UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA
CURSO DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA ANIMAL

LORENZO GARRIDO TEIXEIRA MARTINI SEGABINAZZI

MODULAÇÃO DA RESPOSTA INFLAMATÓRIA NO ÚTERO DE ÉGUAS TRATADAS COM PLASMA RICO EM PLAQUETAS

Botucatu - SP
Abril/2021

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Tese apresentada a Faculdade de Medicina Veterinária e Zootecnia da Universidade Estadual Paulista “Júlio de Mesquita Filho”, Campus de Botucatu, para o Conclusão do curso de Doutorado em Biotecnologia Animal.

Orientador: Prof. Dr. Marco Antonio Alvarenga
Coorientador: Prof. Dr. Igor F. Canisso

Botucatu - SP
Abril/2021

FICHA CATALOGRÁFICA ELABORADA PELA SEÇÃO TÉC. AQUIS. TRATAMENTO DA INFORM.
DIVISÃO TÉCNICA DE BIBLIOTECA E DOCUMENTAÇÃO - CÂMPUS DE BOTUCATU - UNESP
BIBLIOTECÁRIA RESPONSÁVEL: ROSEMEIRE APARECIDA VICENTE-CRB 8/5651

Segabinazzi, Lorenzo Garrido Teixeira Martini.
Modulação da resposta inflamatória no útero de éguas tratadas com plasma rico em plaquetas / Lorenzo Garrido Teixeira Martini Segabinazzi. - Botucatu, 2021

Tese (doutorado) - Universidade Estadual Paulista "Júlio de Mesquita Filho", Faculdade de Medicina Veterinária e Zootecnia
Orientador: Marco Antonio Alvarenga
Coorientador: Igor Frederico Canisso
Capes: 50504002

1. Éguas. 2. Útero - Doenças inflamatórias. 3. Plasma rico em plaquetas. 4. Bactérias. 5. Imunomodulação. 6. Endometrite.

Palavras-chave: Bactéria; Endometrite infecciosa; Imunomodulação; Inflamação uterina; PRP.

Título: MODULAÇÃO DA RESPOSTA INFLAMATÓRIA NO ÚTERO DE ÉGUAS TRATADAS COM PLASMA RICO EM PLAQUETAS

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Data da Defesa: 15 de abril de 2021.

DEDICATÓRIA

Dedico esta conquista a todos que de alguma forma se fizeram presentes na minha vida. Em especial aos meus pais, meus maiores exemplos e meu suporte incondicional.

AGRADECIMENTO

Primeiramente agradecer a Deus e a Nossa Senhora Aparecida, meus fiéis protetores. A eles que me concederam a Vida, saúde, oportunidades e que sempre colocam pessoas maravilhosas no meu caminho.

Aos meus pais e minha família pelo amor incondicional, exemplo, ensinamentos e apoio. Por toda a confiança e expectativas depositadas em mim, as quais me levaram a realizar muitos dos meus sonhos.

Ao meu Orientador e um grande AMIGO que construí nesse período de pós-graduação, Professor Marco Alvarenga. Ele que confiou em mim desde o início, que me oportunizou a realização dos cursos de Mestrado e Doutorado sob sua orientação. Por todos os ensinamentos, oportunidades e exemplos dados durante este período maravilhoso da minha vida. E pela grande amizade construída e que levarei para a vida toda.

Ao meu coorientador, Professor Igor Canisso, por ter me recebido na Universidade de Illinois e ter me ensinado muito durante o período que estive por lá. Além disso, por ter me dado oportunidades ímpares de aprendizado e profissionais, e por ser um grande AMIGO que fiz nesse período de Pós-graduação.

Ao professor Frederico Ozanam Papa, ou “OZAMA” como costumo lhe chamar, hehehehe; que além de grande Mestre e incentivador, posso considerar um grande amigo e um grande EXEMPLO de pessoa e profissional a ser seguido. Igualmente ao Professor José Antônio Dell’Aqua, ou apenas Zé pros conhecidos, por todos os ensinamentos, conversas, conselhos e muitas oportunidades geradas.

Aos meus amigos, os quais não vou listar, pois além de serem muitos tenho medo de esquecer de algum. A eles, por terem me proporcionado lembranças e momentos inesquecíveis, e amizades que levarei comigo para o resto da vida.

Um agradecimento especial a residente da Universidade de Illinois, Giorgia Podico, que realizou várias análises quando eu não pude estar presente devido a pandemia, o que fez possível a finalização do meu doutorado. Nesse

contexto, também agradeço ao meu amigo “Alemão” o Guilherme Novello, a Lais Leal, assim como o Leonardo Barbosa que me acompanharam durante o período de experimento em Illinois e ajudaram muito na finalização deste projeto e experimento,

A instituição “Faculdade Estadual Paulista – UNESP” pela oportunidade de realização dos cursos de Mestrado e Doutorado. Da mesma forma a Universidade de Illinois, IL, EUA pela oportunidade de realização de estágio e parte do projeto de Doutorado na instituição. Em especial, aos funcionários da FMVZ, UNESP Botucatu pelo apoio e ajuda diária.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001.

À Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), pela bolsa de doutorado (Processo n°2017/13883-9), bolsa de Estágio do Exterior (BEPE n°2019/05017-5) e pelo apoio financeiro para a realização do estudo (Processo n°2018/02856-3).

Desta forma, deixo aqui meu AGRADECIMENTO a todos que de alguma forma se fizeram presentes na minha vida. Espero um dia poder retribuir todo o bem que me proporcionaram.

MUITO OBRIGADO

Queda prohibido llorar sin aprender,
levantarme un día sin saber qué hacer,
tener miedo a mis recuerdos,
sentirme sólo alguna vez.

Queda prohibido no sonreir a los problemas,
no luchar por lo que quiero,
abandonarlo todo por tener miedo,
no convertir em realidade mis sueños.

Alfredo Cuervo Barrero

Hay un certo placer en la locura,
qué sólo el loco conoce.

Pablo Neruda

RESUMO

SEGABINAZZI, L.G. **MODULAÇÃO DA RESPOSTA INFLAMATÓRIA NO ÚTERO DE ÉGUAS TRATADAS COM PLASMA RICO EM PLAQUETAS.** 2020, p.148. Tese (Doutorado) – Faculdade de Medicina Veterinária e Zootecnia, Campus Botucatu, Universidade Estadual Paulista, Botucatu – SP.

