HSP90) (BASTOS et al., 2019).

Klein e Troedsson (2011a) observaram expressão reduzida de osteopontina (RNAm) (gene SPP1) em embriões do dia 14 em comparação com os do dia 8, proteína de origem extracelular que se une às integrinas para promover a união e a comunicação célula-célula, sugerindo que este sistema pode contribuir ao prolongado período de pré-implantação nesta espécie. Por outro lado, foi determinado que a intensidade de coloração de receptores de osteopontina no endométrio equino é superior nas amostras obtidas nos dias 21 e 27 quando comparadas com a dos dias 14 e 17 de prenhez (AL-RAMADAN et al., 2002).

Considerando o longo período pré-implantação, o histotrofo é particularmente importante na espécie equina (REILAS, 2001). As principais proteínas encontradas no fluído uterino são a uteroglobina (UGL), a uteroferrina (UF) e uma das maiores proteínas, secretada unicamente pelo endométrio da égua, a uterocalina (UCA ou proteína P19) (BEIER-HELLWIG et al., 1995; HOFFMANN et al., 2009).

Grandes quantidades de UCA são secretadas no lúmen uterino da égua durante o ciclo estral e a prenhez inicial. A detecção mais precoce da mesma se evidencia em biópsias uterinas coletadas dois dias pós-ovulação, e a coloração revelou que se encontrava nas células epiteliais glandulares. A UCA se associa fortemente com a cápsula acelular que circunda o concepto (CROSSETT et al., 1998). O RNAm para UCA diminui à medida que o concepto se desenvolve; possui quantidades moderadas em conceptos do dia 8 e diminui nos embriões dos dias 10, 12 e 14. A diminuição em sua expressão poderia ser a consequência das grandes quantidades de UCA uterinas proporcionadas ao concepto (KLEIN; TROEDSSON, 2011b). Isto coincide com os achados de Merkl et al. (2010) que encontraram altos níveis endometriais de RNAm para UCA no dia 12 e Bastos (2017) que determinou um comportamento decrescente de UCA no liquido endometrial em éguas prenhes entre os dias 7, 10 e 13.

A UCA está envolvida no transporte de lipídios de importância biológica como ácidos graxos poli-insaturados e retinol através da cápsula (CROSSETT et al., 1998). A expressão desta proteína se correlaciona com as concentrações séricas

de P_4 e inclusive pode ser estimulada mediante a administração exógena de P_4 em éguas no anestro (CROSSETT et al., 1998; STEWART; KENNEDY; SUIRE, 2000; HOFFMANN et al., 2009). A UGL e UF também são dependentes da P_4 (BEIER-HELLWIG et al., 1995; HOFFMANN et al., 2009).

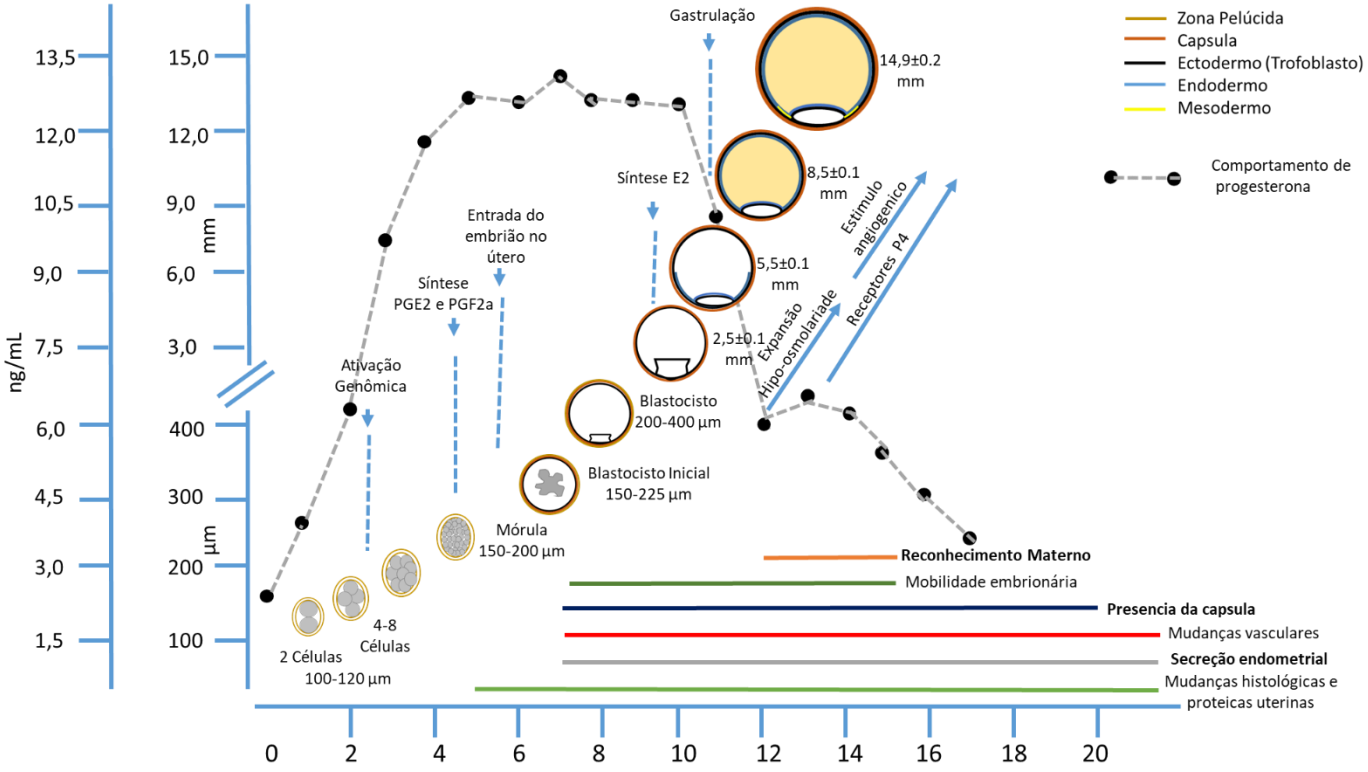

Fig. 2 Esquematização dos processos sofridos na comunicação materno embrionária equina na prenhez inicial. Fonte: Autor.

2.3.4 Sistema imunológico materno e o embrião

Existe uma baixa regulação de genes envolvidos na estimulação do sistema imune (BRCA1 e FGF2), sugerindo que o concepto é protegido do sistema imune da égua (KLEIN; TROEDSSON, 2011b). A alta regulação de fibrinogênio α, β, e γ ainda é motivo de estudo, no entanto, foi sugerido que o sistema de plasminogênio de baixa regulação controla e evita a erosão do endométrio na prenhez equina (KLEIN; TROEDSSON, 2011a).

Além disto, foi desenvolvida pesquisa com interferons. Os interferons são citocinas dos vertebrados com muitos efeitos, como: antivirais, imunoregulatorios e atividade anti-proliferativa. Os interferons são agrupados em 10 diferentes famílias α, β, ω, δ, ε, αω, ν, τ, κ e ζ. Alguns tipos de interferons são secretados pelos embriões na prenhez inicial, este é o caso dos embriões bovinos com o interferon-τ que é o sinal do reconhecimento materno para essa espécie. A expressão de interferon α-1, ômega 1 e 2 foi avaliada em embriões equinos nos dias 13, 15, 20 e 25, sem apresentar expressão nestes dias (BAKER; ADAMS; MCDOWELL, 1991).

Expressão de dois genes de interferon-delta foram identificados no tecido do concepto equino nos dias 16 e 22 (COCHET; VAIMAN; LEFÈVRE, 2009). Além disto, níveis crescentes de expressão de RNA mensageiro (mRNA) para interferon alfa-1 foram detectados em conceptos equinos nos dias 10, 12 e 14 (KLEIN; TROEDSSON, 2011b).

2.3.5 Sistemas Complementar Exossomal

Os exossomas são microvesículas de origem endocítica, secretadas pelas células, que contém mRNA, microRNA (miRNA) e proteínas, que podem levar tecido ou material celular especifico. Os exossomas podem mediar a comunicação intercelular em processos fisiológicos, como a apresentação de antígenos, tendo sido demostrado que os RNA secretados através de exossomos são transferíveis para outras células (VALADI et al., 2007). Os exossomas têm uma importância especial na predição do status reprodutivo na mulher, porque foi comprovada sua liberação via sanguínea e o transporte de miRNAs (LUO et al., 2009). Em soro sanguíneo de éguas prenhes e não prenhes, foram isolados e determinadas diferenças nos conteúdos de micro RNA de exossomas durante os dias do RMP, com os miRNA identificados foram associados com moléculas focais de adesão (CAMERON, 2012). Foi determinado que as moléculas focais de adesão se encontram no epitélio glandular, sugerindo que as mesmas são afetadas pelo embrião e estão envolvidas no RMP na égua (KLOHONATZ et al., 2016).

2.3.6 Angiogênese e remodelação vascular

A vascularização pode ser formada por dois diferentes mecanismos, vasculogenese ou angiogênese. A vasculogenese é o processo de formação de vasos sanguíneos novos de células mesodermais precursoras, onde não há vasos pré-existentes, ocorrendo principalmente no desenvolvimento fetal, evidenciando-se inicialmente no saco vitelino (desenvolvimento cardiovascular embrionário) e no adulto em lesões isquêmicas (TAKAGI et al., 1998; CONWAY; COLLEN; CARMELIET, 2001). Angiogênese é o processo onde se desenvolvem novos vasos sanguíneos e remodelação dos preexistentes. O processo angiogênico acontece normalmente em patologias (tumores, artrite reumatoide, inflamação crônica) ou em condições fisiológicas especiais (osso em fraturas, formação do corpo lúteo, crescimento endometrial, implantação embrionária e placentação) (CHARNOCK-JONES; KAUFMANN; MAYHEW, 2004).

Os processos de regulação da vasculogênese ou angiogênese estão relacionados com as proteínas de Fator Induzido por Hipóxia (HIF-1), Fator de crescimento endotelial vascular (VEGF) e o fator de crescimento de fibroblastos (FGFs). A presença de VEGF, VEGFR1, VEGFR2 e Ki-67 no endométrio equino foi determinada apresentando mudanças no padrão de coloração dos VEGF e Ki-67 entre éguas prenhes e éguas cíclicas (SILVA et al., 2011).

Por outra parte, utilizando ultrassonografia doppler em éguas prenhes, determinou-se um aumento da vascularização do dia 12 a 15 em ambos cornos uterinos comparado com éguas sem embrião, incrementando a perfusão e os pixels em éguas prenhes no corno com embrião. Aparentemente, o endométrio sofre mudanças locais transitórias na perfusão vascular em presença da vesícula embrionária no período prévio à fixação (SILVA et al., 2005). Após a fixação, a perfusão se mantem significativamente mais elevada no corno uterino gravídico, talvez como resultado dos estrógenos e prostaglandinas secretadas pelo embrião. O concepto desempenha um papel ativo na vasculogênese uterina, favorecendo a chegada de nutrientes importantes após a fixação (SILVA et al., 2011).

2.3.7 Transferência de embriões assíncrona e inoculação de substâncias e dispositivos no útero

O desenvolvimento do embrião equino em suas primeiras semanas depende da manutenção da função lútea e do ambiente uterino. A forma esférica do embrião equino, junto com o rápido aumento das concentrações séricas de progesterona pós-ovulação no soro e a implantação tardia possivelmente contribuem para uma maior tolerância da assíncronia embrião-útero (WILSHER; ALLEN, 2009; WILSHER; LEFRANC; ALLEN, 2012). O tempo de contato com a progesterona também foi associado com sobrevivência dos embriões assíncronos transferidos, devido ao que a progesterona estimula a produção de histotrofo e o crescimento exponencial do embrião (NEWCOMBE; CUERVO-ARANGO, 2008; WILSHER; ALLEN, 2009).

Na transferência de embriões assíncronos foi demonstrado que o crescimento embrionário é dependente do estado do útero. Embriões de 10 dias transferidos a receptoras no dia 3 apresentaram crescimento lento em comparação com transferências síncronas (WILSHER et al., 2010; WILSHER; ALLEN, 2016). foi demonstrado que o embrião assíncrono pode evitar a luteólise, provavelmente porque o sinal MRP é secretado por um período de tempo maior (WILSHER; ALLEN, 2009; WILSHER; LEFRANC; ALLEN, 2012).

Assim mesmo o uso de dispositivos intrauterinos para a prolongação de função lútea tem sido estudada determinando uma expressão significativa de uteroferrina nas éguas submetidas ao dispositivo em comparação com as prenhes (KLEIN et al., 2016).

2.3.8 Perda embrionária e Manutenção do corpo lúteo

A perda embrionária tem uma alta prevalência na indústria equina sendo definida como as perdas de prenhez entre a fecundação e os 40 dias gestação (BALL, 1988). O intervalo de tempo com a maior perda embrionária é entre os dias 11-15 após ovulação, onde 18,3% das éguas diagnosticadas como prenhez no dia

11 não tinham evidencia de embrião no dia 15 por ultrassom; 29% das perdas vão a presentar pseudoprenhez atribuído aos vasos embrionários e o sinal do reconhecimento materno presente ainda no útero da égua (GINTHER et al., 1985). A perda embrionária inicial representa 20% das perdas em éguas férteis e 70% das perdas em subférteis (CARNEVALE et al., 2000).

A causa da perda embrionária é multifatorial e frequentemente idiopática, embora existam algumas causas conhecidas (CARNEVALE et al., 2000). Muitos fatores são identificados como causas de perda embrionária, mas as perdas embrionárias que acontecem antes do dia 15 após ovulação são associadas com falhas na sinalização do reconhecimento materno (GINTHER et al., 1985) ou com a luteólise secundária por endometrite (maior causa de perda embrionária neste período) (SQUIRES; GARCIA; GINTHER, 1985; CARNEVALE et al., 2000).

A disfunção primária de corpo lúteo não produz perda embrionária inicial nas éguas, devido a que os níveis de progesterona são similares em éguas prenhas e nas que aconteceram a perda embrionária. Portanto, as concentrações de progesterona mudaram só depois da perda embrionária precoce, o que significa que a queda de progesterona é um efeito, não uma causa, da perda embrionária precoce (PAPA et al., 1998). Outra hipótese é que como o concepto está desencadeando a contratilidade uterina pela PGF, pode estimular muita secreção do hormônio, levando a entrada de PGF na circulação e iniciando a luteólise (ALLEN; STEWART, 2001). Outros fatores de perda embrionária incluem desalinhamento acidental de cromossomos no momento da fecundação, obstrução da mobilidade embrionária pela presença de cistos endometriais (BRACHER; ALLEN, 1992), nutrição da égua, condição corporal, garanhão, anormalidades cromossômicas, deficiências hormonais, estresse, fatores imunes (CARNEVALE et al., 2000), produção inadequada de histotrófo e alterações degenerativas nas glândulas endometriais relacionadas à idade (KENNEY; DOIG, 1986).

2.3.9 Fragmentos embrionários

Tecidos embrionárias homogeneizados (ROWSON; MOOR, 1967), células trofoblasticas (HEYMAN et al., 1987) ou vesículas trofoblasticas sem disco embrionário (HEYMAN et al., 1984) foram depositados no útero de ovelhas e vacas como modelo experimental, tentando melhorar o entendimento do controle antiluteolitico, reconhecimento materno da prenhez, manutenção da prenhez e melhoramento das taxas de prenhez por transferência de embriões. Em equinos, sistemas de cultura de monocamadas com células trofoblasticas e células epiteliais endometriais equinas foram usadas para examinar os eventos associados com o reconhecimento materno da prenhez, demostrando uma comunicação célula a célula (BRADY et al., 1993).

3. ARTIGO 1 SUBMETIDO O JOURNAL OF EQUINE VETERINARY SCIENCE

Histomorphometric and vascular changes in the endometrium after the infusion of dead conceptus fragments

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Abstract

The aim of this experiment was to evaluate ultra-structural, histological and vascular changes of the equine endometrium at day 7 after ovulation in mares in which fragments from day 13 conceptus were infused at day 5 after ovulation. Ten healthy cyclic mares were used. Once estrus was confirmed mares were examined daily to detect ovulation (day 0). After ovulation, mares were examined daily until day 7 by transrectal palpation and B-mode and Doppler ultrasonography. In the first cycle, intrauterine biopsies were collected at day 7 after ovulation, constituting the Cyclic group (*n* = 10). In the second cycle, the same mares were examined daily until ovulation was detected. After ovulation, mares were examined daily by transrectal palpation and, B-mode and Doppler ultrasonography, until day 7. At day 5 after ovulation, fragments from previously collected 13 days old concepti were

infused into the uterus of each mare. Intrauterine biopsies were collected at day 7 in all mares (*n* = 10), constituting the Fragment group. A decrease in the percentage of ciliated and flattened cells was observed in Fragment group in relation to the Cyclic group. Nevertheless, protruded cells, superficial and intraglandular secretion, glandular lumen and diameter, blood vessel diameter, endometrial vascularization and immune cells were higher in Fragment group than in Cyclic group. In summary, equine conceptus fragments infused at day 5 after ovulation signalized histological and vascular changes in the endometrium at the 7th day after ovulation.
Key words: ultrastructure, histological, vascularization, uterus.

1. Introduction

The uterus is a dynamic physiological system in which cellular proliferation and differentiation occurs during pregnancy (DAS, 2009). A swift endometrial adaptation occurs when the embryo enters the uterus. Ultra-structural and histological changes of the endometrium were reported in pregnant mares from the 7th day after ovulation, compared to cyclic mares (CAMOZZATO et al., 2019). In pregnant mares, the number of leukocytes also increased, being associated with a local immunological response (KEENAN et al., 1987). Embryo presence was associated with a rise of blood flow and vascular perfusion from the uterine artery at the 11th day after ovulation (BOLLWEIN; MAYER; STOLLA, 2003) and to local vascular changes related to embryo localization (SILVA et al., 2005).

The horse is one of the few domestic species in which the conceptus-derived pregnancy recognition signal has not been identified (KLEIN; TROEDSSON, 2011a). Persistence of the corpus luteum is seen in a certain percentage of non-pregnant mares after introduction of a glass marble (NIE et al., 2003) or fluid-filled rubber ball (RIVERA DEL ALAMO et al., 2008) into the uterine lumen in the first days after ovulation. Concepti obtained before day 14 after ovulation release a characteristic pattern of proteins, and probably one or more of these proteins may be involved in the antiluteolytic mechanism (MCDOWELL et al., 1990), which prolongs the lifespan of the corpus luteum. These results suggest that the embryonic signal for maternal

recognition of pregnancy in the horse might be at least in part secretory rather than mechanical (AURICH; BUDIK, 2015).

Conceptus fragments have been used as study models to improve our understanding of anti-luteolytic control, maternal pregnancy recognition, pregnancy maintenance, and the maintenance of corpus luteum lifespan in mares (BALL; ALTSCHUL; HILLMAN, 1989) and in ruminants (ROWSON; MOOR, 1967, CAMOUS et al., 1984; HEYMAN et al., 1984;1987). Culture systems with trophoblastic vesicles and monolayers of equine uterine epithelial cells have been used to examine events associated with maternal recognition of pregnancy in the mare, determining a cell-cell communication (BRADY et al., 1993).

It is hypothesized that proteins present in fragments of a 13th day dead conceptus infused into the equine uterus 5 days after ovulation will induce changes in the equine endometrial ultra-structure, histology and vascularization, that can be detected at day 7 after ovulation. The aim of this experiment was to verify the above mentioned ultra-structural, histological and vascular changes of the equine endometrium at day 7 after ovulation, using cyclic mares not infused with concepti fragments as controls.

2. Material and Methods

2.1 Animals

The study was performed in the southern hemisphere breeding season. Ten healthy cyclic Quarter Horse type mares (mean age 6.8, ranging between 4 to 10 years old), and weighting 450-550 kg were used. Mares were kept in natural pastures with free access to mineral supplementation and water. Throughout the experiment, mares remained healthy and with an average body condition score of 3.5 (scale 1 to 5) (MALSCHITZKY et al., 2001). This study was carried out with a protocol approved by the Animal Ethical Use Committee from Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil (protocol number 34572).

Mares were examined for reproductive soundness by evaluation of perineal conformation, transrectal palpation, ultrasound of the genital tract (Sonoscape S8V, China) and endometrial biopsy. Only cyclic and clinically normal mares, with endometrium classified as category I or IIA (KENNEY; DOIG, 1986), without evidence of endometritis were selected.

2.2 Experimental Design

Mare's reproductive tracts were routinely examined by transrectal palpation and B-mode ultrasonography until estrus was detected. Once estrus was confirmed (ovarian follicle > 35 mm in diameter and marked uterine edema), mares were examined daily to detect ovulation, considered day 0. After ovulation, mares were examined daily by transrectal palpation, B-mode and Doppler ultrasonography, until day 7. In this first cycle, endometrial biopsies were collected at day 7 after ovulation in all mares (*n* = 10). This constituted the Cyclic group (CG).

In the second cycle the same mares were examined daily until ovulation was detected (day 0). After ovulation, the mares were examined daily by transrectal palpation, B-mode and Doppler ultrasonography, until day 7. At day 5 after ovulation, fragments from dead concepti recovered previously from pregnant mares at day 13 post ovulation, were infused into the uterus to each mare. Intrauterine biopsies were collected at day 7 in all mares (*n* = 10). This constituted the Fragment group (FG).

2.3 Conceptus Fragments

Conceptus fragments were obtained from previous embryo collections performed at day 13 after ovulation, snap frozen with 2.5 mL Ringer solution and stored in liquid N_2. Before their infusion in the FG mares, concepti were thawed at room temperature. Cryopreservation in temperatures below -79˚C and the thawing process used keep the proteins active (GARBER COHEN; CASTELLO; FLECHA, 2010). Thawed dead conceptuses were divided equally in two parts, and infused to the mares (*n* = 10), each mare receiving half an embryo suspension. Infusion of the

fragments was carefully performed at day 5 after ovulation with a nonsurgical trans-cervical procedure, using a standard artificial insemination pipette, protected with a sterile outer chemise. Dead conceptus fragments (suspended in 2 mL) were deposited into the uterine body.

2.4 Endometrial Biopsies

Two biopsies were obtained per mare at day 7 after ovulation, one from the dorsal wall of each uterine horn, close to the bifurcation, at each cycle. Each biopsy sample from the Cyclic (*n* = 20) and Fragment (*n* = 20) groups was bisected and stored separately in 5 mL tubes containing 2.5% glutaraldehyde solution (0.01 mol/L phosphate buffer, pH 7.3) for scanning electron microscopy studies, and 4% buffered paraformaldehyde solution used for light microscopy and morphometry studies.

2.5 Scanning Electron Microscopy

Scanning Electron Microscopy (SEM) gives a three-dimensional aspect of tissue surface ultrastructure, exhibiting the epithelium of the uterus which is folded into long ridges and troughs. The samples for SEM were dehydrated through a graded series of acetone. The tissue was dried in a critical-point drier (CPD030 – Balzers) using carbon dioxide. The dried pieces were attached to stubs with double-sided adhesive tape and were sputter-coated with gold/palladium (Sputter Coater SCD050 – Balzers). Samples were scanned and photographed with a JEOL (JSM 6060) digital scanning electron microscope. For image analysis, five pre-defined squares were superimposed to SEM images. For each mare in each group, five images of each horn were evaluated, totalizing 100 areas/group. Ciliated cells, polygonal microvilli secretory cells (protruded and flattened) and apical blebs were counted, and the percentage of area occupied by the different cells and by the superficial secretion was calculated. Quantification was performed using ImageJ analyzing software (ImageJ, National Institutes of Health, Maryland, USA).

2.6 Light Microscopy

Light microscopy provides transverse images of the tissue, showing structures in the *stratum compactum* and *spongiosum* of endometrium, including luminal epithelium, endometrial glands, stroma and blood vessels. Samples conserved in paraformaldehyde were processed to be included in paraffin. Paraffin blocks were cut with an automated microtome (Leica, RM165) at 5 µm thickness, adhered in histology slides and kept on a 60ºC incubator. After deparaffinization, cuts were stained using routine techniques for tissue samples (TOLOSA et al., 2003), using H-E. All sample slides were analyzed by an experienced pathologist blinded from any information, under light microscopy.

The morphological features were photo-documented using an optic microscope (DM500, Leica Microsystems GmbH, Germany), with an attached capture camera (ICC500 HD, Leica Microsystems GmbH, Germany), and an image acquisition software (LAS EZ, Leica Microsystems GmbH, Germany). Measurements and counting were done using the ImageJ image analyzing software (ImageJ, National Institutes of Health, Maryland, USA).

The following measures were performed in both uterine horns:

a) Intraglandular secretion: quantified using the mean of the two largest diameters of the secretion inside 10 randomly selected spherical glands at 400x magnification from each horn (*n* = 20/mare).

b) Glandular diameter: obtained using the mean of two perpendicular diameters of each gland (from a basement membrane to the opposite one). Measured in 10 randomly selected spherical *stratum spongiosum* glands at 400x magnification from each horn (*n* = 20/mare).

c) Glandular lumen: measured in the same way as the previous variable, measuring the space between the apical membranes of the epithelial cells, recorded in 10 randomly selected spherical *stratum spongiosum* glands at 400x magnification from each horn (*n* = 20/mare).

d) Glandular density: 3 fields from each horn (*n* = 6/mare) were observed at 100x magnification to evaluate glandular density in both the *stratum spongiosum* and the *stratum compactum;* counting the glands per stratum in each field.

e) Height of the glandular epithelium: measured from the basal lamina of the cells to the apical membrane of the cells recorded at 400x magnification in 10 randomly selected spherical *stratum spongiosum* glands from each horn (*n* = 20/mare).

f) Height of the luminal epithelium: measured from the basal lamina to the apical membrane of the cells, recorded at 400x magnification in five randomly selected fields from each horn (*n* = 10/mare).

g) Immune cells: lymphocytes, eosinophils, and neutrophils counted in five fields from each horn (*n* = 10/mare) at 1000x magnification in the *stratum spongiosum* and the *stratum compactum.*

h) Blood vessel diameter: measured in five fields from each horn (*n* = 10/mare) at 1000x magnification. Total blood vessel area was calculated multiplying the vessel diameter by the number of vessels observed in the studied field.

An average of the records of each variable for each uterine horn was calculated.

2.7 Doppler uterine hemodynamics

The analysis of hemodynamics was performed using a Doppler ultrasound (SonoScape® model S8V) with a transrectal linear probe (5-10 mHZ) in Power Doppler and Spectral Doppler modes, using a frequency of 7.2 mHZ, a filter of 100 HZ and 5.5 cm/s of flow detection.

2.7.1 Power Doppler

Vascular perfusion of the endometrium was evaluated using the Power Doppler mode. The transducer was placed over the uterine body and over a cross-section of the middle segment of each uterine horn (Fig. 3). Vascular perfusion was

estimated subjectively and objectively. The subjective evaluation scored the extent of colored areas in the endometrium for 30 seconds, videos were recorded as Windows Media Video (WMV). The score ranged from 1 to 4, being 1 absence of perfusion, and 4 maximal perfusion (SILVA et al., 2005).

The objective evaluation was performed by off-line measurement of the area and intensity (pixels) of blood flow of the endometrium, to give a quantitative measure of the extent of perfusion and relative velocity (GINTHER, 2007). The above-mentioned videos were transformed and saved as GIF image using Total Video Converter 3.11 (Copyright© EffectMatrix Ltd. Company from 2002 - 2016). From each group five still images from cross-sections of the middle segment of each horn and uterine body from each mare were used for determination of area and intensity, and the average was used in the analyses. The measurement of area and intensity were performed with ImageJ 1.31v software (National Institutes of Health, Maryland, USA) (SILVA et al., 2005).

2.7.2 Spectral Doppler

Both the left and right uterine arteries were examined transrectally as previously described (BOLLWEIN et al., 1998), in all the mares in each group. The transrectal pulsed Doppler ultrasound examinations were always performed once daily between 07:00 and 10:00 h and lasted about 20 min for each mare.

All blood flow velocity waveforms were displayed on-line and saved. Following the collection of all data, the Doppler calculations were performed off-line by using three similar consecutive flow velocity waveforms with maximum end-diastolic frequency shift. Analysis was based on the envelope of the Doppler shift spectrum.

The following parameters were measured, in the Spectral Doppler:

a) Time-averaged maximum velocity (TAMV): Average of the maximum velocity values (upper surface of spectrum) over the time of a cardiac cycle.

b) Pulsatility index (PI): Expression of the extent of the difference between the peak systolic velocity and end diastolic velocity in the Time-averaged maximum velocity, giving a negative relationship between the extent of pulsatility in the tissues and the extent of the vascular perfusion. Increasing PI values indicate decreasing perfusion of the distal tissues.

c) Resistance index (RI): Expression of the extent of the difference between the peak systolic velocity and end diastolic velocity in the peak systolic velocity, giving a negative relationship between the extent of resistance in the tissues and the extent of the vascular perfusion. Decreasing RI values indicate increasing blood perfusion.

Changes of the RI and PI are highly correlated. Data was collected with the Software of the Doppler ultrasound (SonoScape® model S8V).

2.8 Statistical Analysis

Variables were evaluated using the Statistical Analysis System (SAS, Cary, NC, USA). Percentage of area occupied by ciliated cells, polygonal microvilli secretory cells (protruded and flattened), apical blebs, superficial secretion, glandular diameter, glandular lumen, glandular density, height of glandular and luminal epithelium, diameter of endometrial blood vessels, total blood vessel area, immune cells, subjective vascular perfusion, objective area and intensity of blood-flow, TAMV, RI and PI, were considered as dependent variables. Groups (Cyclic or Fragment) and uterine horn (ipsi or contralateral to corpus luteum) were considered as independent variables.

Variables were evaluated for normality using the PROC UNIVARIATE procedure, those not meeting normal distribution were transformed using natural logarithm and those still not meeting normality by this method were evaluated by non-parametric statistics.

Variables with normal distribution were analyzed using the PROC GLM procedure, which evaluates non-balanced variables, testing for interactions between treatment (Cyclic or Fragment), and if these had a statistical effect on the variables. Means were evaluated using Tukey's test, using the LSMEANS procedure. Variables

not meeting normal distribution were analyzed by the PROC NPAR1WAY procedure, to evaluate the means by Wilcoxon and Kruskal-Wallis tests. Data are presented as means ± standard deviation. Differences $P \leq 0.05$ were considered significant. Differences > 0.05 and ≤ 0.09 were considered tendency. Interactions were evaluated between horns, arteries and days.

3. Results

Biopsies from the 10 mares were classified as Category I (70%) and as Category IIA (30%). The percentages of ciliated, secretory protruded and flattened cells, apical blebs, superficial secretion, intraglandular secretion, glandular diameter, glandular lumen, glandular density and height of glandular epithelium did not differ between the contralateral horn to the corpus luteum and the ipsilateral horn ($P > 0.05$).

The percentage of ciliated cells per field showed a significant decrease ($P = 0.001$) in the Fragment group (11.0 ± 8%) in comparison with Cyclic group (33.7 ± 17%) (Fig. 4A, 4E, 4F). However, the percentage of protruded cells was higher ($P = 0.002$) in the Fragment group (76.9 ± 13 %) than in the Cyclic group (32.9 ± 14%) (Fig. 4B, 4E, 4F). In the infused mares, the percentage of flattened cells decreased ($P = 0.001$) (11.3 ± 9%) compared with cyclic mares (32.9 ± 17%) (Fig. 4C, 4E, 4F). No difference was detected in apical blebs ($P = 0.416$) between Cyclic and Fragment group (Fig. 4D).

In the cyclic mares, superficial secretion occupied a lower endometrial area per field (0.2 ± 0.1%) whereas in the infused mares (0.3 ± 0.1%), secretion was more abundant ($P = 0.003$) (Fig. 5A, 5C, 5D). Likewise in the Cyclic group, intraglandular secretion (1.3 ± 0.4 µm) was less abundant than in the Fragment group (3.2 ± 0.7 µm) ($P < 0.001$) (Fig.5B, 5E, 5F).

A higher glandular diameter was observed in Fragment group (50.4 ± 4 µm) than in Cyclic group (41.9 ± 4 µm) ($P < 0.001$) (Fig. 6A, 6C, 6D). Glandular lumen increased in the group infused with conceptus fragments (18.9 ± 3 µm), in comparison with the Cyclic group (9.4 ± 2 µm) ($P < 0.001$) (Fig. 6B, 6C, 6D).

Glandular density did not differ between groups (*P* = 0.291), neither in the *stratum compactum,* nor in the *stratum spongiosum.* The height of glandular epithelium and luminal epithelium were similar between Fragment and Cyclic groups (*P* = 0.501 and *P* = 0.099 respectively).