A endometrite é a causa mais comum de subfertilidade em éguas. Todas as éguas apresentam uma inflamação endometrial fisiológica pós-cobertura; entretanto, 15-20% das éguas têm endometrite persistente pós-cobertura (EPPC). Recentemente, o plasma rico em plaquetas (PRP) vêm se tornando popular no manejo reprodutivo de éguas susceptíveis a EPPC. Atualmente, não existe uma padronização dos métodos de preparação do PRP para uso intrauterino em éguas. Desta forma, este estudo teve como objetivo comparar três métodos de preparação de PRP para infusão intrauterina em éguas e avaliar a resposta inflamatória pós-cobertura, cultura endometrial e recuperação embrionária em éguas suscetíveis a EPPC tratadas com PRP. No primeiro estudo, o PRP foi produzido por três métodos. Método 1, o sangue foi coletado em bolsa de transfusão, transferido para tubos Falcon e duplamente centrifugadas; Método 2, sangue foi coletado em tubos *vacutainer* e centrifugado uma única vez; Método 3 sangue foi coletado em uma seringa de 60 mL contendo anticoagulante, a qual foi mantida em posição vertical para sedimentar por 4h. Após o processamento, a contagem de células e a viabilidade plaquetária foram avaliadas. Em um subgrupo de éguas (n = 6), o PRP foi avaliado 6 e 24h pós-refrigeração a 5 °C. No estudo 2, éguas (n = 12) suscetíveis a EPPC tiveram três ciclos atribuídos aleatoriamente para receber infusões intrauterinas de LRS (controle), ou PRP autólogo ou plasma pobre em plaquetas (PPP) antes (48 e 24h) e após a inseminação artificial (IA) (6 e 24h). Tanto o PRP quanto o PPP foram obtidos pelo Método 1 no experimento 2. As éguas foram inseminadas com sêmen fresco de um garanhão com fertilidade conhecida. O acúmulo de líquido intrauterino (IUF) e as células polimorfonucleares endometriais (PMNs) foram avaliados a cada 24h até 96h pós-inseminação. Citocinas uterinas (Ilβ, IL6, CXCL8 e IL10) foram avaliadas antes (0h), 6 e 24h após IA, e cultura endometrial três e nove dias após IA. A lavagem do embrião foi realizada 8 dias após a ovulação. Os dados foram analisados com modelo misto, teste post-hoc de Tukey's e regressão multivariada. No estudo 1, o Método 1 resultou no maior e o método 3 nas menores concentrações de plaquetas, e o último teve maior concentração de leucócitos que os outros ($P<0,05$). A viabilidade das plaquetas foi semelhante entre os tratamentos. O resfriamento por 24h não afetou a contagem de plaquetas. No entanto, a viabilidade plaquetária foi reduzida após o resfriamento do PRP produzido pelo método 3. Estudo 2, o tratamento com PRP reduziu os PMNs endometriais, IUF pós-reprodução e citocinas pró-inflamatórias quando comparados aos ciclos atribuídos pelo controle, mas não significativamente diferente do PPP. Os controles tiveram uma porcentagem significativamente maior de culturas bacterianas positivas (33%) em comparação com os ciclos atribuídos a PRP (0%), enquanto os ciclos tratados com PPP não foram significativamente diferentes dos outros grupos (25%). Os ciclos atribuídos ao PRP tiveram taxas de recuperação embrionária significativamente maiores (83%) do que o controle (33%), embora não significativamente diferente do PPP (60%). Em conclusão, o Método 1 resultou nas maiores concentrações de plaquetas, enquanto o método 3 resultou em maiores contagens de leucócitos. O resfriamento afetou a viabilidade das plaquetas no PRP obtido com o método 3. Resta determinar se os diferentes métodos e o resfriamento afetariam a eficácia clínica do PRP. A infusão de plasma reduziu a duração e intensidade da resposta inflamatória pós-IA e melhorou a recuperação embrionária em éguas suscetíveis a EPPC. As plaquetas parecem ter uma propriedade antimicrobiana e anti-inflamatória dependente da dose.

Palavras-chave: PRP, endometrite infecciosa, bactéria, inflamação uterina, imunomodulação, equino.

ABSTRACT

SEGABINAZZI, L.G. **MODULATION OF UTERINE INFLAMMATORY RESPONSE IN MARES TREATED WITH PLATELET-RICH PLASMA.** 2020, p.148. Tese (Doutorado) – Faculdade de Medicina Veterinária e Zootecnia, Campus Botucatu, Universidade Estadual Paulista, Botucatu – SP.

Endometritis is the most common cause of subfertility in mares. All mares display a physiological post-breeding endometrial inflammation; however, 15-20% of mares have persistent breeding-induced endometritis (PBIE). Recently, platelet-rich plasma (PRP) is becoming popular in mare practice to mitigate post-breeding induced endometritis and improve fertility. Currently, there is no standardization of methods to prepare PRP for intrauterine use in mares. This study aimed to compare three methods to prepare PRP for intrauterine infusion in mares and to assess the post-breeding inflammatory response, endometrial culture and embryo recovery in mares susceptible to PBIE treated with PRP. In the first study, PRP was produce by three methods. Method 1, blood was collected in blood transfusion bag, transferred to Falcon tubes and double centrifuged; Method 2, blood was collected in vacutainer tubes and centrifuged once; Method 3, blood was collected in a syringe containing anticoagulant, which was kept in an upright-position to sediment for 4h. After processing, cell counts and platelet viability were assessed. In a subset of mares (n=6), PRP was evaluated at 6 and 24h post-cooling at 5°C. In study 2, mares (n=12) susceptible to PBIE had three cycles randomly assigned in a crossover design to receive intrauterine infusions of LRS (control), or autologous PRP or platelet-poor plasma (PPP) pre- (48 and 24h) and post-breeding (6 and 24h). Both PRP and PPP were obtained by Method 1. Mares were bred with fresh semen from one stallion. Intrauterine fluid accumulation (IUF) and endometrial polymorphonuclear cells (PMNs) were assessed every 24h up to 96h post-breeding. Uterine cytokines (Ilβ, IL6, CXCL8 and IL10) were evaluated before (0h), 6 and 24h post-breeding, and endometrial culture three and nine-days after breed. Embryo flushing was performed 8d post-ovulation. Data were analyzed with mixed model, Tukey's post-hoc test, and multivariate regression. In study 1, Method 1 resulted in the greatest and method 3 in the fewest platelet concentrations, and the latter had greater WBC than the others ($P<0.05$). Platelet viability was similar across treatments. Cooling for 24h did not affect platelet counts. However, platelet viability was reduced after cooling PRP produced by method 3. Study 2, PRP treatment reduced endometrial PMNs, post-breeding IUF and pro-inflammatory cytokines when compared to control-assigned cycles, but not significantly different than PPP. Controls had a significantly higher percentage of positive bacterial cultures (33%) in comparison to PRP-assigned cycles (0%), whereas cycles treated with PPP were not significantly different from the other groups (25%). The PRP-assigned cycles had significantly higher embryo recovery rates (83%) than the control (33%), though not significantly different than PPP (60%). In conclusion, Method 1 resulted in the greatest platelet concentrations, while method 3 resulted had greater WBC counts. Cooling affected platelet viability in PRP obtained with method 3. It remains to be determined whether the different methods and cooling would affect PRP's clinical efficacy. Plasma infusion reduced the duration and intensity of the post-breeding inflammatory response and improved embryo recovery in mares susceptible to PBIE. Platelets incrementally downregulate PBIE and appear to have a dose-dependent antimicrobial property.

Keywords: PRP, infectious endometritis, bacteria, uterine inflammation, immunomodulators, equine.

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LISTA DE ABREVIATURAS E SÍMBOLOS

ACP	Células apresentadoras de antígenos
BAFF	Fator de ativação de células B
C1q	Componente de complemento 1
C3a	Complemento 3a
C3b	Complemento 3b
C4a	Complemento 4a
C4b	Complemento 4b
C5a	Complemento 5a
CD14	Cluster de diferenciação 14
CD40	Cluster de diferenciação 40
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
CXCL8	Ligante da quimiocina 8
EC	Células epiteliais
ECM	Matriz extracelular
EDTA	Ácido etilenodiamino tetra-acético
FoxP3	Proteína Forkhead P3
GnRH	Hormônio liberador de gonadotrofina
IFN	Interferon
IFNα	Interferon I α
IgA	Imunoglobulina A
IgG	Imunoglobulina G
IgM	Imunoglobulina M
IL1	Interleucina 1
IL1RN	Antagonista da Interleucina 1
IL10	Interleucina 10
IL13	Interleucina 13
IL17	Interleucina 17
IL1α	Interleucina 1alpha
IL1β	Interleucina 1Beta
IL4	Interleucina 4