Number of counted lymphocytes was higher (*P* < 0.001) in Fragment group (8.3 ± 2 cells/field) than in Cyclic group (3.4 ± 1 cells/field) (Fig. 7A). Similarly, the number of eosinophils and neutrophils was higher (*P* < 0.001) in the Fragment group (1.5 ± 1.0 cells/field and 0.3 ± 0.3 cells/field respectively) in comparison with the Cyclic group (0.2 ± 0.1 cells/field and 0.04 ± 0.04 cells/field respectively) (Fig. 7B, 7C). Presence of immune cells was similar in *stratum compactum* and *stratum spongiosum*. There was neither influence nor interaction between horn and treatment.

Histological analysis at the 7th day after ovulation showed endometrial blood vessels with higher (*P* = 0.022) diameter in the Fragment group (18.5 ± 3 μm) than in the Cyclic group (15.9 ± 3 μm) (Fig. 8A).

On day 6 after ovulation, one day after the infusion of the conceptus fragments, the blood-flow area tended (*P* = 0.06) to rise in Fragment group in comparison to the Cyclic group (Fig. 8B). At day 6, Fragment group also presented higher blood flow intensity in the objective evaluation (*P* = 0.038) (Fig. 8C) and larger extent of colored areas in the subjective evaluation (*P* = 0.048) (Fig. 8D) than the Cyclic group. At day 7, no differences (*P* > 0.05) were observed in blood flow evaluated by objective and subjective methods between Fragment and Cyclic groups. Objective and subjective measurments of uterine vascularization did not differ between the uterine body, the contralateral horn to the corpus luteum and the ipsilateral horn (*P* > 0.05).

The RI, PI and TAMV values measured by Spectral Doppler for the two uterine arteries between the days 0 to 7 after ovulation, did not show differences between groups (*P* > 0.05), neither interactions (*P* = 0.722) between arteries and group.

4. Discussion

Proper utero-embryonic communication is essential for the development and maintenance of pregnancy, this crosstalk is orchestrated by a complex network of humoral interactions with local and systemic implications. In the present study, rapid endometrial changes were evident two days after the infusion of fragments of a 13-day old equine conceptus in mares at the 5th day post ovulation.

The snap frozen 13 day old concepti were selected due to the higher content of proteins in the yolk sac (SMITS et al., 2018), before maternal recognition of pregnancy (SHARP et al., 1989). Probably, one or more of these proteins may be involved in the antiluteolytic mechanism (MCDOWELL et al., 1990). The proteins present in the conceptus tissues remain inactive with unfolded conformations during storage in low temperatures, resulting in active molecules after the thawing process (GARBER COHEN; CASTELLO; FLECHA, 2010). The days of the infusion (5th day) and of the biopsy (7th day) were selected because it is the time lapse when the peak of the serum progesterone concentration occurs and does not differ between pregnant and cyclic mares (CAMOZZATO et al., 2019).

During pregnancy, the presence of the conceptus in the uterus alters the secretion of histotroph and growth factors to create a more favorable uterine environment for early embryonic survival (WILSHER; GOWER; ALLEN, 2011). Probably the proteins contained in the concepti fragments infused in the present study, can induce these changes to produce a satisfactory milieu; and therefore proteins and transcripts produced can lead to an increase endometrial vascular perfusion (SILVA et al., 2011) and the production of histotroph from the endometrial glands (BAZER; ROBERTS; THATCHER, 1979), altering gene expression (MERKL et al., 2010).

In the present study, the cellular profiles 2 days after the infusion of the conceptus fragments differ to those observed in the endometrium of cyclic mares at 7th day of diestrus. Moreover, cellular profiles of the infused mares show similarity with to those observed in the pregnant mares at the 7th day of pregnancy: loss of ciliated cells, presence of protruded cells, existence of

histotrophic material and large glandular diameter (CAMOZZATO et al., 2019). These modifications were probably induced by the protein content of the material placed into the uterus.

The possibility of corpus luteum lysis due to cervical manipulation during the infusion procedure may be excluded. High pregnancy rates are obtained after cervical manipulation (> 70%) (CUERVO-ARANGO; CLAES; STOUT, 2018). Endometrium of mares in estrus present large number of apical blebs and the superficial cells are flattened in majority (SAMUEL et al., 1979), profiles that were not observed in the infused mares. Moreover, the presence of protruded cells, glandular secretion and large glandular diameter observed in the infused mares has not been detected in mares in estrus.

In the present study vessel diameter was higher in the infused mares in comparison to the cyclic mares. This rise in vessel diameter in the endometrial stroma is similar to the observed in pregnant mares in comparison with cyclic mares (CAMOZZATO et al., 2019). The complex regulation of angiogenesis and the vascular remodeling process is fundamental for the success or failure of the maintenance of the pregnancy (TAYADE et al., 2007). Conceptus secretory products stimulate vasodilatation and angiogenesis to increase uterine blood-flow and substrate delivery to the pregnant uterus (BAZER et al., 1986). Several studies have identified genes and proteins involved in the regulatory systems of the angiogenesis process such as the VEGF system, the angiopoietin family, different regulators of endothelial cells, and hypoxia-induced genes (MERKL et al, 2010; KLEIN; TROEDSSON, 2011a; SILVA et al., 2011; BASTOS et al 2019). This remodeling of vascularization is likely to play a role in maternal support of conceptus' growth, preparing the uterus for the prospective pregnancy and facilitate exchange of gases and nutrients (MERKL et al, 2010; KLEIN, 2016).

A rise in local vascular perfusion was detected in infused mares in contrast with cyclic mares. These increments suggest that proteins of the conceptus fragments produced changes in the endometrial vascularization at day 6, though these alterations disappeared by the 7th day, probably due to the limited amount of

protein content, denaturalization of proteins to the body temperature and the lack of the live embryo or its motility. Increment in endometrial vascular perfusion of pregnant mares has been reported since the 7th day after ovulation in relation with non-pregnant mares (NIETO-OLMEDO et al., 2018).

No differences were observed between cyclic mares and infused mares in the hemodynamics of the uterine arteries. This result probably is due to limited amount of proteins, and therefore lack a more prolonged stimulus. However, in pregnant mares conceptus promote changes in the hemodynamic of the uterine arteries starting at the 11th day after ovulation (BOLLWEIN; MAYER; STOLLA, 2003).

An increased number of immune cells was observed in the Fragment group in relation to Cyclic group. The increase of lymphocytes suggests an adaptive immunologic response from the uterus, caused by contact with proteins of the dead conceptus, where lymphocytes probably induce a immunological response and eosinophils may be involved in moderating this response (KEENAN et al., 1987; MARTÍNEZ PEREIRA, 2016). Therefore, the conceptus produces inflammatory mediators that aid in the embryonic-maternal cross talk, and avoid rejection (PEARSON, 2002; WACLAWIK, 2011; WACLAWIK et al., 2017), and immune cells are mediators of this communication.

An increase in the population of neutrophils was observed in the endometrium of the Fragment group, in relation to the Cyclic group. These endometrial changes observed in the present study are probably not associated with inflammation. Mares with endometritis show luminal epithelial degeneration, damaged ciliated cells with holes in the surface, ulcers around the mouths of the glands, cellular debris and neutrophilic migration (RICKETTS et al., 1978). These cellular alterations were not observed in the present study. In a recent study in women, a role for maternal neutrophils in maintaining normal pregnancy through their interactions with T cells was detected, suggesting immune cells population aid and regulated angiogenic process (NADKARNI et al., 2016).

The observed results confirm the hypothesis. In summary, dead equine conceptus fragments caused histological and vascular changes in the endometrium at the 7th day after ovulation, two days after their infusion, showing differences to the endometrial characteristics at the 7th day in the same cyclic mares. These alterations are similar to those observed in the endometrium of mares with live conceptus at 7 days of pregnancy.

Conflict of interest

None of the authors has any conflict of interest to declare**.**

Acknowledgements

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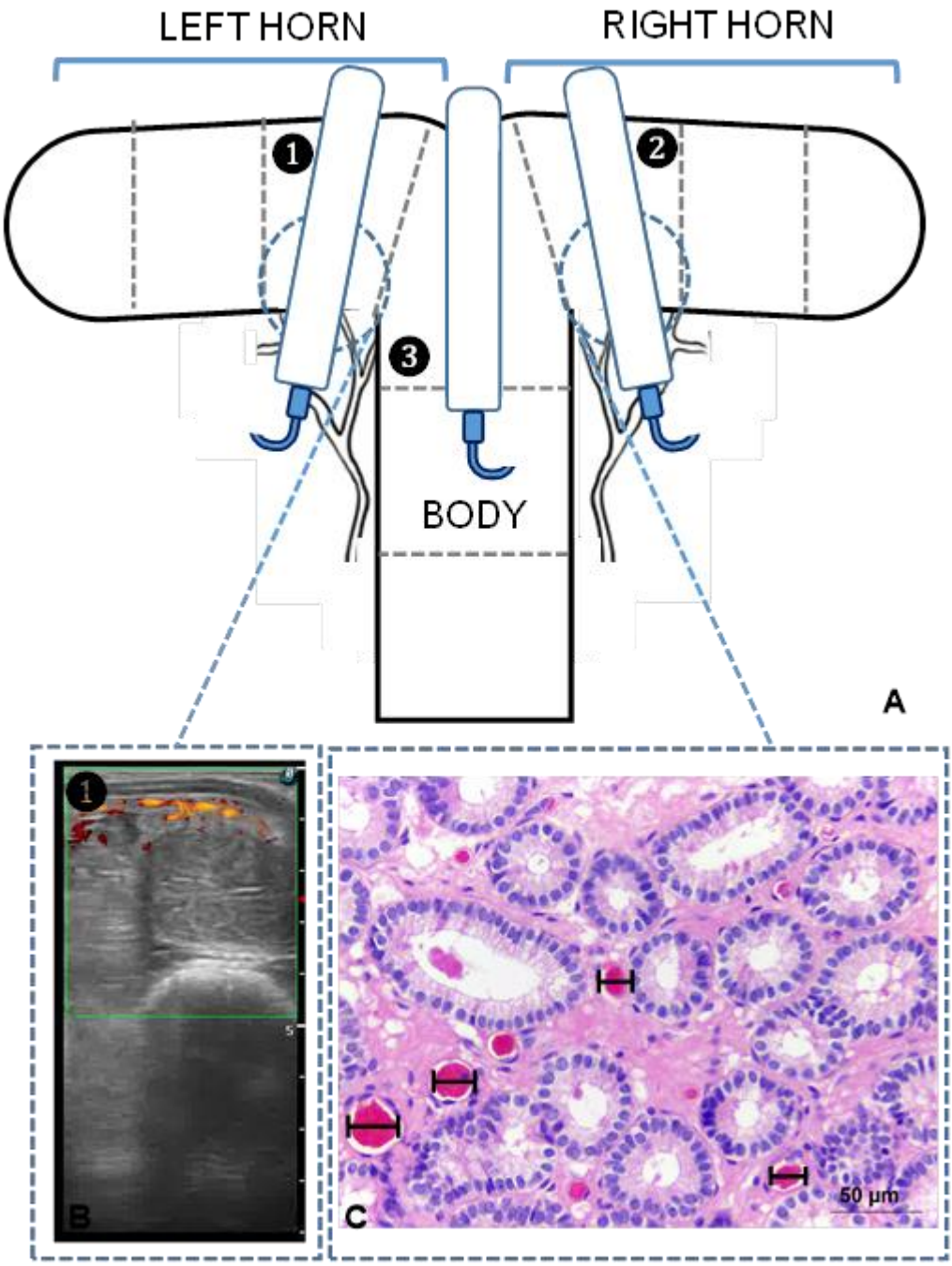

Fig. 3. **(A)** Diagram of the transrectal placement of a linear-array ultrasound transducer, showing the spatial relationships between the uterine horns and transducer. The numbers show the three uterine segments used in experiment. **(B)** Ultrasonography image. The colored spots are Power-Doppler indicators of blood flow. **(C)** Histological section of an endometrial biopsy, black bars show blood vessel diameter.

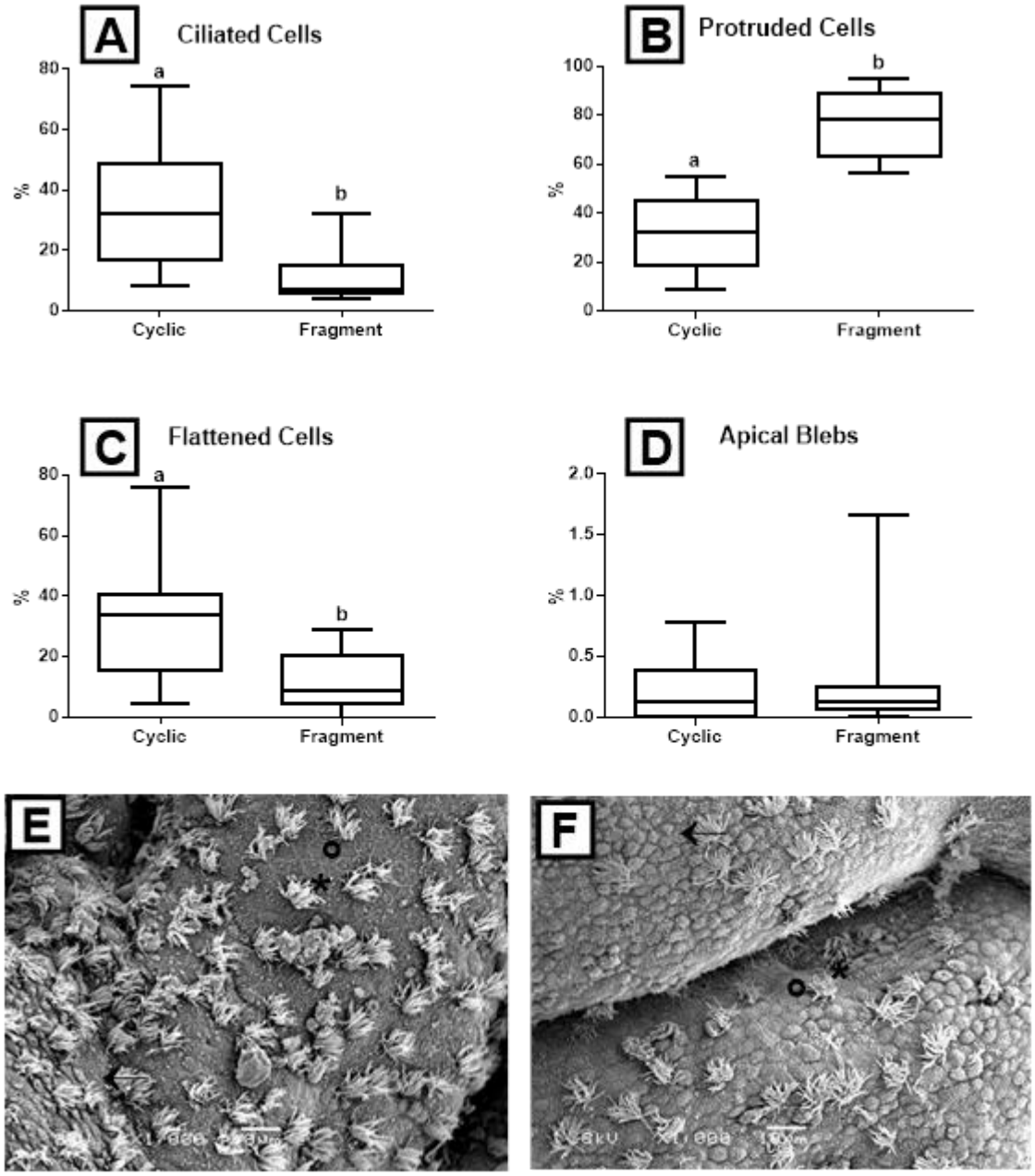

Fig. 4. Box-plots (whiskers represent min to max) of **(A)** Ciliated Cells, **(B)** Protruded Cells, **(C)** Flattened Cells, **(D)** Apical blebs of Cyclic and Fragment groups. Different letters (a, b) represent significant difference ($P < 0.002$). **(E)** Scanning Electron Micrograph (1000x, bar = 10 µm) of endometrium of the Cyclic group. **(F)** Scanning Electron Micrograph (1000x, bar = 10 µm) of endometrium of the Fragment group. Asterisks (*) indicate ciliated cells; black arrows indicate protruded cells, and circles show flattened cells.