IL6	Interleucina 6
LH	Hormônio luteinizante
LPS	Lipopolissacarídeos
LRS	Riger com lactato
MAC	Complexo de ataque à membrana
MCWE	Extrato de parede celular de Mycobacterium phlei
MMP-3	Metaloproteinase-3
MMPs	Matrix metaloproteinases
MSCs	Células tronco mesenquimais
MyD88	Resposta primária de diferenciação mieloide 88
NETs	Armadilhas extracelulares de neutrófilos
NF-κB	Fator nuclear kappa-B
NK	Células natural killer
NLR	Receptores NOD-like
NO	Óxido nítrico
NOD	Domínio de ligação de nucleotídeo e oligomerização
NSAIDs	Anti-inflamatório não esteroidal
PABA	Ácido para-aminobenzóico
EPPC	Endometrite persistente pós-cobertura
PBPs	Proteínas de ligação à penicilina
PGF2α	Prostaglandina 2α
PMNs	Neutrófilos polimorfonucleares
PRP	Plasma rico em plaquetas
PRRs	Pattern recognition receptors
SAA	Soro amilóide A
TIMPs	Inibidores de tecido de MMPs
TLR2	Receptores Toll-like tipo 2
TLR4	Receptores Toll-like tipo 4
TLRs	Receptores Toll-like
TNFα	Fator de necrose tumoral α
TRAF6	Fator 6 associado ao receptor
TRIF	Interferon-β indutor de adaptador contendo domínio TIR
WBC	Glóbulos brancos

SUMÁRIO

Capítulo 1

1. Introdução

A endometrite, inflamação e/ou infecção endometrial, é considerada a principal causa de subfertilidade e a terceira doença mais recorrente em equinos (TRAUB-DARGATZ; SALMAN; VOSS, 1991; TROEDSSON, 1999). Clinicamente, as fêmeas são classificadas como susceptíveis ou resistentes à endometrite persistente pós-cobertura (EPPC), conforme sua capacidade de eliminar a inflamação/infecção em 48 horas após a cobertura (LEBLANC et al., 1994; TROEDSSON *et al.*, 1994a). Consideram-se éguas susceptíveis, as que apresentam dificuldade em modular à inflamação, podendo apresentar alterações na conformação vulvar e útero penduloso (SCOGGIN, 2015), sendo esta condição comumente associada à fêmeas idosas (ALLEN; PYCOCK, 1988). Em um programa comercial de reprodução equina, cerca de 25% das éguas têm mais de 16 anos, idade em que a fertilidade das fêmeas começa a reduzir (ALLEN; PYCOCK, 1988). Estima-se que 10-15% das éguas em programas reprodutivos são consideradas susceptíveis ao desenvolvimento da EPPC (ZENT; TROEDSSON; XUE, 1998).

A inflamação pós-cobertura pode ser causada por agentes infecciosos como bactérias e fungos, além de agentes não-infecciosos como o espermatozoide (TROEDSSON; LIU; CRABO, 1998). Todas as éguas desenvolvem essa resposta inflamatória uterina de forma transitória em até 30 minutos após a monta natural ou inseminação artificial (KATILA, 1995a; TROEDSSON, 1997; ALGHAMDI *et al.*, 2005). Essa reação fisiológica ocorre para eliminar o plasma seminal, excesso de espermatozoides, microrganismos e debris do lúmen uterino, e assim preparar a chegada do embrião (TROEDSSON; LIU; CRABO, 1998). Contudo, éguas susceptíveis à EPPC apresentam um início retardado e um prolongamento desse processo inflamatório (TROEDSSON; LIU, 1991; TROEDSSON, 1997; CARNEVALE *et al.*, 2000), o que resulta no acúmulo de neutrófilos polimorfonucleares (PMNs) e líquido intrauterino após a cobertura, prejudicando a sobrevivência embrionária e o estabelecimento da gestação (ZENT; TROEDSSON; XUE, 1998; BUCCA *et al.*, 2008; WOODWARD *et al.*, 2013b). Além disso, com a resposta imune inata prejudicada, os microrganismos introduzidos no útero durante a cobertura não são eliminados de forma eficiente, levando a uma infecção em potencial.

O manejo das éguas susceptíveis à EPPC é uma tarefa difícil. O embrião equino migra da tuba uterina para o lúmen uterino entre as 144 e 168 horas após a ovulação

(FREEMAN *et al.*, 1991). Este evento ocorre de forma concomitante com o aumento da produção de progesterona e tônus cervical, promovendo um tempo hábil para que esta inflamação seja debelada. A neutrofilia persistente, acúmulo de fluído intraluminal excessivo, e a produção prolongada de citocinas pró-inflamatórias são considerados eventos embriotóxicos, diminuindo o potencial de fertilidade das éguas quando submetidas à monta natural ou inseminação artificial (ROBERTSON *et al.*, 2018). A inflamação endometrial persistente cria um ambiente uterino hostil para a entrada do embrião no útero, comprometendo a recuperação embrionária e manutenção da gestação (TROEDSSON; LIU, 1991; TROEDSSON, 1997).

Tradicionalmente a endometrite é tratada utilizando uma terapia multimodal, combinando lavagens uterinas, agentes ecbólicos, anti-inflamatórios e antibióticos. Infelizmente, algumas éguas não respondem aos tratamentos tradicionais (CANISSO; STEWART; COUTINHO DA SILVA, 2016; SCOGGIN, 2016). A falha na eficácia dos tratamentos, com o aumento da resistência aos agentes antimicrobianos, conduziu ao desenvolvimento de terapias alternativas para éguas com EPPC (SCOGGIN, 2016).

Enquanto a endometrite equina foi amplamente estudada nos últimos 40 anos, sendo publicadas diversas revisões sobre o assunto (TROEDSSON, 2006, 2014; LIU; TROEDSSON, 2008; WOODWARD; TROEDSSON, 2015; KATILA, 2016; TROEDSSON; WOODWARD, 2016), muito progresso foi obtido na última década referente à compreensão dos aspectos moleculares envolvidos na EPPC e os caminhos de regulações negativas e positivas em resposta à indução experimental e tratamento (FREEMAN *et al.*, 1991; FUMUSO *et al.*, 2003, 2007; NASH *et al.*, 2010; CHRISTOFFERSEN *et al.*, 2012a; WOODWARD *et al.*, 2013b, 2015; MARTH *et al.*, 2015, 2018). Com isso, esse trabalho tem como objetivo revisar os aspectos clínicos, moleculares e microbiológicos da patogênese, diagnóstico e tratamento da endometrite em éguas.

2. Etiologia e patogênese da endometrite

A endometrite pode ser classificada conforme sua etiologia em infecciosa ou não-infecciosa, as quais na prática clínica, comumente observam-se em associação. Os sinais clínicos entre os dois tipos de endometrite são indistinguíveis, exceto pelo envolvimento de microrganismos. Entretanto, até o presente momento, existem poucos estudos

comparando a endometrite infecciosa e não-infecciosa em éguas (KOTILAINEN; HUHTINEN; KATILA, 1994). Éguas com alterações anatômicas no trato reprodutivo (ex., má conformação vulvar, lacerações do esfíncter vestíbulo-vaginal, útero penduloso, contratilidade uterina ineficiente, incompetência cervical e dobras endometriais atrofiadas) são mais predispostas à aspirarem ar e acumularem fluído ou urina na vagina e no útero, o que propicia ao desenvolvimento de endometrite infecciosa e/ou não-infecciosa (TROTTER; MCKINNON, 1988; CANISSO; STEWART; COUTINHO DA SILVA, 2016). Éguas com resposta imune competente e anatomia funcional do trato reprodutivo são aptas a limparem-se de uma infecção de forma espontânea (i.e., éguas resistentes à endometrite), enquanto que éguas com uma resposta imune deficiente podem ser incapazes de combater o desenvolvimento de uma infecção ou ainda, apresentarem uma inflamação persistente (FUMUSO et al., 2003, 2007; CHRISTOFFERSEN et al., 2012a, 2015b).

2.1. Endometrite infecciosa

A endometrite infecciosa é a principal responsável pela subfertilidade em equinos (WINGFIELD DIGBY; RICKETTS, 1982; RIDDLE; LEBLANC; STROMBERG, 2007). Microrganismos, incluindo bactérias patogênicas e oportunistas e fungos, podem ser introduzidos no útero durante a cobertura. Enquanto éguas resistentes devem responder rapidamente à presença dos microrganismos, uma resposta imune inadequada e drenagem uterina prejudicada (ex., na presença de útero penduloso, cérvix deficiente, ou contratilidade miometrial deficiente) pode predispor à infecção (LEBLANC; CAUSEY, 2009; OVERBECK; WITTE; HEUWIESER, 2011). A endometrite é mais associada à bactérias aeróbicas (RIDDLE; LEBLANC; STROMBERG, 2007), entretanto, bactérias anaeróbicas também podem invadir o útero (RICKETTS; MACKINTOSH, 1987). De interesse, pesquisas retrospectivas identificaram que 25-60% das éguas incapazes de manterem uma gestação apresentam uma infecção uterina bacteriana (COLLINS, 1964; BAIN, 1966). Nos casos clínicos, as bactérias mais comuns isoladas em éguas com endometrite são as espécies de Streptococcus, seguidas pelos Coliformes, *Pseudomonas aeruginosa* e *Staphylococcus aureus* (LEBLANC; MAGSIG; STROMBERG, 2007; LEBLANC; CAUSEY, 2009; BELTAIRE; CHEONG; COUTINHO DA SILVA, 2012; WALTER et al., 2012; CANISSO; STEWART; COUTINHO DA SILVA, 2016) (Tabela 1). Entre todos, *Streptococcus equi subsp.*

zooepidemicus (*Streptococcus zooepidemicus*) e *Escherichia coli* são os maiores causadores de endometrite aguda e crônica, respectivamente (ALBIHN; BÅVERUD; MAGNUSSON, 2003; FRONTOSO et al., 2008). É importante ressaltar que o *Streptococcus zooepidemicus* também demonstrou causar infecções latentes e profundas no endométrio de éguas, fazendo-as resistentes às terapias tradicionais (PETERSEN et al., 2015).

Os fungos são mais incomuns de serem associados com a endometrite (1-5%), e podem ocorrer sozinhos ou associados à bactérias (DASCANIO; SCHWEIZER; LEY, 2010). Aspergillus e Candida são os gêneros mais comuns, mas outras espécies também são menos identificadas (e.g., *Mucor sp*) (DASCANIO; SCHWEIZER; LEY, 2010; SILVA; ALVARENGA, 2011; BELTAIRE; CHEONG; COUTINHO DA SILVA, 2012) (Tabela 1). Pode-se notar que a endometrite fúngica comumente ocorre como uma infecção oportunista e é observada após a utilização de antimicrobianos intrauterinos (HINRICHS; SPENSLEY; MCDONOUGH, 1992; STOUT, 2008; SILVA; ALVARENGA, 2011).

Éguas susceptíveis à EPPC são propensas ao desenvolvimento de infecções crônicas, e, algumas destas infecções ocorrem em decorrência da capacidade das bactérias e dos fungos produzirem biofilme (RYAN A FERRIS, 2014; BEEHAN et al., 2015; FERRIS, 2017). Biofilme é um agregado complexo de microrganismos e suas secreções (matriz extracelular de substâncias poliméricas) (STOODLEY et al., 2002), o qual confere a habilidade dos microrganismos evadirem o sistema imune (JENSEN et al., 1990; MUSTOE, 2004; SHAH et al., 2006; THURLOW et al., 2011). O biofilme funciona como uma barreira para a difusão dos antimicrobianos, com sua penetração limitada, resulta em uma resistência à terapia antimicrobiana, particularidade quando comparada à infecção por bactérias planctônicas (bactérias sem biofilme) (BROWN; ALLISON; GILBERT, 1988; ANWAR; STRAP; COSTERTON, 1992; MAH; O'TOOLE, 2001).

A mudança entre bactérias do estado planctônico para o biofilme ocorre via sinalização celular de molécula cíclica di-GMP (HICKMAN; TIFREA; HARWOOD, 2005; ROMLING; GALPERIN; GOMELSKY, 2013), que regula a produção de exopolisacarídeos, alginato, Pel e Psl (KUCHMA; CONNOLLY; O'TOOLE, 2005; MERIGHI et al., 2007; HAY; REMMINGHORST; REHM, 2009). Se sabe que tanto Pel quanto Psl estão envolvidos na fixação de uma bactéria em um substrato celular ou não-

celular, e na fixação de uma microcolônia e estabilização do DNA extracelular para dar suporte ao biofilme (VASSEUR et al., 2005; MA et al., 2006; GHAFOOR; HAY; REHM, 2011; CHEW et al., 2014; JENNINGS et al., 2015). Aproximadamente 80% das bactérias isoladas do útero de éguas são capazes de produzirem biofilme (FERRIS et al., 2014, 2016; RYAN A FERRIS, 2014; BEEHAN et al., 2015). A imunidade do hospedeiro e o microambiente são conhecidos por apresentarem uma relação na formação do biofilme em outro sistemas do corpo como a cavidade oral (BERGER et al., 2018; MORSE et al., 2018); Contudo, resta serem determinados como estes fatores contribuem à formação do biofilme e patogênese da endometrite no útero equino (FERRIS et al., 2014, 2017; BEEHAN et al., 2015).

Tabela 1. Bactérias e fungos comumente isolados do útero de éguas com endometrite infecciosa. G+: Gram-positiva; G-: Gram-negativa.

	Microrganismo	Superfamília	Características
Bactéria	*Streptococcus zooepidemicus*	Lactobacillales	G+, agente oportunista com potencial venéreo
	Escherichia coli	Enterobacterales	G-, oportunista, anaeróbico facultativo
	Pseudomonas aeruginosa	Pseudomonadales	G +, potencial venéreo, aeróbico
	Klebsiella pneumoniae	Enterobacterales	G-, agente oportunista, anaeróbico facultativo, potencial venéreo
	Staphylococcus spp	Bacillales	G+, oportunista, anaeróbico facultativo
	Taylorella equigenitalis	Burkholderiales	G-, venéreo, microaerofílico, causa endometrite severa e purulenta
	Enterobacter cloacae	Enterobacterales	G-, oportunista, anaeróbico facultativo
	Proteus spp	Enterobacterales	G-, oportunista, anaeróbico
Fungo	*Candida spp.*	Saccharomycetales	Levedura, 58–69% das endometrites fúngicas
	Aspergillus spp.	Eurotiales	Hifa septada, 25–26% das endometrites fúngicas
	Mucor spp.	Mucorales	Hifas asseptadas, 5–12% das endometrites fúngicas

Adaptado de Canisso, Segabinazzi & Fedorka (2020).