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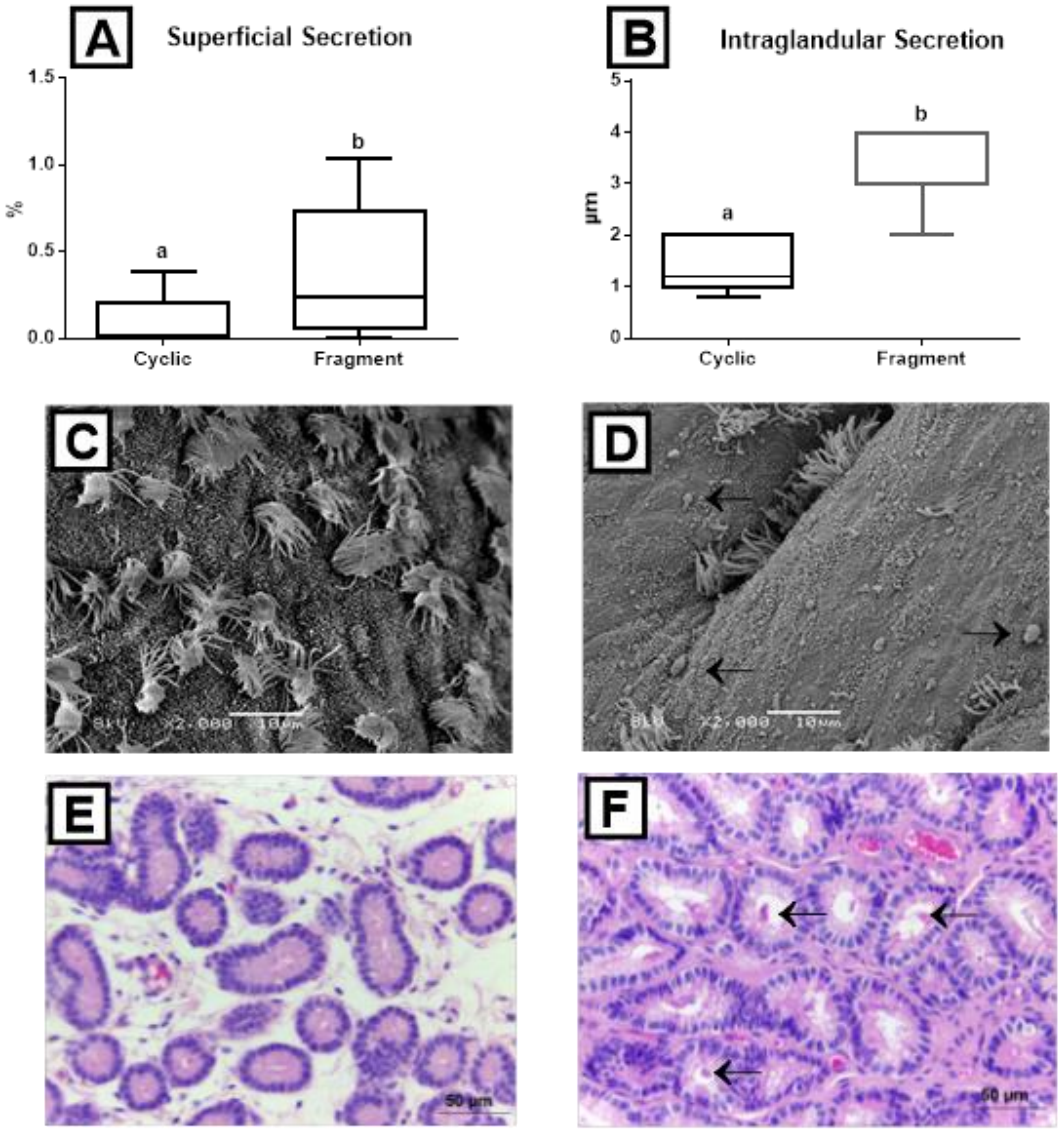

Fig. 5. Box-plots (whiskers represent min to max) of **(A)** superficial secretion, **(B)** intraglandular secretion, of Cyclic and Fragment groups. Different letters (a, b) represent significant difference ($P < 0.03$). **(C)** (Cyclic Group) Scanning Electron Micrograph (2000x, bar = 10 µm) of endometrium showing ciliated cells, protruded secretory cells, flattened secretory cell and secretion. **(D)** (Fragment group) Scanning Electron Micrograph (2000x, bar = 10 µm) of endometrium showing ciliated cells, protruded secretory cells, flattened secretory cell and secretion. **(E)** (Cyclic group) Histological section of endometrial glands (400x, bar = 20 µm). **(F)** (Fragment group) Histological section of endometrial glands (400x, bar = 20 µm). Black arrow shows superficial and intraglandular secretion.

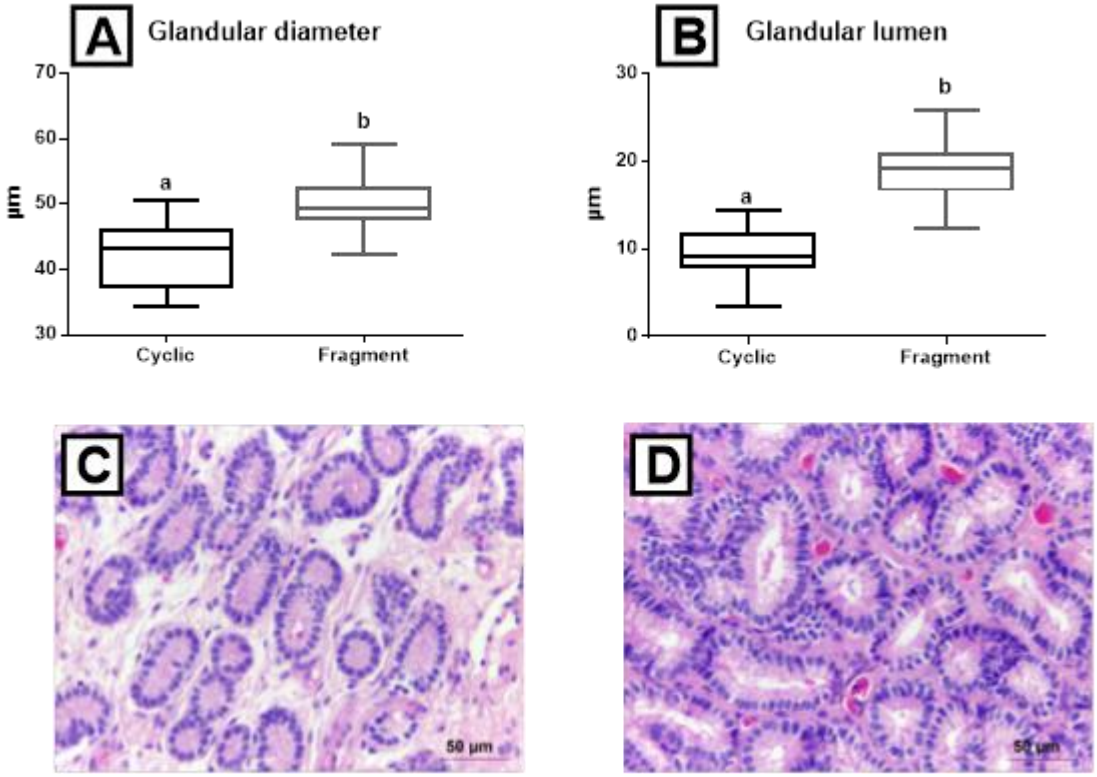

Fig. 6. Box-plots (whiskers represent min to max) of (A) Glandular diameter, (B) Glandular lumen of Cyclic and Fragment groups. Different letters (a, b) represent significant difference ($P < 0.001$). (C) (Cyclic Group) histological section of endometrial glands (400x, bar = 20 µm). (D) (Fragment group) histological section of endometrial glands (400x, bar = 20 µm).

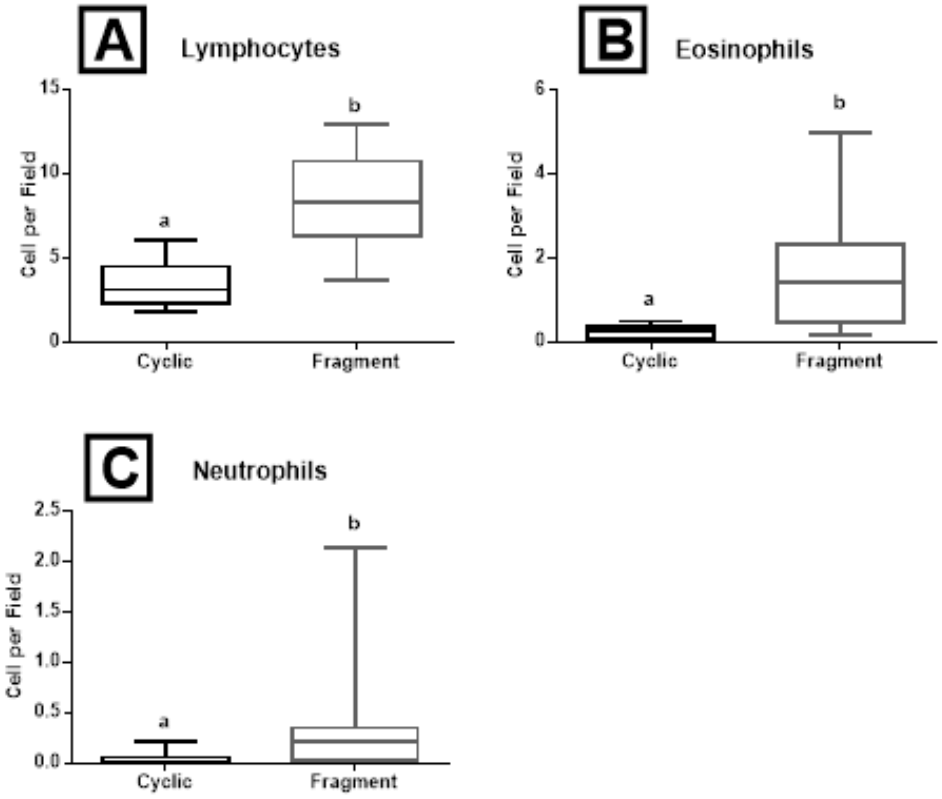

Fig. 7. Box-plots (whiskers represent min to max) of **(A)** Lymphocytes, **(B)** Eosinophils, **(C)** Neutrophils of Cyclic and Fragment groups. Different letters (a, b) represent significant difference (P = 0.001).

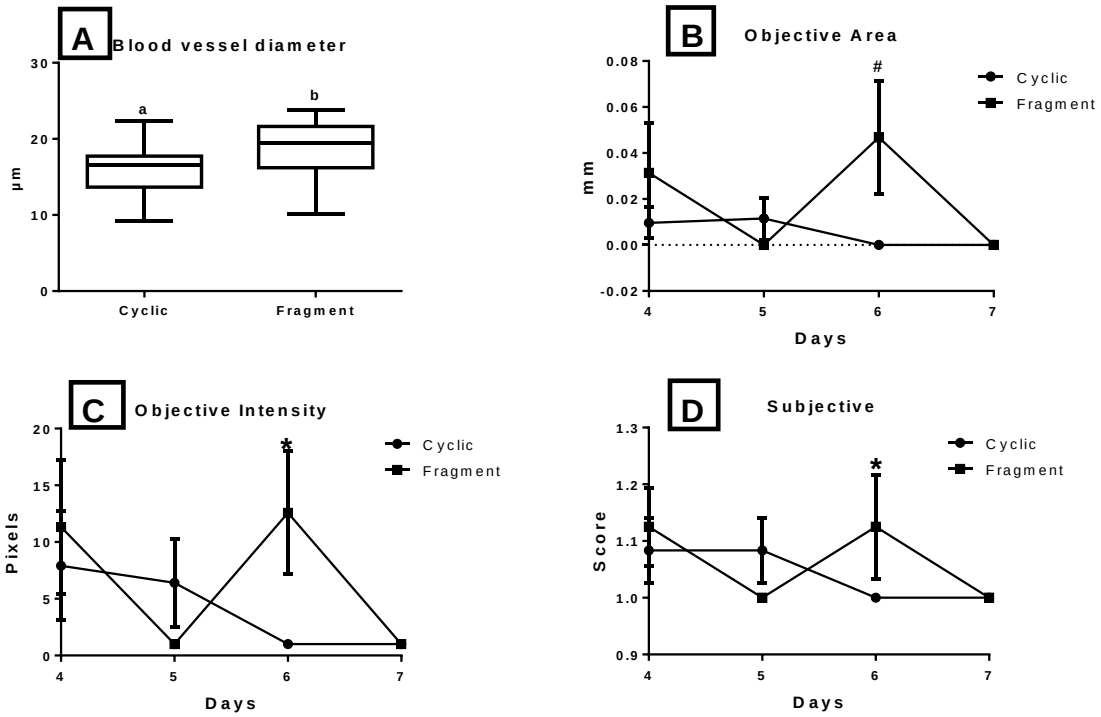

Fig. 8. Box-plots (whiskers represent min to max) of **(A)** Blood vessel diameter of Cyclic and Fragment groups (7th day), by histological analysis. Different letters (a, b) represent significant difference (P = 0,022). **(B)** (4th to 7th day) Power Doppler objective endometrial vascularization area. **(C)** (4th to 7th day) Power Doppler objective intensity of endometrial vascularization **(D)** (4th to 7th day) Subjective endometrial vascularization. Asterisks (*) represent significant difference (P < 0.05), Hashtag symbols (#) represent a tendency (P = 0.06).

4 ARTIGO 2

Uterine infusion of conceptus fragments changes the protein profile from cyclic mares

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Abstract

The aim of this experiment was to compare the protein profile of uterine fluid at day 7 after ovulation, in mares in which two days before conceptus fragments of day 13 were infused in comparison to cyclic mares. Ten healthy cyclic mares were used. Once estrus was confirmed mares were examined daily to detect ovulation (day 0). At day 7 after ovulation, uterine fluid was collected, constituting the Cyclic group (n = 10). In the second cycle, the same mares were examined daily until ovulation was detected. At day 5 after ovulation, fragments from a previously collected 13th day old concepti were infused into the uterus of each mare. Two days after infusion, uterine fluid was collected, constituting the Fragment group (n = 10). Uterine fluid samples were processed by two-dimensional electrophoresis technique. A total of 373 spots were detected. Twenty spots with differences in abundance between the cyclic (7) and fragment (13) mares were identified by MALDI-TOF/TOF and NanoUHPLC-

QTOF mass spectrometry. Thirteen proteins were identified with different abundance between the groups. Identified proteins may be related to the embryo-maternal communication and these are involved in process as adhesion, nutrition, endothelial cell proliferation, transport and immunological tolerance. In conclusion, conceptus fragments signalized changes in the protein profile of the uterine fluid 7 day after ovulation, in relation to the observed at the 7th day in the same cyclic mares.

Key words: Maternal recognition, Two-dimensional electrophoresis, Mass spectrometry, Embryo-maternal communication

1. Introduction

Establishment and maintenance of pregnancy are critically dependent of the embryo-maternal communication during the preimplantation period (KLEIN; TROEDSSON, 2011b). Crosstalk between the conceptus and the reproductive tract results in maintaining progesterone production by the ovaries, preparing the uterus for embryo implantation (WACLAWIK et al., 2017). The equine early pregnancy is poorly understand (SWEGEN et al., 2017) because, its reproductive physiology shows remarkable differences with other domestic species (ALLEN, 2005; KLEIN, 2016). Thereupon, the conceptus-derived pregnancy recognition signal has not been identified (KLEIN; TROEDSSON, 2011a)

Many approaches have been used for the improvement of the understanding of the stage of the estrous cycle, uterine receptivity, embryo-maternal communication and embryo metabolism. Thus, histomorphometric (KEENAN et al., 1987; CABALLEROS et al., 2019; CAMOZZATO et al., 2019) and molecular analysis (MERKL et al., 2010; KLEIN; TROEDSSON, 2011b; SWEGEN et al., 2017; SMITS et al., 2018; BASTOS et al., 2019) has been performed using equine endometrial tissues, uterine fluid and embryos or conceptus fragments.

Infusion of conceptus fragments into the mare uterus influenced endometrial and vascular changes, with an increase of the glandular secretion, suggesting that proteins present in the conceptus can alter the uterine fluid (CAMACHO et al., 2018).

Such endometrial changes the rise in number of immune cells and differentiate cellular pattern were considered similar to the observed in pregnant mares (KEENAN et al., 1987; CAMOZZATO et al., 2019).

It is hypothesized that the infusion of conceptus fragments promotes changes in the content of protein of the uterine fluid. The aim of this experiment was to compare the protein profile of uterine fluid at day 7 after ovulation, in mares in which two days before conceptus fragments of day 13 were infused in relation to cyclic mares on day 7.

2. Materials and methods

2.1 Animals

The study was performed in the southern hemisphere-breeding season. Ten healthy cyclic Quarter Horse type mares (mean age, 6.8 and range between 4 to 10 years old), and weighting 450-550 Kg were used. Mares were kept in natural pastures with free access to mineral supplementation and water. Throughout the experiment, mares remained healthy and with an average body condition score of 3.5 (scale 1 to 5) (MALSCHITZKY et al., 2001). This study was carried out with an Animal Ethical Use Committee approved protocol at the Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil (protocol number 34572).

Mares were examined for reproductive soundness by evaluation of perineal conformation, transrectal palpation, ultrasound of the genital tract (Sonoscape S8V, China) and endometrial biopsy. Only cyclic and clinically normal mares, with endometrium classified as category I or IIA (KENNEY; DOIG, 1986), without evidence of endometritis were selected.

2.2 Experimental Design

Mare’s reproductive tracts were routinely examined by transrectal palpation and ultrasonography until estrus was detected. Once estrus was confirmed (ovarian

follicle > 35 mm in diameter and marked uterine edema), mares were examined daily to detect ovulation, considered day 0. In this first cycle, uterine fluid samples from 10 cyclic mares were collected at day 7 after ovulation. This constituted the Cyclic group (CG).

In the second cycle, the same mares were examined daily until ovulation was detected (day 0). At day 5 after ovulation, fragments from dead concepti recovered previously from pregnant mares at day 13 post ovulation, were infused into the uterus to each mare. Uterine fluid samples were collected at day 7 in all mares (*n* = 10). This constituted the Fragment group (FG).

2.3 Conceptus Fragments

Conceptus fragments were obtained from previous embryo collections performed at day 13 after ovulation, snap frozen with 2.5 mL of Ringer solution and stored in liquid N2. Before their infusion in the FG mares, concepti were thawed at room temperature. Cryopreservation in temperatures below -79˚C and the thawing process used keep the proteins active (GARBER COHEN; CASTELLO; FLECHA, 2010). Thawed dead conceptus were divided equally in two parts and infused posteriorly to the mares (*n* = 10), each mare receiving the content of a half embryo. Infusion of the fragments was carefully performed at day 5 after ovulation with a nonsurgical trans-cervical procedure, using a standard artificial insemination pipette, protected with a sterile outer chemise. Dead conceptus fragments (2 mL) were deposited into the uterine body.

2.4 Uterine fluid samples

Uterine fluid samples were collected using commercial vaginal tampons (Mini OB; Johnson & Johnson Industrial Ltda, São José dos Campos, São Paulo, Brazil). Tampons were aseptically introduced into the uterus, according to REILAS, (2001), modified by MALSCHITZKY et al., (2008). The tampon was inserted through the cervix, protected by a palpation glove (double-glove technique). The distal part of a

palpation glove was cut to form a plastic tube, and the gloved hand with the tampon was introduced in the plastic tube, closing its end with a finger. The plastic tube was removed at the moment of the tampon's introduction in the uterus, where it remained for 30 min. The tampon was then removed, protected by a palpation glove, and immediately inserted in a sterile plastic sack and centrifuged (1500 x g, for 10 min) for fluid recovery. The recovered fluid (> 0.5 mL) was transferred to conic tubes and immediately centrifuged at 4 °C (10.000 x g, for 1 h). The supernatant was transferred to cryovials in 500 µL aliquots and stored at -80°C until further analyses.

2.5 Electrophoresis

Protein content from the uterine samples was determined using the Bradford method (BRADFORD, 1976), using 1 mg/mL of BSA (A7906; Sigma-Aldrich, St. Louis, MO, USA) as standard. Proteins were separated using two dimensional gel electrophoresis in duplicate. In summary, samples containing 250 µg of total protein were mixed with buffer (7-M urea, 2-M thiourea), 0,5% free ampholytes (IPG buffer, pH 3-10 [GE Life Sciences, Piscataway, NJ, USA]), 2% dithiothreitol (DTT), 2% CHAPS, and traces of bromophenol blue. Initially, the strips were rehydrated for 16 h in 250 µL rehydration solution at room temperature in IPG Box (GE Life Sciences). Then, samples were incubated in 13 cm IPG Strips (pH 3-10 linear, GE Life Sciences). Isoelectric focusing was carried out in Ettan IPGphor III System (GE Life Sciences) with the following conditions: 100 V (100 V/h), 150 V (75 V/h), 200 V (200 V/h), 500 V (500 V/h), 1000 V (800 V/h), 8000 V (11300 V/h), 8000 V (14412 V/h), 8000 V (7900 V/h), by total of 35000 V. The current limit was 50 mA per strip. Finished the isofocalization the strips were stored to -80 °C.

For the second dimension, strips containing endometrial proteins were thawed at room temperature and incubated for 15 min in equilibration buffer I (75 mM Tris-HCl, pH 8.8, 6-M urea, 29.3% glycerol, 2% SDS, 2% DTT) and equilibrated for an additional 15 min in buffer II (similar to buffer I, but containing 2.5% iodoacetamide instead of DTT), by the second dimension. Subsequently, strips were placed on top of 1.5 mm thick 12.5% SDS polyacrylamide gels and fixed with

Agarose sealing solution (25 mM Tris base, 192 mM glycine, 0.1% SDS, 0.5% agarose, 0.002% bromophenol blue). Proteins were separated using SE 600 Ruby system (GE Life Science) at 20 °C. Electrophoresis was performed with 15 mA for 15 min per plate starting at 90 V followed by 40 mA per plate at 250 V, for 4 h with 30 Watts (Electrophoresis Power Supply 301; Amersham Pharmacia Biotech).

Gels were stained in colloidal Coomassie blue (KANG et al., 2002) with modifications (DYBALLA; METZGER, 2009). Briefly, gels were washed three times (20 min each) with Milli-Q water. After, the gels were placed in a solution with phosphoric acid 85% (2%), ethanol (10%), ammonium sulfate (5%) and Coomassie Blue G-250 solution (0.02%), in agitation on a shaker for 24 h. Later, gels were washed three times (20 min each) with Milli-Q and placed in destaining solution (phosphoric acid (2%) and ethanol (30%) for 12 h and stored in acetic acid 5%. Two-dimensional gels were scanned using the ImageScanner III (GE Life Sciences) at 300 dpi and analyzed to determine relative volume of each spot considering the volume over all the spots in the image using software ImageMaster™ 2D Platinum (version 7.0; GE Life Sciences). Proteins in key regions were used as landmarks, and final spot matches were organized by checking each spot in each gel with the respective pattern.

2.6 Spot selection criteria

Spots were selected by: (a) presence in at least 80% of gels in one of the groups (Cyclic or Fragment), (b) significant abundance ($P \leq 0.01$) of relative volume in one of the groups (Cyclic or Fragment), and (c) a minimum of 1.5-fold magnitude difference between groups using ImageMaster™ 2D Platinum (version 7.0; GE Life Sciences).

2.7 In-gel tryptic digestion and mass spectrometry

Spots were manually excised, in-gel tryptic digestion (SHEVCHENKO et al., 2007). Tryptic peptides were dried by centrifugation in vacuum (model AG-22331

Eppendorf, Germany) and samples were re-suspended in 10 µL of 0.1% trifluoroacetic acid solution and desalted in ZIPTIP, model ZTC18S096 (Millipore, USA) and eluted in 3 µL of 50% acetonitrile, acidified with 0.1% trifluoroacetic acid. 1 µL peptide solution eluted was placed in the matrix of αcyano-4-hydroxycinnamic acid-HCCA (Bruker Daltonics, Germany), solubilized in the same solution from which tryptic peptides were eluted, to a final concentration of 10 mg/mL. Calibration of the analysis method MS1 were used peptides mix (*Peptide Calibration Standard II*) (Bruker Daltonics, Germany), placed in the matrix.

2.7.1 Protein identification using MALDI-TOF/TOF

The spectra of MS1 and MS2 were acquired in a MALDI-TOF/TOF spectrometer, Ultraflex III model (Bruker Daltonics). To obtain the MS1 data, the reflective and positive mode were used with a detection range of 500 - 3400 Da. For the MS2, using the positive mode LIFT method, the ratio mass/charge (m/z) of the ions with the highest intensity was selected.

All data obtained were managed by Flexcontrol software, version 3.3 (Bruker Daltonics, Germany). The spectra resulting from the MS1 and MS2 analyzes were processed using the FlexAnalysis software, version 3.3 (Bruker Daltonics, Germany). The peak lists of MS2 were generated in the mascot generic format by the BioTools application, version 3.2 (Bruker Daltonics, Germany).

2.7.2 Protein identification using NanoUHPLC-QTOF

The tryptic peptides from the excisions were solubilized in 70 µL of 0.1% formic acid solution (v/v). The peptides were analyzed using Liquid Chromatography—Mass Spectrometry (LC–MS) using the nanoACQUITY UPLC system (Waters, Milford, MA, USA) and a mass spectrometer micrOTOF QII® (Bruker Daltonics, Bremen, Germany), with a microESI ionization needle. Chromatographic analysis of the samples were executed in a trap column and a

capillary column ProteCol GHQ303 C18 3.0 µm–300 µm × 150 mm, operating at a flow rate of 4.5 µL min, using mobile phase solutions.

Mobile phase solutions was carried with the following solutions: water and 0.1% formic acid (v/v) (A) and acetonitrile and 0.1% formic acid (v/v) (B). Gradient program started in the desalted, in B at 5% by 14 min, linear rising ramp of B at 5% for 50% in 30 min, maintaining in B at 50% by 5 min, linear rising ramp of B at 50% for 90% in 3 min, maintaining of B at 90% by 2 min, linear gradient descent of B at 90% for 10% in 3 min, and maintaining of B at 10% by 3 min. The acquisition of data lasted approximately 60 min. The scanning of ions for MS1 spectra in positive mode was carried out for masses ranging between 100 and 2000 m/z, and between 70 and 2000 m/z for the MS2 spectra. The mass spectrometer micrOTOF QII® analysis were realized by the Hystar software program, version 3.2 (Bruker Daltonics, Bremen, Germany), and the spectra were processed through the Data Analysis software program, version 4.0 (Bruker Daltonics, Bremen, Germany). The mass list was generated in the format extensible mark-up language (*.mzXML).

2.8 Protein identification and characterization

The mass lists were compared with the protein sequences of Equidae family database, available at the UNIPROT Knowledgebase (UniProtKB, http://www.uniprot.org/; downloaded on 13/12/2018, with 29,702 entries), using the Mascot Daemon software, version 2.4.0 (Matrix Science, London, UK). The Mascot search parameters were: enzymatic digestion by the trypsin with one missed cleavage (allowing an error tolerance of 0.2 Da for the parental ion and 0.5 Da for the fragments), carbamidomethylation of cysteine as fixed modification and oxidation of methionine as a variable modification. The mass list in format mzXML was analyzed using software PEAKS version 7.0 (Bioinformatics Solutions Inc., Canada) (MA et al., 2003). The following parameters were used in the search: enzymatic digestion by the trypsin with one missed cleavage, carbamidomethylation of cysteine as fixed modification and oxidation of methionine as a variable modification; error tolerance was of 30 ppm MS/MS for the parental ion and 0.02 Da for the fragments.

Proteins were considered like identified when these presented at least one unique peptide with FDR (False Discovery Rate) < 1%.

The Scaffold software, version 3.6.4 (Proteome Software INc., Portland, OR) was used to validate Mascot results by applying the Peptide Prophet (KELLER et al., 2002) and the Protein Prophet (NESVIZHSKII et al., 2003) algorithms. Proteins and peptides were statistically validated when the probability of identity was equal or above 90%. The estimation was performed based on the complete amino acid sequence of the protein deposited in the database selected as reference for the identification.

Functional information of the proteins identified were retrieved from the UNIPROT Knowledgebase (THE UNIPROT CONSORTIUM, 2014). Proteins sequences were also functionally annotated using Blast2GO version 5.2.5 (GÖTZ et al., 2008). In this analysis, the proteins were aligned with the sequences of the Protein database (https://www.ncbi.nlm.nih.gov/protein) using the tool blastp of Blast version 2.8.1 (ALTSCHUL et al., 1990) (E-value threshold 1 e^{-25}).

A functional classification of the identified and statistically validated proteins was also performed by comparing their sequences with the Eukaryotic Orthologous Groups of proteins database (KOG) (KOONIN et al., 2004). In this classification, a KOG ID was assigned to each protein that significantly aligned (E-value threshold 1 e^{-25}) with those deposited in that database, using the *Reverse Position Specific* BLAST (RPS-BLAST).

Theoretical molecular weight and isoelectric point (pI) for each protein were estimated using ProtParam tool (https://web.expasy.org/protparam/). The estimation was performed based on the complete amino acid sequence of the protein deposited in the selected database as a reference for identification.

The interaction networks between the identified and validated proteins were reconstructed using the software STRING (*Search Tool for the Retrieval of Interacting Genes*) version 10.0 (http:// http://string-db.org/) (SZKLARCZYK et al., 2015).

2.9 Statistical analysis

The relative volume of each spot (optical density x area) was normalized to the total volume of spots detected on each gel for comparison groups. Software ImageMaster™ 2D Platinum (version 7.0; GE Life Sciences) was used to detect the differential abundance. Spots data were submitted to ANOVA to evaluate the relative volume of each protein spot as a dependent factor and the Groups (Cyclic or Fragment) were considered as independent variables using the software GraphPad Prism (Version 7.03). False discovery rate was calculated and corrected with a significance level by Benjamini-Hochberg corresponding to $P < 0.05$ was $q^*=0.013$.

3. Results

The protein concentration recovered from the uterus showed a median of 2.36 ± 0.21 µg/µl for the Cyclic group and of 2.5 ± 0.3 µg/µl for the Fragment group, without differences between groups ($P = 0.255$).

A total of 373 spots were detected, with molecular weights ranging from 12 to 225 kDa and Isoelectric point of 3 to 10. The spots mean number observed in the gels of the Fragment group (280 ± 30) did not differ ($P = 0.763$) with the mean number of the Cyclic group (291 ± 25). The total matches in the 2 groups were 299 spots (Fig 9). 52 and 22 spots were identified only in the gels of cyclic mares and infused mares, respectively. A total of twenty-eight spots satisfied the selection criteria, being two present exclusively in the Fragment group. Eight spots were discarded by the low intensity and twenty spots were carried for identification (Fig. 10).

Using the software MASCOT and PEAKS, 13 proteins were identified in the 20 spots with more abundance in the Cyclic (Table 1) and Fragment (Table 2) groups. Five proteins were identified as “Uncharacterized”, where three of these proteins presented the same access number (48, 162 and 173) (Table 1 and 2). Protein functional classification generated by the software Blast2GO are listed in Table 3.

Proteins with more abundance in Cyclic group were Serotransferrin-like (TF/ Spots 70), Serum albumin (ALB/ Spots 106), Immunoglobulin lambda light chain

variable region (IGL/ Spot 130, 133), Apolipoprotein A1 (APOA1/ Spots 130, 291), Immunoglobulin Gamma heavy chain precursor (IGHCp/ Spot 162), and Hemopexin (HPX/ Spot 166) (Fig. 11).

Proteins with more abundance in Fragment group were Leukocyte elastase inhibitor (SERPINB1/ Spot 32), Fibrinogen beta chain (FGB/ Spot 33), Immunoglobulin gamma heavy chain precursor (IGHCp/ Spots 48, 173), Immunoglobulin gamma 1 heavy chain constant region (IGHC1/ Spots 48, 173), Serotransferrin (TF/ Spots 73, 181, 272, 273), Serum albumin (ALB/ Spots 107), Complement factor B (CFB Spot 149) and Fibrinogen gamma chain (FGG/ Spots 184, 273). Lipocalin 2 (LCN2/ Spots 377, 378) was only identified in the Fragment group (Fig. 12).

Two proteins, ALB and TF showed higher abundance in Cyclic and Fragment group. In four spots were identified two proteins (48-173 IGHCp, IGHC1; 130 IGL, APOA1; 273 FGG, TF) (Table 1 and 2).

Table 4 shows functional classification of the statistically validated proteins based on the KOG Database. Analysis of Protein-protein interactions (PPI) network via STRING 10 is showed in the Fig 13.

4. Discussion

The present study was focused in the abundance differences between the proteins of the uterine fluid 7 days after ovulation of cyclic mares and infused mares with conceptus fragments. In the equine early pregnancy, many transcripts and proteins have been identified showing a great diversity of the regulatory mechanisms that underlie early development (PILLAI et al., 2018). This study used an experimental design expecting to identify relevant proteins in the embryo-maternal communication. A large number of spots were observed in both groups, indicating a considerable diversity of proteins and probably a range of post-translational changes.