No passado, acreditava-se que as fêmeas mamíferas apresentavam um ambiente uterino estéril (VERSTRAELEN et al., 2016; MORENO; FRANASIAK, 2017; BAKER; CHASE; HERBST-KRALOVETZ, 2018); entretanto, essa afirmação foi alterada após a publicação do Projeto do Microbioma Humano (2007), que mostrou que a cavidade uterina abriga uma microbiota única (MORENO; FRANASIAK, 2017; PELZER et al., 2017; BAKER; CHASE; HERBST-KRALOVETZ, 2018). Nos equinos, o útero suporta uma microbiota diversa, e sua composição aparenta ser extremamente semelhante à encontrada na região externa da cérvix (HEIL et al., 2018). Essa correlação entre a microbiota do lúmen uterino e da região cranial da vagina, pode ser explicada pela abertura da cérvix durante o estro (HEIL; PACCAMONTI; SONES, 2019). A microbiota uterina muda conforme a fase do ciclo estral conforme as pesquisas (ROCK et al., 2011; SUBRAMANIAM et al., 2016). Proteobacterias são as primeiras bactérias a ambientar o útero, com uma diversidade de microrganismos incluindo Firmicutes, Bacteriodates e Actinobacteria (SWARTZ et al., 2014; HEIL et al., 2018).

A microbiota uterina da mulher e das vacas com endometrite difere das fêmeas saudáveis (BENNER et al., 2018), sugerindo que a endometrite é associada à uma disbiose comensal da microbiota (HEIL; PACCAMONTI; SONES, 2019). A invasão da cavidade uterina em éguas e outros mamíferos geralmente ocorre por via ascendente pela vagina (CANISSO et al., 2014; PELZER et al., 2017), em associação a um ou mais problemas já descritos anteriormente. No entanto, a relação entre a microbiota residente no útero e a prevenção do desenvolvimento da infecção e a potencial relação com a perda gestacional não estão totalmente elucidados em equinos. Assim como, a relação entre as terapias utilizadas em infecções uterinas e o reestabelecimento da saúde da microbiota uterina não foram estudados nessa espécie.

2.2. Endometrite não-infecciosa

No passado, acreditava-se que a endometrite era causada unicamente por infecções bacterianas ou fúngicas. Entretanto, estudos encontraram uma resposta neutrofilica similar em éguas desafiadas com espermatozoides, solução salina ou bactérias (KOTILAINEN; HUHTINEN; KATILA, 1994; TROEDSSON, 1997; TROEDSSON et al., 2001b). O sistema imune da mucosa do trato reprodutivo consiste em duas respostas: a inata e a adaptativa. A imunidade adaptativa responde de forma seletiva e gradual, conforme a identificação dos antígenos, e é mediada pelos linfócitos T

(LIEBERMAN, 2003). Em contraste, a resposta à cobertura é ditada pela imunidade inata (NASH et al., 2010; MARTH et al., 2018). Esta consiste em uma resposta inespecífica, rápida e transitória (MURAILLE; GORIELY, 2017). Iniciada pelos receptores Toll-like (TLRs), imunoglobulinas e complemento, conduz a digestão leucocítica e eliminação do material estranho, independentemente da patogenicidade (MEDZHITOV; JANEWAY, 1998, 2000; JANEWAY; MEDZHITOV, 2002). A predominância da resposta imune inata permite que ocorram desafios recorrentes com espermatozoides e embriões sem que se desenvolvam anticorpos anti-espermatozoides e anti-embriões. Mais especificamente, células T regulatórias (células Treg) reconhecem os antígenos masculinos e desenvolvem um ambiente imunológico tolerogênico pela supressão da inflamação e resposta de rejeição imunológica (MOLDENHAUER et al., 2009; SCHJENKEN; ROBERTSON, 2015). As células Treg são a população de linfócitos T com propriedades imunosupressivas que compreendem tanto CD4+ quanto CD8+ subtipos (JØRGENSEN; PERSSON; HVIID, 2019). Células Treg geralmente agem suprimindo a síntese de citocinas e a função efetora em macrófagos, T-, B-, natural killer (NK) e células dendríticas (SAKAGUCHI, 2000; SHEVACH, 2002).

As células Treg ainda apresentam uma relação extrema mediando a tolerância imune requerida pela implantação embrionária pelo aumento circulante de CD4+ CD25+ altamente enriquecidas com proteína P3 do fator de transcrição Treg (FoxP3) (ALUVIHARE; KALLIKOURDIS; BETZ, 2004; SOMERSET et al., 2004; MOLDENHAUER et al., 2009). Se sabe que a FoxP3 foi identificada como um potencial marcador de Tregs em ratos, sendo essencial para atividade imunosupressiva das células Treg (HORI; NOMURA; SAKAGUCHI, 2003; KHATTRI et al., 2017). Além disso, transcrições relacionadas à Treg encontram-se aumentadas durante a gestação quando comparadas com éguas não-gestantes em diestro, e células FoxP3+ na unidade fetoplacentária estão associadas com a prenhez e idade gestacional em éguas (FEDORKA et al., 2020). Contudo, ainda não está determinado se a susceptibilidade à EPPC está associada com a interrupção deste mecanismo nas éguas e consequentemente comprometer o estabelecimento gestacional. Fatores solúveis, incluindo fator de crescimento transformador beta, prostaglandina E, e receptores TLR4, presentes no fluído seminal do macho, e também contribuem para o desvio da resposta imune que dirige o desenvolvimento deste ambiente tolerogênico à imunidade em modelos *in vivo* de ratos e *in vitro* humanos (CHEN et al., 2003; BARATELLI et al., 2005; MOLDENHAUER et

al., 2009). Curiosamente, por um longo tempo especula-se que éguas cobertas pelo mesmo garanhão diversas vezes durante a mesma temporada reprodutiva, especialmente em programas de transferência de embriões, são predispostas ao desenvolvimento de EPPC e não emprenharem. Contudo, índices de prenhez satisfatórios são encontrados em éguas submetidas à machos que não foram usados previamente na temporada reprodutiva. A alteração de alguns destes efeitos fisiológicos pode influenciar na resposta imunológica uterina após repetidas exposições ao mesmo macho, entretanto, esta hipótese ainda não foi criticamente evidenciada em equinos.

2.3. Resposta imune inata

A resposte imunológica é imediatamente ativada após o reconhecimento do antígeno e sinalização pela células epiteliais presentes no endométrio (NASH et al., 2010; MARTH et al., 2018). A detecção de partículas estranhas induz a ativação do sistema imune inato, o qual é considerado a primeira linha de defesa. As maiores funções da resposta imune inata são: 1) recrutar células imunes aos sítios de infecção através da ativação de citocinas como quimiocinas; 2) ativar a cascata de complemento para promover a limpeza das células mortas; 3) induz a ativação do sistema imune adaptativo contra a presença dos antígenos, e 4) age como uma barreira física para os microrganismos invasores e partículas. Muitas destas funções estão sendo estudadas pelo seu envolvimento no desenvolvimento da endometrite e serão apresentadas aqui.

Acredita-se que o braço mais antigo do sistema imune, o sistema complemento, é utilizado de forma inespecífica na limpeza fagocítica de células danificadas e microrganismos (KUMAR, 2019). O complemento é um contribuinte importante na opsonização de secreções uterinas e quimiotaxia de células inflamatórias, precedente à uma resposta inflamatória (HAKANSSON; ALBIHN; MAGNUSSON, 1993). No caminho clássico do complemento, a subunidade C4 liga-se ao IgM/IgG associado a C1q, iniciando a clivagem enzimática de C4 em C4a e C4b e C2 em C2a e C2b (GROSSMAN et al., 2016) (Figura 1). A associação entre C4b e C2b ativa C3 convertase. Após a C3 convertase cliva C3 em C3a e C3b (WEILER et al., 1976) e a C3a auxilia no recrutamento dos leucócitos e futura ativação do complemento (PANGBURN; SCHREIBER; MÜLLER-EBERHARD, 1977; CONRAD; CARLOS; RUDDY, 1978). Ainda, a ligação de C3b e C4b ativa a convertase (AHEARN; FEARON, 1989; KRYCH-GOLDBERG; ATKINSON, 2001). Tanto C4b quanto C3b são aptas a ligarem-se à superfície de

microrganismos ou imunoglobulinas adicionais (NONAKA, 2014). A C5 convertase quebra C5 em C5a e C5b, resultando em mudanças conformacionais e ativação do caminho final do complemento, que promove a formação de um complexo de ataque de membrana (MAC) (WALPORT, 2001). A MAC é sintetizada pelas interações entre C5b e outros componentes terminais (ex., C6, C7, C8 e C9) e lisa as células alvo através da criação de poros em suas membranas plasmáticas (RUS; CUDRICI; NICULESCU, 2005). A cascata do complemento também pode ser desencadeada por um caminho alternativo, o qual envolve a ligação direta de C3b com os antígenos (MERI, 2016). Curiosamente, a ativação deste sistema foi encontrada em resposta à cobertura em equinos. O espermatozoide equino foi citado por induzir a cascata do complemento, resultando em um aumento de C3b e C5a, leucotrienos e prostaglandinas, e quimiotaxia de PMNs no útero (WATSON; STOKES; BOURNE, 1987b; PYCOCK; ALLEN, 1988, 1990).

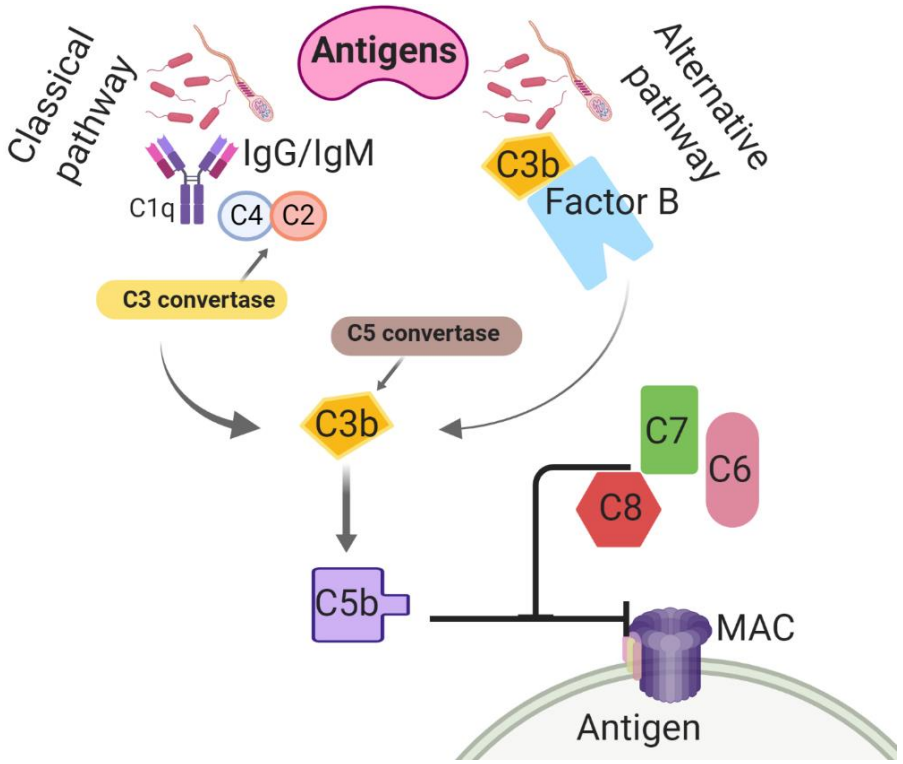

Figura 1. Ativação clássica e alternativa da cascata do complemento no lúmen uterino de éguas pós-cobertura. C1q: componente de complemento 1; C2: componente de complemento 2; C3: complemento 3; C4: complemento 4; C5: complemento 5; C6: complemento 6; C7: complemento 7; C8: complemento 8; IgG: imunoglobulina G; IgM: imunoglobulina M; MAC: complexo de ataque de membrana. (Adaptado de Canisso, Segabinazzi & Fedorka, 2020).

Um fator de clivagem do complemento C3b foi encontrado nas secreções uterinas tanto em populações de éguas resistentes, quanto susceptíveis à endometrite (ASBURY; GORMAN; FOSTER, 1984; WATSON; STOKES; BOURNE, 1987a; WATSON, 1988). As diversas classes de imunoglobulinas (ex., IgA, IgG e IgM) também foram identificadas nas secreções uterinas de éguas. Associado ao complemento, estas moléculas exercem um papel importante na apresentação dos antígenos às células T (ASBURY et al., 1980; MITCHELL et al., 1982; WILLIAMSON et al., 1983; WIDDERS et al., 1984; TROEDSSON; LIU; THURMOND, 1993). Curiosamente, não foram encontradas diferenças entre éguas resistentes e susceptíveis quanto à produção de subunidades do complemento e imunoglobulinas, sugerindo que a influência destas moléculas é limitada na patogenicidade e susceptibilidade à EPPC (TROEDSSON, 1999; DELL'AQUA JR et al., 2006).

Microrganismos e partículas estranhas (ex., espermatozoides e proteínas do plasma seminal) também são detectadas através da apresentação dos antígenos para receptores de reconhecimento de padrões (PRRs) localizados nas células epiteliais do endométrio (KAWAI; AKIRA, 2010; CRONIN et al., 2012; MARTH et al., 2015). Estas células, ao lado das células imunes (ex., macrófagos teciduais, células NK e neutrófilos) que elas recrutam, produzem vários tipos de citocinas, incluindo quimiocinas. As quimiocinas são responsáveis pela mobilização dos leucócitos até o sítio de inflamação, enquanto outras citocinas permitem a diferenciação e ativação de outras células imunes quimiotaxadas (WIRA et al., 2005; KITAYA; YAMADA, 2011). Coletivamente, estas células formam uma barreira física e imunológica na mucosa uterina (FARAGE et al., 2011).

Receptores tipo Toll são uma família de proteínas transmembranas expressas nas células de mamíferos (AN et al., 2002; SILVA et al., 2010; SWANGCHAN-UTHAI et al., 2012; CHEN et al., 2014), sendo as principais responsáveis no reconhecimento de antígenos (TURNER; HEALEY; SHELDON, 2012). Se sabe que o TLR2 e TLR4 encontram-se aumentados no útero de éguas resistentes à EPPC após a inoculação de *Escherichia coli* (MARTH et al., 2015). Além disso, pesquisas em outras espécies indicam que o esperma humano e de roedores pode estimular a transcrição de citocinas pró-inflamatórias, incluindo quimiocinas (TNFα, IL1β, CXCL8) e prostaglandina E, ativando o sistema complemento local (C3), mediado via TLR/4 (ELWEZA et al., 2018; AKTHAR et al., 2019; EZZ et al., 2019).

Outro grupo de PRRs são os receptores NOD-like (NLR), os quais são responsáveis pela detecção de patógenos intracelulares (FRANCHI et al., 2009). Os NRLs são expressos em diferentes células, incluindo células imunes e epiteliais, embora certos membros da família de NLR sejam expressos primariamente em fagócitos, incluindo macrófagos e neutrófilos. Uma variação genética destes genes pode predispor humanos à desenvolverem doenças inflamatórias severas (INOHARA et al., 2005; FRANCHI et al., 2009), contudo, a relação entre os NLRs e a patogênese da endometrite ainda não foi elucidada em equinos.