Infusion with conceptus fragments showed endometrial and vascular changes, associated with a protein stimulus (CAMACHO et al., 2018), suggesting

similarity with equine early pregnancy process (CABALLEROS et al., 2019; CAMOZZATO et al., 2019). Identified proteins in the present research have been reported in current studies of protein and transcripts of endometrial tissues, embryonic tissues and uterine fluid (BASTOS et al., 2019; KLEIN et al., 2010; MALONEY et al., 2018; SMITS et al., 2018; SWEGEN et al., 2017) (Fig. 14). However, some proteins identified in the present study are different to the detected with the same methodology, with a live conceptus (BASTOS et al., 2019).

In the Protein-protein interaction network constructed by STRING 10, some proteins identified in the present study showed an independent role (Lipocalin 2, Complement factor B and Immunoglobulin gamma heavy chain precursor).

Lipocalin 2 (LCN2) or Neutrophil gelatinase-associated lipocalin (NGAL) was detected only in the Fragment group. This protein has been identified in previous studies in the uterine fluid, increasing in the early pregnant mare and in the yolk sac in comparison with the cyclic mare (HAYES et al., 2012; HANEDA et al., 2017; SMITS et al., 2018). LCN2 is known as a multifunctional protein, playing a key role in homeostasis and iron transport (MIYAMOTO et al., 2011). Lipocalin 2 shows an iron delivery pathway that is crucial to the survival, growth and maturation of cells, that can carry to apoptosis regulation, by interaction with interleukins (DEVIREDDY et al., 2001; LIN et al., 2011). Lipocalin 2 also has been correlated in the processes of embryogenesis (ZHANG et al., 2012), and, it is suggested as a mediator of innate immunity in the reproductive process by the stimulation of cytokines (TADESSE et al., 2011). Studies in women suggest that LCN2 plays an important role in the control of the invasion of extra-villous trophoblasts in the early placentation under hypoxia, by MPP-9 pathway stimuli (KOBARA et al., 2013).

Complement factor B (CFB) was another protein identified in the present study that showed an independent role. The gene of CFB is localized in the major histocompatibility complex (MHC) and belongs to the C3-convertase complex. In human, it is has been suggested that the oviduct possesses C3-convertase and in presence of preimplantation embryos may be involved in the production of embryotrophic inactivated complement-3b (iC3b), which stimulates blastocyst development (TSE et al., 2008). In early pregnancies of cows and ewes a decrease

of the CFB was detected, likely indicating that the protein is being used by the embryos to generate iC3b (KOCH; RAMADOSS; MAGNESS, 2010; MUÑOZ et al., 2011). In the present study, CFB increased in the Fragment group, probably the proteins of conceptus fragments signalized the production of CFB, but in the absence of a “viable embryo” the protein increased in the uterine lumen, without modifications. Dysregulation of the CFB has been suggested in pregnant women with preeclampsia (LYNCH et al., 2016).

In the present study, several immunoglobulins were identified with different molecular weight and/or isoelectric point: IGL (2), IGHCp (3) and IGHC1 (2). IGL increased in the Cyclic group in comparison with Fragment group. However, IGHCp presented increase in the Cyclic and Fragment group, showing a major number of spots in the infused mares, suggesting a relation with the higher increase of the IGHC1 in the Fragment group than in Cyclic group. These rise of the IGs can be associated to the increase of the immune cells reported after infusion of conceptus fragments (CAMACHO et al., 2018). Increase of IGs in the uterine fluid has been detected in pregnant mares in comparison with cyclic mares. (HAYES et al., 2012; SMITS et al., 2017). The IGs presence could be probably explained by the interaction of the maternal immunity with conceptus fragments, by the exposition to the conceptus’ antigens (HANSEN, 2011) or as anti-microbial protection for the “pregnancy” (PARR; PARR, 1985).

IGHC1 showed interactions with the FG units in the Protein-protein interaction network. The higher functional connection was identified between the Fibrinogens (FG) Alpha and Gamma. These proteins showed a greater abundance in the Fragment group, than in the Cyclic group. In equine early pregnancy, fibrinogen proteins have been detected in embryo capsule (KLEIN et al., 2010), in the blastocoel (SWEGEN et al., 2017), intrauterine secretion (SMITS et al., 2018; BASTOS et al., 2019) and in the conceptus´ yolk-sac (SMITS et al., 2018). FG has been hypothesized to promote cessation of the conceptus mobility and fixation, pathways integrins (KLEIN, 2016). This abundance of the fibrinogen subunits in the Fragment group could be involved in the increase of the diameter vessel and vascular endometrial changes previously reported after infusion of the conceptus

fragments (CAMACHO et al., 2018). In mice has been documented that fibrinogen subunits plays a critical role in the maintenance of pregnancy, supporting proper development of fetal-maternal vascular communication and stabilization of embryo implantation, moreover, aiding the correct formation of the yolk sac (IWAKI et al., 2002).

FG also showed functional connection with the Hemopexin and Apolipoprotein A1. Hemopexin (HPX) is an acute phase reactant induced mainly in the liver following inflammation, this protein has been identified in the uterine fluid of humans (DESOUZA et al., 2005; AL-RUMAIH et al., 2006) and equines (HAYES et al., 2012; MALONEY et al., 2018; SMITS et al., 2018). Increment of hemopexin has been detected with higher presence of E2 (AL-RUMAIH et al., 2006) and in blood during the normal pregnancy (CHEN; KHALIL, 2017), showing an association of HPX with the matrix metalloproteinases-9 (MMP-9) by dimerization patterns promoting vascular remodeling (CHEN et al., 2017). Interestedly, diameter vessel and vascular index by Doppler increased after the infusion of conceptus fragments (CAMACHO et al., 2018). Hemopexin in the present study showed a higher abundance in the Cyclic group suggesting that HPX could be conjugated in the blood vessels and probably these vascular changes are mediated with aid of the FGB.

Transcripts of apolipoproteins as APOA1 have been previously reported in equine conceptus and endometrium (KLEIN; TROEDSSON, 2011b). Apolipoprotein A1 has been identified in the equine conceptus and uterine fluid (SWEGEN et al., 2017; SMITS et al., 2018), suggesting that APOA1 is likely involved in guarantee the nutritional demands of the embryo, ensuring the adequate transport of lipids by endocytosis pathway (SMITS et al., 2018). In humans embryos has been suggested that, the secretion of low level of APOA1 can be reflective of viability and metabolic competence of the embryo (NYALWIDHE et al., 2013). Curiously, in the present study APOA1 showed higher abundance in the Cyclic group than Fragment group, probably due to the stimuli of the conceptus fragments.

The FG subunits, APOA 1 and Hemopexin converged in functional connections with Serum Albumin (ALB), an important protein in the immunological role, transport and nutrition. ALB plays a fundamental immunological role in the

embryo states, crossing from the maternal blood to the uterine fluid, passing into the free living blastocysts aiding its expansion (KULANGARA; CRUTCHFIELD, 1973). In the present study, Albumin showed abundance differences in cyclic and infused mares suggesting post-translational changes by differences in the isoelectric point and molecular weight. Different molecular weight and isoelectric point of ALBs were been previously reported in mares in the follicular fluid (FAHIMINIYA et al., 2011) and uterine fluid (BASTOS et al., 2019).

Serum albumin has been related to Serotransferrin in the embryo metabolism of mice (MCARDLE; PRISCOTT, 1984). Serotransferrin (TF), also known as siderophilin, is a protein that transports iron from sites of storage to regions of iron metabolism (MACGILLIVRAY et al., 1998). TF was proposed as a protein to protect uterine and conceptus tissues from the lipid peroxidation activity, which may occur as a consequence of the transport of iron via UF secretion (VALLET, 1995; VALLET; CHRISTENSON; MCGUIRE, 1996).

TF and ALB are negative acute phase proteins (GRUYS et al., 2005), and showed differences in abundance in the Cyclic as in Fragment groups, probably these proteins were undergone to post-translational changes and acted actively in the cyclic and the pregnant mare, as reported in many studies (SWEGEN et al., 2017; SMITS et al., 2018; BASTOS et al., 2019). Therefore, these proteins are of importance in the maintenance of the concepti homeostasis and the cellular patterns. The differences in the ALB and TF presence with higher abundance in the Cyclic or the Fragment group are probably to the lack of the functional and live conceptus that realizes endocytosis of proteins.

Albumin also show simples functional connection with two proteins of defense, Leukocyte elastase inhibitor (SERPINB1) and Immunoglobulin lambda light chain variable region (IGL). The SERPINB1 is a protein that has been associated with the function to inhibit proliferation of leukocytes (PADUA; HANSEN, 2008; ULBRICH et al., 2009). Serpin's transcripts has been detected in the inner cell mass and trophectoderm of the equine embryo (IQBAL et al., 2014). SERPINB1 was identified in endometrial fluid of pregnant mare and in the yolk sac, being associate with innate immune system (SMITS et al., 2018). The uterine SERPINs likely

performs diverse biological functions which include direct nutrition of the conceptus, growth control, inhibition of proteolytic activities, suppression of the local maternal immune system for sustaining of the pregnancy (KUMAR et al., 2013) and control of the MMP-2 expression (HUASONG et al., 2015). SERPINB1 belongs to proteins classified as Ovalbumin (REMOLD-O'DONNELL; CHIN; ALBERTS, 2006). The ovalbumin has been used in murine as a model to investigate immune mechanisms of allergic lung inflammation, resulting in increased levels of prostaglandins, cytokines, immune cells and secretions (MOORE; PEEBLES, 2006). Histomorphometric changes, glandular secretion increase and immune cells rise in the uterus has been reported after infusion of conceptus fragments (CAMACHO et al., 2018). These alterations could be partially mediated by the SERPINB1 signalization and probably can influence also the histomorphometric changes in the normal early pregnancy (KEENAN et al., 1987; MARTÍNEZ PEREIRA, 2016; CAMOZZATO et al., 2019).

The results revealed changes in the pattern of adhesion, nutrition, endothelial cell proliferation, transport and immunological tolerance proteins, suggesting that these proteins are functionally important in the embryo-maternal communication. Acute phase proteins and immunoglobulins were identified, suggesting a high influence of the immunological system. In conclusion, conceptus fragments signalized changes in the protein profile of the uterine fluid 7 day after ovulation, in relation to the observed at the 7th day in the same cyclic mares.

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Conflict of interest

None of the authors has any conflict of interest to declare**.**

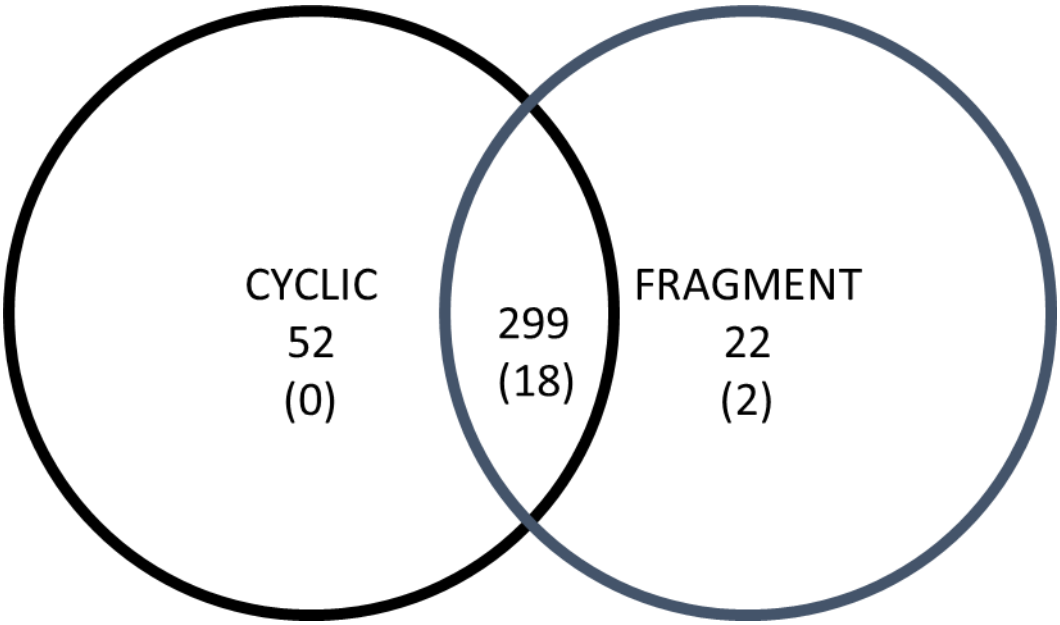

Fig. 9. Venn diagram depicting the matches between Cyclic and Fragment group. Without brackets the number of detected spots and into brackets the number identified spots.

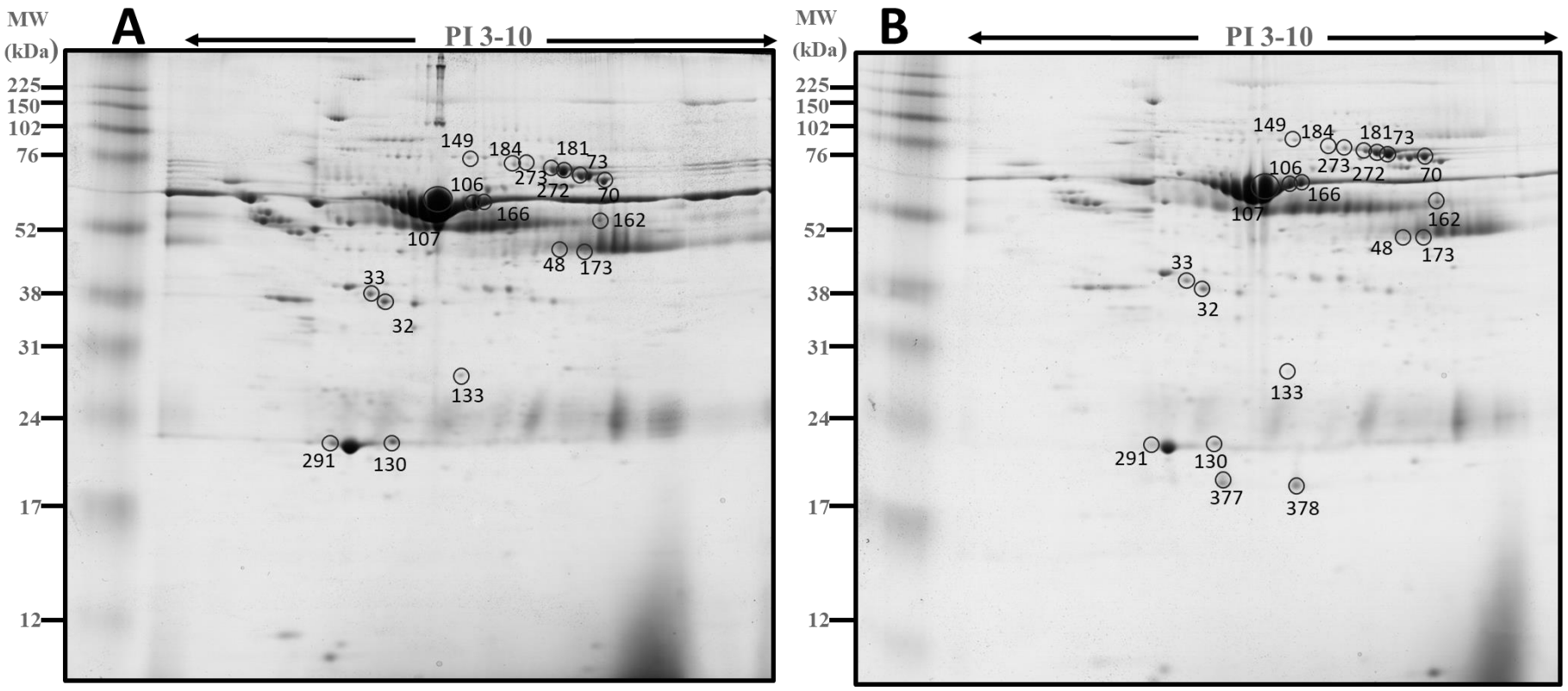

Fig. 10. Representative 2D PAGE gel with spots from the uterine fluid of Cyclic (A) and Fragment (B) group. Numbers in the figure correspond to the Spot ID shown in Tables 1 and 2.

Tabela 1. Proteins identified with higher abundance in the uterine fluid of the Cyclic group using MALDI TOF/TOF and LC-MS/MS, with MASCOT and PEAKS software, and compared with Database Equidae / Uniprot.