Receptores de reconhecimento de padrões podem responder à diversas matrizes de antígenos e assim ativar citocinas pró-inflamatórias (MARIATHASAN et al., 2006; SCHRODER; TSCHOPP, 2010; BARBÉ; DOUGLAS; SALEH, 2014) para regularem a resposta imune (BENKO et al., 2010; CUI et al., 2010). A ativação de TLRs é um evento chave para a iniciação da cascata inflamatória (TAKEDA; AKIRA, 2004), a qual estimula o fator nuclear kappa beta (NF-κB). O NF-κB é composto por cinco subunidades (RelA {p65}, RelB, ReL, p50, p52), e pode ser ativado pelas respostas imunes inatas (canônica) e adaptativas (alternativa) (LAWRENCE, 2009). A via canônica é desencadeada pelos microrganismos e citocinas pró-inflamatórias (IL1 e TNFα) (LAWRENCE, 2009). O desencadeamento desta via inicia a ativação de RelA- ou cRel- (KARIN; BEN-NERIAH, 2000), que são regulados pela IkB quinase β (IIK β) através da fosforilação de inibidores de kB (IkB) (ZANDI et al., 1997). As IkB quinases são as chaves regulatórias da via de NF-κB (YAMAMOTO; GAYNOR, 2004). De forma controvérsia, na via alternativa, NF-κB é ativado por outros bioprodutos como linfotoxina β, CD40, fator de ativação de célula B e receptor ativador de NF-κB (MATSUSHIMA et al., 2001; SENFTLEBEN et al., 2001; DEJARDIN et al., 2002; NOVACK et al., 2003; BONIZZI et al., 2004; BONIZZI; KARIN, 2004). A ativação do complexo RelB/p52 e IkB quinase α é requerida nesta via para a fosforilação e processamento dos precursores p52 e p100 (SENFTLEBEN et al., 2001) (Figura 2).

A via NF-κB assim ativa os genes codificando citocinas pró-inflamatórias, incluindo quimiocinas e cicloxigenase-2 (COX-2) (TAKEDA; AKIRA, 2004; GIRLING; HEDGER, 2007). As citocinas e COX-2 geram um sinal que modula as células imunes à gerarem uma resposta inflamatória aguda (CHANDRASEKHARAN; SIMMONS, 2004). A expressão de genes tanto de famílias NLR e TLR são regulados positivamente após a inoculação intrauterina de *Escherichia coli* em éguas. Além disso, vias de transdução de

sinal imune severo e vias de interação entre citocinas também são estimuladas após a inoculação com *Escherichia coli* (MARTH et al., 2015). A determinação se os espermatozoides realizam uma resposta similar aos microrganismos no sistema imune ainda deve ser elucidada.

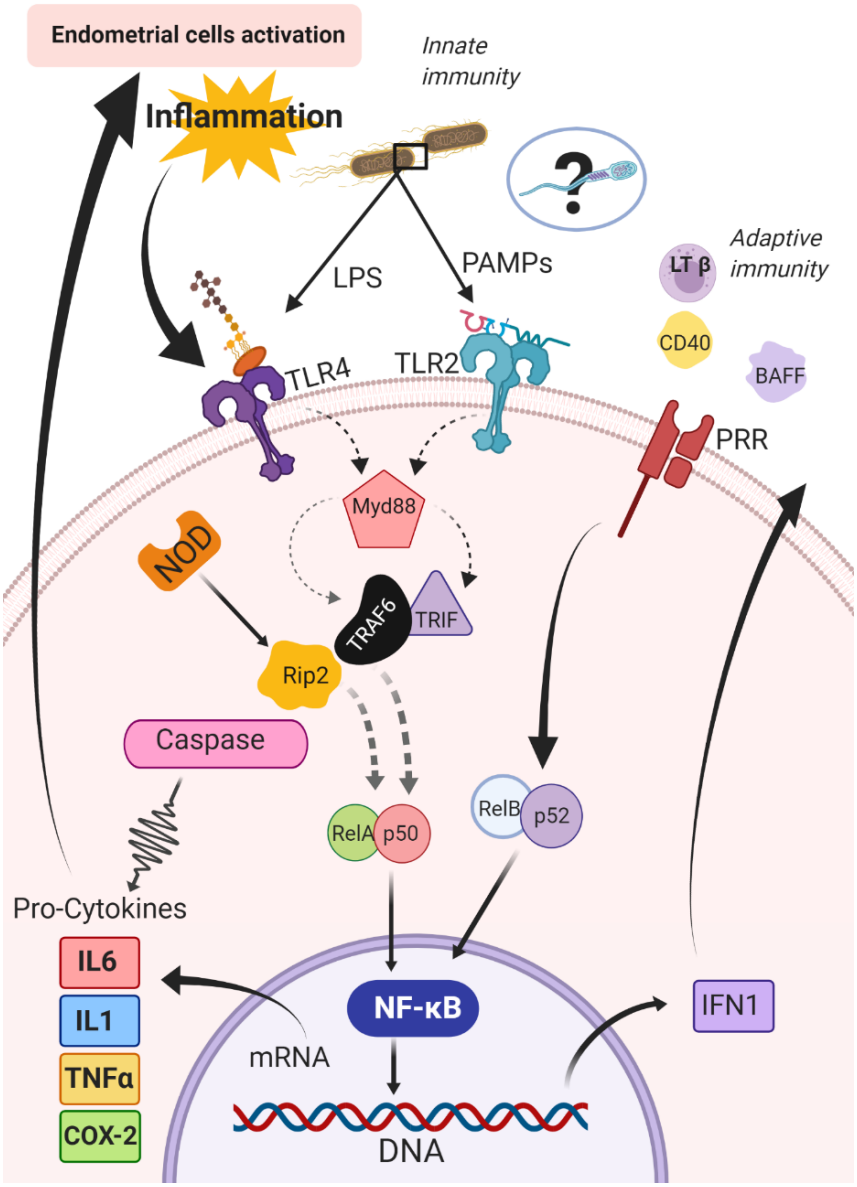

Figura 2. Vias canônica e alternativa para ativação de fator kappa beta (NF-kB) no endométrio de éguas. BAFF: fator de ativação de células B; CD40: Grupo de diferenciação 40; COX-2: cicloxigenase-2; IFN1: interferon tipo 1; IL1: interleucina 1; IL6: interleucina 6; LPS: lipopolissacarídeos; LTβ: linfotoxina β; MyD88: resposta primária de diferenciação de mieloide 88; NF- κB: fator nuclear kappa beta; NOD: ligação de nucleotídeo e oligomerização; PAMPs: padrões moleculares associados à patógenos; PRR: receptores de reconhecimento de padrões; RelA/p50 e RelB/p52: subunidades do complexo NF-κB; Rip2: proteína que interage com o receptor 2; TLR2: receptores Toll-like tipo 2; TLR4: receptores Toll-like tipo 4; TNFα: fator de necrose tumoral α; TRIF: interferon beta indutor de adaptador contendo domínio TIR; TRAF6: fator associado ao receptor 6. (Adaptado de Canisso, Segabinazzi & Fedorka, 2020).