Spot[a]	Protein name	GENE[b]	Access number	Protein score[c]	Coverage[d]	Theoretical/Experimental		Peptide sequence	Peptide score[e]
						MW (kDa)	pI		
70	Uncharacterized protein	TF	F6ZEH8	156.12# 88†	11%	109.1/71.0	8.39/8.0	TEPQTHYYAVAVVK (*)	36.38≈
								SKDFHLFSSPHGK (*)	42.65≈
								HCEFDKFFR (*)	52.56≈
								EGCAPGYR (*)	32.74≈
								YYGYTGAFR (**)	97%‡
								CLVEKGDVAFVK (*)	32.75≈
								SGNFQLLCPDGTRK (*)†	31.75≈
								AVTEFESCNLAK (*)†	55.38≈
								APNHAVVSR (*)	44.63≈
								YLTAVANLR (*)	46.49≈

(Continues)

Spot[a]	Protein name	GENE[b]	Access number	Protein score[c]	Coverage[d]	Theoretical/Experimental		Peptide sequence	Peptide score[e]
						MW (kDa)	pI		
106	Serum albumin	ALB	F7BAY6	226.52# 336†	39%	68.3/61.0	5.78/6.59	SEIAHR (*)	43.87≈
								FNDLGEK (*)	44.74≈
								LVNEVTEFAKK (*)	44.90≈
								SLHTLFGDK (*)	43.47≈
								LCTVATLR (*)	27.11≈
								DDHPNLPK (*)	26.39≈
								YLYEVAR (***)	54.18≈
								ADFTECCPADDKAGCLIPK (*)	43.22≈
								CSSFQNFGER (*)	71.73≈
								ECCHGDLLECADDRADLAK (*)	42.15≈
								YICEHQDSISGK (*)	62.78≈
								DVFLGTFLYEYSR (**)	95%‡
								RHPDYSVSLLLR (***)	41.42≈
								IAKTYEATLEK (*)	44.51≈
								KAPQVSTPTLVEIGR (*)	65.06≈
								LPESER (*)	43.82≈
								LPCSENHLALALNR (*)	63.71≈
								LCVLHEK (*)	34.36≈
								TPVSEK (*)	49.51≈
								RPCFSALELDEGYVPK (***)	53.01≈
								KQSALAELVK (*)	38.35≈
								TVLGNFSAFVAK (*)	45.67≈
130	Immmunoglobulin lambda light chain variable region	IGL	A0A0A1E6K7	87.25#	11%	23.1/23.0	7.61/5.8	VNDAVTTDGVQTTR (*)	65.93≈
								SYSSVSCQVK (*)	27.03≈
	Apolipoprotein A1	APOA1	F6Z2L5	234†	11%	30.3/23.0	5.65/5.8	VAPLSDEFR (**)	99%‡
								VNLAPFSEELR (**)	99%‡
								AHPALEDLR (**)	97%‡

(Continues)

Spot[a]	Protein name	GENE[b]	Access number	Protein score[c]	Coverage[d]	Theoretical/Experimental		Peptide sequence	Peptide score[e]
						MW (kDa)	pI		
133	Immmunoglobulin lambda light chain variable region	IGL	A0A0A1E6K7	84.36#	10%	23.1/29.0	7.61/6.5	VNDAVTTDGVQTTR (*)	54.17≈
								YAASSYLTR (*)	47.38≈
162	Uncharacterized protein	IGHCp*	H9GZT5	76.96#	7%	36.4/56.0	8.27/8.13	VVSILAIQHK (*)	41.71≈
								ALPAPVER (*)	31.20≈
								LTVETNR (*)	35.92≈
166	Hemopexin	HPX	F6X1I8	101.93#	7%	51.3/62.0	7.58/6.7	NFIGPADAAFR (*)	43.79≈
								FNPVSGEVPPK (*)	41.65≈
								GGHTLVDGYPK (*)	56.53≈
291	Apolipoprotein A1	APOA1	F6Z2L5	130.67# 70†	18%	30.3/23.0	5.65/5.12	EYVAQFEASALGK (*)	59.21≈
								DTEGLRQELNK (*)	43.92≈
								VAPLSDEFREGAR (*)	42.98≈
								VNLAPFSEELR (**)	99%‡

[a] Spot ID correspond to the numbers shown in Fig. 10.
[b] Gene designation in UniProt. N/A Gene not characterized.
[c] Score Protein of PEAKS(#) or MASCOT (†)
[d] Protein coverage calculated (identified amino acids/total amino acids).
[e] Peptide score data obtain of PEAKS (P-value of probability of correspondence ≈) or SCAFFOLD (sequence probability percentage ‡)
(*) Acquisition of mass spectra of the peptide identified by LC-MS / MS.
(**) Acquisition of mass spectra of the peptide identified by MALDI TOF/TOF.
(***)Acquisition of mass spectra of the peptide identified by the both, LC-MS/MS e MALDI TOF/TOF.
Unique peptides of similar proteins †.

Tabela 2. Proteins identified with higher abundance in the uterine fluid of the Fragment group using MALDI TOF/TOF and LC-MS/MS, with MASCOT and PEAKS software, and compared with Database Equidae / Uniprot.

Spot[a]	Protein name	GENE[b]	Access number	Protein score[c]	Coverage[d]	Theoretical/Experimental		Peptide sequence	Peptide score[e]
						MW (kDa)	pI		
32	Leukocyte elastase inhibitor	SERPINB1	P05619	79.39# 196†	16%	43.0 /39	5.78/5.68	ADLSGMSGAR (*)	54.12≈
								LGVQDLFNR (*)	50.55≈
								VLELPYQGK (*)	48.81≈
								IPELLVK(*)	34.25≈
								ALYFDTVEDIHSR (**)	98%‡
								HNPSANILFLGR (**)	100%‡
33	Fibrinogen beta chain	FGB	F6PH38	71.57# 185†	10%	55.7/40.0	8.63/5.5	ALYEGFTVK (***)	41.50≈
								QGFGNIATNADGK (*)	28.89≈
								IRPYFPQQ (*)	27.64≈
								QDGSVDFGR (**)	92%‡
								EDGGGWWYNR (**)	97%‡
48	Uncharacterized protein	IGHCp*	H9GZT5	91.65# 129†	19%	36.4/47.0	8.27/7.5	VVSILAIQHK (*)	47.13≈
								DVLMISR (*)	37.62≈
								SQTYICNVAHPASSTK (*)	29.70≈
								VSVTCLVK (*)	28.74≈
								LTVETNR (*)	27.43≈
								EPQVYVLAPHRDELSK (**)	99%‡
	Immunoglobulin gamma 1 heavy chain constant region	IGHC1	Q95M34	97.46#	12%	37.4/47.0	7.68/7.5	FNWYMDGVEVR (*)	55.97≈
								IQHQDWLSGK (*)	39.67≈
								VNNQALPQPIER (*)	42.14≈
								VSVTCLVK (*)	28.74≈

(Continues)

Spot[a]	Protein name	GENE [b]	Access number	Protein score[c]	Coverage[d]	Theoretical/Experimental		Peptide sequence	Peptide score[e]
						MW (kDa)	pI		
73	Serotransferrrin	TF	P27425	211.04# 76†	24%	78.0/71.0	6.83/7.7	SIVPAPPLVACVK (*)	55.82≈
								RTSYLECIK (*)	38.76≈
								KNSNFQLNQLQGK (*)	44.23≈
								CLADGAGDVAFVK (*)	70.04≈
								SKDFHLFSSPHGK (*)	54.06≈
								DSALGFLR (*)	45.89≈
								SSSDPDLTWNSLK (*)	55.81≈
								HCEFDKFFR (*)	55.43≈
								YYGYTGAFR (***)	51.49≈
								CLVEKGDVAFVK (*)	59.20≈
								HQTVEQNTDGRNPDDWA K (*)	54.48≈
								SCYLAR (*)†	38.27≈
								AACVCQELHNQQASYGK (*)†	57.02≈
								YLTAVANLR (*)	52.77≈
								LLEACTFHRV (*)†	50.24≈

(Continues)

Spot[a]	Protein name	GENE [b]	Access number	Protein score[c]	Coverage[d]	Theoretical/Experimental		Peptide sequence	Peptide score[e]
						MW (kDa)	pI		
107	Serum albumin	ALB	F7BAY6	247.80#	50%	68.3/58.0	5.78/6.3	DTHKSEIAHR (*)	53.72≈
								FNDLGEK (*)	25.96≈
								LVNEVTEFAKK (*)	38.01≈
								CAADESAENCDK (*)	66.75≈
								SLHTLFGDKLCTVATLR (*)	61.95≈
								ATYGELADCCEK (*)	48.10≈
								DDHPNLPK (*)	46.72≈
								ADFTECCPADDKAGCLIPK (*)	31.66≈
								LDALKER (*)	34.17≈
								LSQKFPK (*)	39.65≈
								ADFAEVSKIVTDLTK (*)	47.17≈
								ECCHGDLLECADDRADLAK (*)	28.69≈
								YICEHQDSISGK (*)	43.12≈
								ACCDKPLLQK (*)	43.84≈
								SHCIAEVK (*)	43.75≈
								DAKDVFLGTFLYEYSR (*)	42.59≈
								RHPDYSVSLLLR (***)	58.66≈
								IAKTYEATLEK (*)	41.89≈
								KAPQVSTPTLVEIGR (*)	66.02≈
								TLGKVGSR (*)	42.99≈
								LPESERLPCSENHLALALNR (*)	48.49≈
								LCVLHEKTPVSEK (*)	54.06≈
								CCTDSLAER (*)	43.70≈
								RPCFSALELDEGYVPK (***)	48.74≈
								KQSALAELVK (*)	34.81≈
								ATKEQLK (*)	36.04≈
								TVLGNFSAFVAK (*)	60.22≈
								CCGAEDKEACFAEEGPK (*)	60.69≈
								LVASSQLALA (*)	38.07≈

(Continues)

Spot[a]	Protein name	GENE[b]	Access number	Protein score[c]	Coverage[d]	Theoretical/Experimental		Peptide sequence	Peptide score[e]
						MW (kDa)	pI		
149	Uncharacterized protein	CFB	F6RMD0	76.57#	3%	85.9/89.0	6.75/7.03	LEDSVTYYCSR (*)	30.83≈
								DISAVVTPR (*)	61.15≈
173	Uncharacterized protein	IGHCp*	H9GZT5	154.02# 67†	24%	36.4/47.0	8.27/7.79	SQTYICNVAHPASSTK (*)	69.58≈
								DVLMISR (*)	44.61≈
								VVSILAIQHK (*)	57.55≈
								TISKPTGQPR (*)	58.01≈
								EPQVYVLAPHR (**)	95%‡
								VSVTCLVK (*)	46.80≈
								DFYPTDIDIEWK (*)	43.58≈
								LTVETNR (*)	40.49≈
	Immunoglobulin gamma 1 heavy chain constant region	IGHC1	Q95M34	97.01#	11%	37.4/47.0	7.68/7.79	IQHQDWLSGK (*)	53.81≈
								SQEPQVYVLAPHPDELSK (*)	42.66≈
								VSVTCLVK (*)	46.80≈
181	Serotransferrin	TF	P27425	178.76# 147†	11%	78.0/72.0	6.83/7.62	RTSYLECIK (*)	33.82≈
								TEPQTHYYAVAVVK (*)	70.57≈
								SKDFHLFSSPHGK (*)	52.55≈
								YYGYTGAFR (***)	55.17≈
								SCYLAR (*)†	39.43≈
								APNHAVVSR (*)	47.29≈
								YLTAVANLR (*)	40.12≈
								LLEACTFHR (*)†	48.50≈
184	Fibrinogen gamma chain	FGG	F6W2Y1	241†	8%	49.7/75.0	5.41/7.16	YEALVVTHESTIR (**)	99%‡
								IHLISTQTTIPYVLR (**)	99%‡
								VGPENDKYR (**)	98%‡
272	Serotransferrin	TF	P27425	118†	3%	78.0/73.0	6.83/7.7	YYGYTGAFR (**)	93%‡
								APNHAVVSR (**)	92%‡

(Continues)

Spot[a]	Protein name	GENE [b]	Access number	Protein score[c]	Coverage[d]	Theoretical/Experimental		Peptide sequence	Peptide score[e]
						MW (kDa)	pI		
273	Fibrinogen gamma chain	FGG	F6W2Y1	124.40#	12%	49.7/74.0	5.41/7.49	YEALVVTHESTIR (*)	74.60≈
								VAQLEAK (*)	34.80≈
								LDGSVDFKK (*)	32.32≈
								IHLISTQTTIPYVLR (**)	96% ‡
								VQLEDWNGK (*)	49.87≈
	Serotransferrin	TF	P27425	70.76#	2%	78.0/74.0	6.83/7.49	DSALGFLR (*)	35.62≈
								LLEACTFHR (*)	47.13≈
377	Lipocalin 2	LCN2	F6TIR2	280†	17%	23.1/20.0	5.94/5.8	LIPAPPLDR (**)	98%‡
								YFGVQSYIVR (**)	100% ‡
								VADTDYNQFAIVFFR (**)	100%‡
378	Lipocalin 2	LCN2	F6TIR2	154.20# 326†	44%	23.1/20.0	5.94/6.65	LIPAPPLDR (**)	99%‡
								VPLQPDFKDDQFQGK (*)	57.29≈
								KEEQGQFTMYTTTYELK (*)	48.29≈
								DQNCDHWIR (**)	97%‡
								YFGVQSYIVR (***)	63.22≈
								NQEYFKTTLYR (*)	41.76≈
								TKELTPELR (**)	94%‡
								EKFISFAK (***)	41.10≈

[a] Spot ID correspond to the numbers shown in Fig. 10.
[b] Gene designation in UniProt or Abbreviation. *Abbreviation of the protein without gene identification.
[c] Score Protein of PEAKS(#) or MASCOT (†)
[d] Protein coverage calculated (identified amino acids/total amino acids).
[e] Peptide score data obtain of PEAKS (P-value of probability of correspondence ≈) or SCAFFOLD (sequence probability percentage ‡)
(*) Acquisition of mass spectra of the peptide identified by LC-MS / MS.
(**) Acquisition of mass spectra of the peptide identified by MALDI TOF/TOF.
(***)Acquisition of mass spectra of the peptide identified by the both, LC-MS/MS e MALDI TOF/TOF.
Unique peptides of similar proteins †.

Tabela 3. Functional classification of the proteins identified in uterine fluid of Cyclic and Fragment mares by Blast2GO with “Uncharacterized protein’.

ID Spots	GENE / ABV[a]	UniProtKb ID[b]	Length[c]	Description	e-Value[d]	Similarity mean (%)[e]	GOs[f]
106, 107	ALB	F7BAY6	607	Serum albumin	0	90,86	17
32	SERPINB1	P05619	379	Leukocyte elastase inhibitor	0	92,74	4
33	FGB	F6PH38	490	Fibrinogen beta chain	0	92,19	29
48, 162, 173	IGHCp*	H9GZT5	335	Immunoglobulin gamma heavy chain precursor	0	83,9	0
48, 173	IGHC1	Q95M34	337	Immunoglobulin gamma 1 heavy chain constant region	0	84,41	9
70	TF	F6ZEH8	990	Serotransferrin-like	0	84,99	8
73, 181, 272, 273	TF	P27425	706	Serotransferrin	0	89,09	7
130, 133	IGL	A0A0A1E6K7	221	Immmunoglobulin lambda light chain variable region	1,78E-159	94,97	0
130, 291	APOA1	F6Z2L5	266	Apolipoprotein A-I	0	90,28	59
149	CFB	F6RMD0	768	Complement factor B	0	93,08	7
166	HX	F6X1I8	462	Hemopexin	0	88,88	14
184, 273	FGG	F6W2Y1	437	Fibrinogen gamma chain	0	93,36	21
377, 378	LCN2	F6TIR2	199	Neutrophil gelatinase-associated lipocalin	2,89E-147	90,14	1

[a] Gene or Abbreviation. *Abbreviation of the protein without gene identification.
[b] Identification in the UniProtKb database.
[c] Protein Length.
[d] E-Value: the number of alignments expected by chance (The lower the E value, the more significant the score and the alignment).
[e] Probability Similarity mean (%) for validation by Blast2GO software of the proteins statistically.
[f] number of Gene Ontology

Tabela 4. Functional classification of proteins identified in uterine fluid of Cyclic and Fragment mares by KOG Database

Spot	GENE	UniProtKb ID	KOG ID	KOG Group	Description	KOG class	e-Value
32	SERPINB1	P05619	KOG2392	V	Serpin	Defense mechanisms	7,66E-146
33	FGB	F6PH38	KOG2579	R	Ficolin and related extracellular proteins	General function prediction only	9,55E-117
70	TF	F6ZEH8	KOG0090	U	Signal recognition particle receptor, beta subunit (small G protein superfamily)	Intracellular trafficking, secretion, and vesicular transport	2,04E-96
149	CFB	F6RMD0	KOG3627	E	Trypsin	Amino acid transport and metabolism	8,52E-30
166	HX	F6X1I8	KOG1565	O	Gelatinase A and related matrix metalloproteases	Posttranslational modification, protein turnover, chaperones, Extracellular structures	1,03E-60
273	FGG	F6W2Y1	KOG2579	R	Ficolin and related extracellular proteins	General function prediction only	4,30E-109

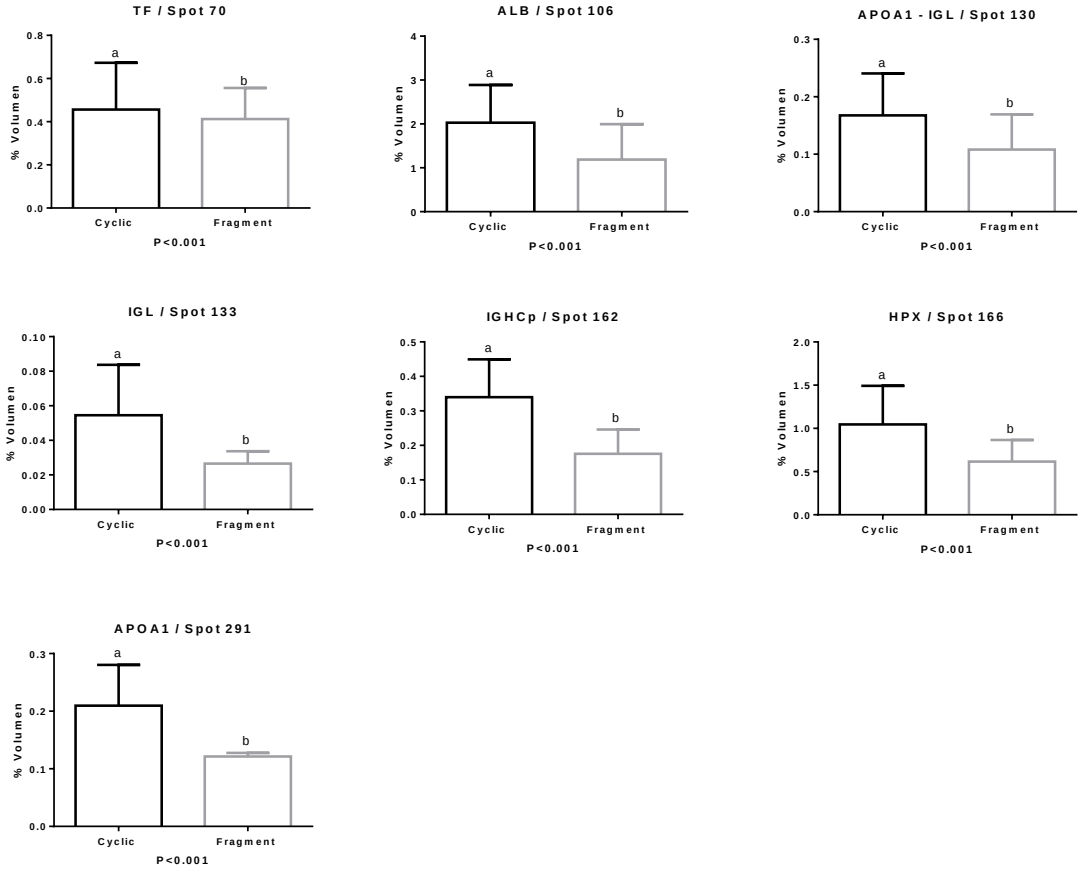

Fig. 11. Relative volume (%) of proteins with higher abundance in the uterine fluid of the Cyclic group. Different letters (a,b) represent significant differences ($P < 0.01$)

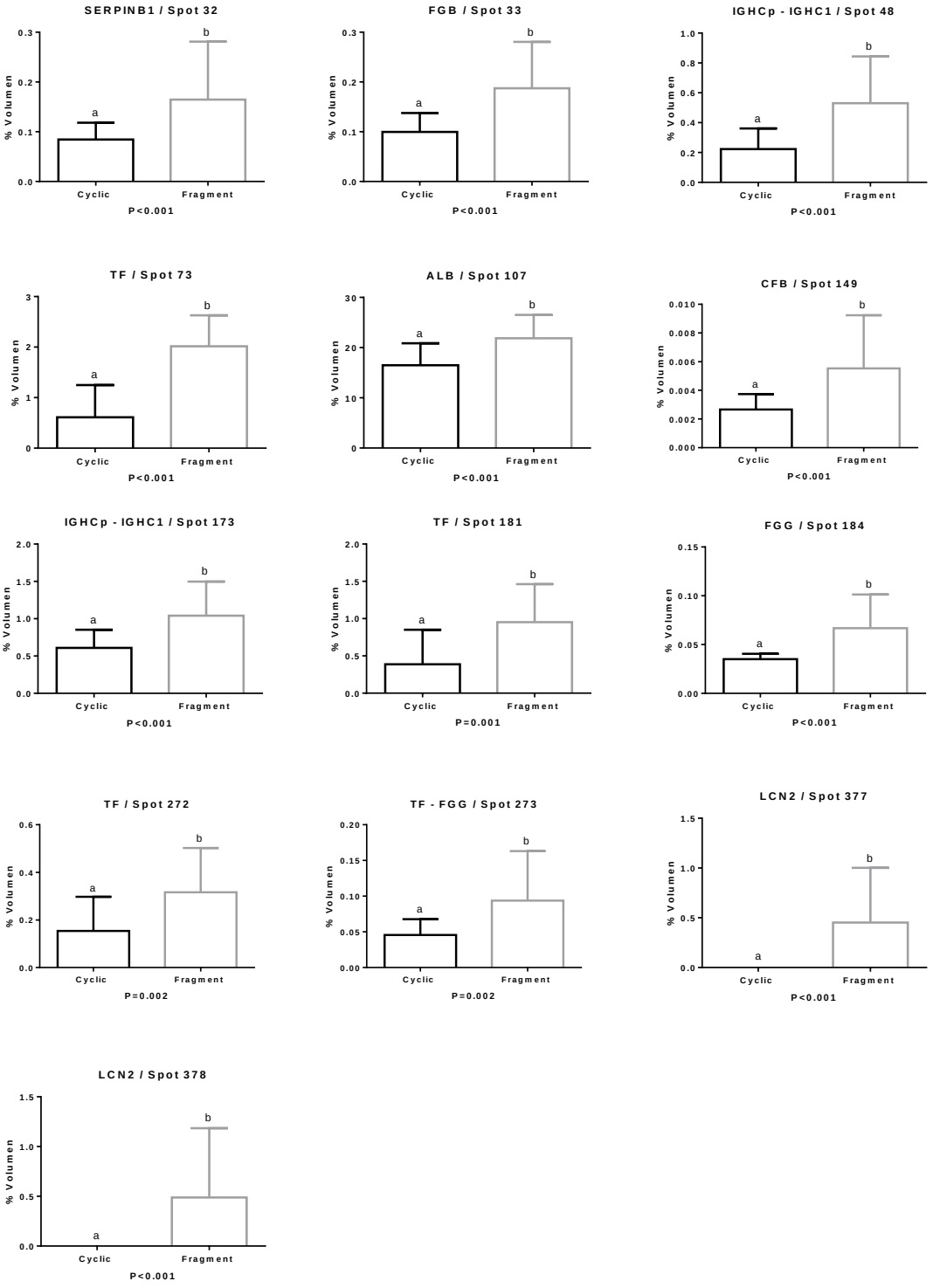

Fig. 12. Relative volume (%) of proteins with higher abundance in the uterine fluid of the Fragment group. Different letters (a,b) represent significant differences (P < 0.01).

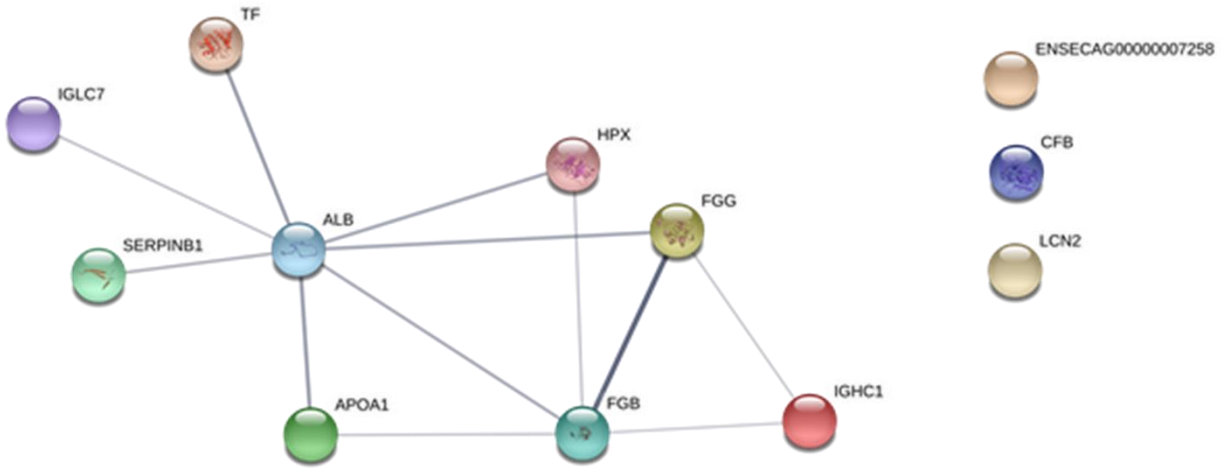

Fig. 13. Protein-protein interactions (PPI) network via STRING 10. Interactions of proteins that differed significantly in their abundance. IGLC7 is the identification of IGL and ENSECAG00000007258 is the identification of IGHCp for STRING.

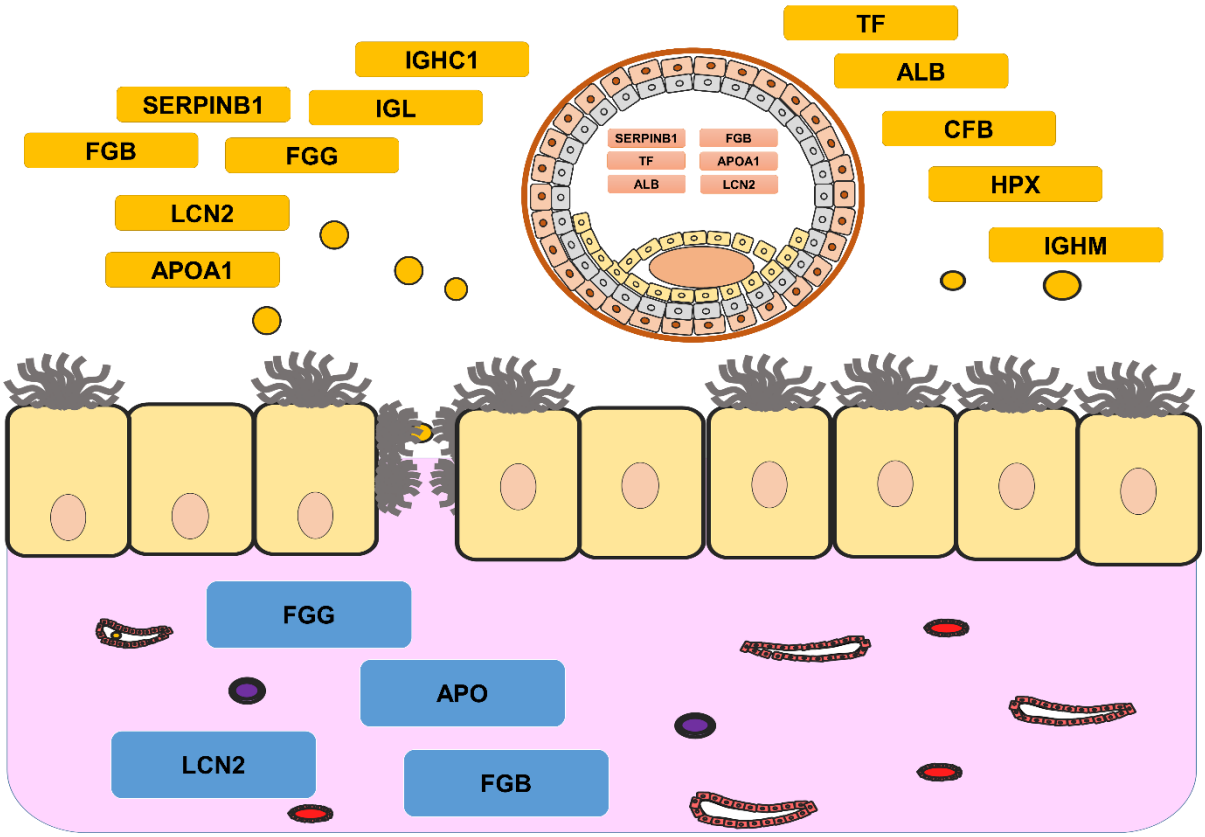

Fig. 14. Schematization of the uterine interaction with the proteins and transcripts identified, based on experiments (KLEIN; TROEDSSON, 2011a; HAYES et al., 2012; SWEGEN et al., 2017; SMITS et al., 2017, 2018; MALONEY et al., 2018). Proteins in the red box are proteins detected into the embryo, proteins in the yellow box are proteins detected in the uterine fluid and proteins in the blue box are proteins detected in the endometrium accorded to the studies above referenced.

5 CONSIDERAÇÕES FINAIS

O estímulo de componentes dos fragmentos de conceptos provavelmente induziram uma reorganização de padrões celulares e vasculares similares à égua prenhe, estimulando a secreção glandular que possivelmente modificou as proteínas do líquido endometrial, possibilitando a detecção de proteínas que podem estar envolvidas na comunicação materno-embrionária inicial.

O presente trabalho apresentou algumas limitações devido que não foi previamente analisada a composição dos conceptos, porém os presentes resultados sugerem que o presente desenho experimental pode ser usado para melhorar a compressão da interação materno-embrionárias e perda embrionária inicial. Outra limitação do presente estudo foi a falta de avaliação do perfil hormonal da éguas e dos receptores hormonais, além de avaliar se a manipulação comprometeu ou aumento a vida do corpo lúteo.

Devido à complexidade da comunicação materno embrionária inicial é necessário realizar futuros estudos para caracterizar as interações histológicas, vasculares e proteicas com o fim de elucidar a fisiologia, abordando diferentes faixas de assincronia tanto com fragmentos de conceptos como com conceptos vivos. Assim, as proteínas identificadas no presente estudo e suas interações podem ser um ponto de início na pesquisa.

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ANEXO 1 PARTICIPAÇÃO EM EVENTOS

POSTER

- AUTOR: CORRELAÇÕES HISTOMORFOMÉTRICAS VASCULARES E PROTEICAS DO ENDOMÉTRIO EQUINO NA PRENHEZ INICIAL. MODELO FRAGMENTOS DE CONCEPTOS. Conferencia ABRAVEQ. 2019-

- AUTOR: CHANGES IN THE PROTEIN PROFILE OF THE UTERINE FLUID AFTER THE TRANSFER OF CONCEPTUS FRAGMENTS. XXIII Congresso Brasileiro de Reprodução Animal. 15 a 17 de maio de 2019 – Centro de Eventos UFRGS/FAURGS, Gramado – Rio Grande do Sul. Abstracts. Anim. Reprod.

- AUTOR: VASCULAR ENDOMETRIAL ALTERATIONS AFTER THE TRANSFER OF EQUINE EMBRYONIC FRAGMENTS. VII International Symposium on Animal Biology of Reproduction, November 6-9, 2018, Aracaju, SE, Brazil. Abstracts. Anim. Reprod., v.16, n.1, p.124, Jan./Mar. 2019.

- AUTOR: ENDOMETRIAL ALTERATIONS AFTER THE TRANSFER OF EMBRYONIC REMAINS. INTERNATIONAL SYMPOSIUM ON EQUINE REPRODUCTION. CAMBRIDGE, INGLATERRA. 23 a 27 de Julho de 2018. Journal of Equine Veterinary Science 66 (2018) 119. DOI: https://doi.org/10.1016/j.jevs.2018.05.164.

APRESENTAÇÃO ORAL

- AUTOR: ALTERAÇÕES HISTOLÓGICAS E VASCULARES NO ENDOMÉTRIO EQUINO ASSOCIADAS À COMUNICAÇÃO MATERNO EMBRIONÁRIA, MODELO COM FRAGMENTOS DE CONCEPTOS. 2° Congresso Reprolab De Reprodução Equina. Porto Alegre, RS. 7 de abril de 2019.