Parte destes PRRs exigem uma ligação simultânea à outras superfícies de receptores celulares endógenos para serem ativados. Por exemplo, quando TLR4 liga-se à CD14 a subsequente detecção de LPS diminui a resposta inflamatória (GIRLING; HEDGER, 2007; CRONIN et al., 2012). Após o reconhecimento do antígeno, o recrutamento de proteínas adaptadas junto com a cascata MYD88 dependente, inicia a secreção de citocinas pró-inflamatórias ou Interferon-β indutor de adaptador contendo domínio TIR (TRIF-), que resulta na produção de interferon tipo 1 (IFN) associado ao aumento de citocinas inflamatórias como as quimiocinas (GIRLING; HEDGER, 2007; CRONIN et al., 2012). A cascata MyD88 dependente induz famílias de quinases associadas à IL-1R e consequentemente a ativação de quinase dependente de ubiquitina por fator 6 associado ao TNFR (GOHDA; MATSUMURA; INOUE, 2004; LU; YEH; OHASHI, 2008). A quinase dependente de ubiquitina induz a ativação de NF-κB e a resposta imune inespecífica pela transcrição de NF-κB (SATO et al., 2005).

Inicialmente, citocinas são sintetizadas como moléculas precursoras que necessitam de uma ativação. Diversos tipos de moléculas podem ativar as citocinas (ex., elastase, catepsinas, metaloproteinases e tripsina). Entretanto, as caspases, uma família grande de proteases, exercem um papel mais abrangente que outras moléculas (VAN DE CRAEN et al., 1999). Por exemplo, a família caspase-1 (caspase 1, 4, 5, 11, 12 e 14) é primariamente envolvida na regulação da ativação de citocinas (VAN DE CRAEN et al., 1999), e a família caspase-3 (caspase 3 e 7) é capaz de ativar as citocinas pró-inflamatórias (BLACK et al., 1988; HAZUDA et al., 1990; ITO et al., 1996). Especificamente, caspase-1 ativa a interleucina 1 beta (IL1β), que é constitutivamente expressa e estimulada no endométrio de éguas após experimentos com inoculação bacteriana (MARTH et al., 2015), e também é sintetizada por estimulação de NF-κB (CHANDRASEKHARAN; SIMMONS, 2004). Sob a ação da prostaglandina endoperóxido-sintase, especialmente COX-2 durante a inflamação, ocorre a síntese de prostaglandinas (BOERBOOM et al., 2004). Nos equinos, um aumento de COX-2 foi observado no endométrio após exposição ao plasma seminal ou diluente (PALM et al., 2008), bem como, ocorreu uma maior expressão de prostaglandina F2 alfa (PGF2 α) no útero de éguas normais 16 horas após a cobertura (NASH et al., 2010).

Para a síntese de prostaglandinas e citocinas pró-inflamatórias, principalmente interleucina 1 (IL1), interleucina 6 (IL6), fator de necrose tumoral alfa (TNFα), ocorre a ativação das células endoteliais vasculares. Isso conduz para a formação de arteríolas e

dilatação de vênulas nos locais afetados, assim, aumentando a permeabilidade vascular e extravasamento de exsudato intersticial, causando edema (COLLINS, T.; CONTRAN, R. S.; KUMAR, 1999). A expressão endometrial de várias interleucinas pró-inflamatórias, incluindo IL1β, quimiocina ligante 8 (CXCL8, formalmente conhecida como IL8), e TNFα é aumentado em éguas susceptíveis à EPPC quando comparado às éguas resistentes, mesmo antes da exposição a algum antígeno. Este aumento em éguas susceptíveis também observado após os desafios com patógenos ou espermatozoides (FUMUSO et al., 2006, 2007; PALM et al., 2008).

Com alterações na permeabilidade do endotélio vascular, iniciam-se as respostas celulares. As células vasculares endoteliais aumentam com a expressão de selectina P através do estímulo inflamatório, que se liga à selectina L na superfície dos neutrófilos, induzindo a quimiotaxia (DORÉ; SIROIS, 1996). Assim, neutrófilos produzem integrinas para ligar-se à moléculas de adesão em células endoteliais até prenderem-se e aderirem-se à parede dos vasos sanguíneos (TIZARD, 2008). Após a detecção dos corpos estranhos, os neutrófilos migram do endométrio para o lúmen uterino em 30 minutos (KATILA, 1995b), ocorrendo um pico da resposta inflamatória entre 6 e 12 horas após o contato com antígeno (TROEDSSON, 1999) (Figura 3). Éguas susceptíveis à EPPC geralmente demonstram um aumento de neutrófilos entre 2 e 12 horas após a cobertura em comparação com as demais fêmeas (WOODWARD et al., 2013b). Além de realizarem a fagocitose, os neutrófilos também secretam citocinas e mediadores quimiotáticos adicionais, estimulando ainda mais o processo inflamatório (TIZARD, 2008). Leucócitos então secretam prostaglandinas, que promovem a contratilidade endometrial e auxiliam na limpeza mecânica do útero em éguas saudáveis (TROEDSSON et al., 1995).

Os neutrófilos são as primeiras células imunes a responderem após o reconhecimento de um antígeno através da imunidade inata. Além de realizarem a fagocitose e a secreção de enzimas líticas em resposta à estes patógenos, os neutrófilos formam armadilhas extracelulares de neutrófilos (NETs). As armadilhas extracelulares de neutrófilos são associados à moléculas de DNA tendo propriedades antimicrobianas e imunomoduladoras (LÖGTERS et al., 2009), que são desencadeadas por diferentes agentes inflamatórios como espécies reativas de oxigênio (FUCHS et al., 2007), complexo antígeno-anticorpo (GARCIA-ROMO et al., 2011), CXCL8, lipopolissacarídeos e forbol-miristato-acetato (BRINKMANN et al., 2004). A atividade antimicrobiana de NETs, chamada de NEToses, ocorre com a ruptura dos neutrófilos e

liberação dos grânulos, permitindo que a cromatina entre em contato com os antígenos e outras células imunes (BRINKMANN, 2011). Diversas enzimas (ex., elastase, proteína 3, catepsina G e mieloperoxidase) e histonas, exercem funções na NETose (WEINRAUCH et al., 2002; WARTHA et al., 2007; MARIN-ESTEBAN et al., 2012; VAN DER WINDT et al., 2012). A formação de NETs é um mecanismo complementar para eliminação de bactérias, sendo estas capazes de desencadearem endometrite em éguas (REBORDÃO et al., 2014b).

Quando exacerbado, este aumento nos sinais pró-inflamatórios e o recrutamento das células imunes podem gerar danos teciduais. Assim, mecanismos se fazem necessários para encerrar o processo de resolução da inflamação. Um aumento nas citocinas anti-inflamatórias ou pleiotropicas é observado rapidamente entre 2 e 6 horas após a cobertura em éguas resistentes (WOODWARD et al., 2013b) (Figura 3). Eles secretam suas propriedades através da inibição de mediadores pró-inflamatórios, competindo por receptores pró-inflamatórios ou causando morte celular (OPAL; DEPALO, 2000). A interleucina 10 (IL-10), antagonista 1R (IL1RN), interleucina 4 (IL4) e interleucina 13 (IL13) são consideradas anti-inflamatórias e exercem um papel importante no término deste processo inflamatório (AREND; GUTHRIDGE, 2000; COUPER; BLOUNT; RILEY, 2008; CHRISTOFFERSEN et al., 2012b; WOODWARD et al., 2013b). Sabe-se que a IL1RN exerce uma função no balanço pró- e anti-inflamatório porque esta citocina compete com IL1 pela ligação aos seus receptores IL1, que impede a ligação de IL1 α e IL1 β (DRIPPS et al., 1991). Comumente, a IL10 é sintetizada relativamente tarde durante a resposta inflamatória, e age com um efeito anti-inflamatório generalizado através da redução da transcrição de citocinas pró-inflamatórias liberadas por monócitos e macrófagos (FIORENTINO et al., 1991; CASSATELLA et al., 1994).

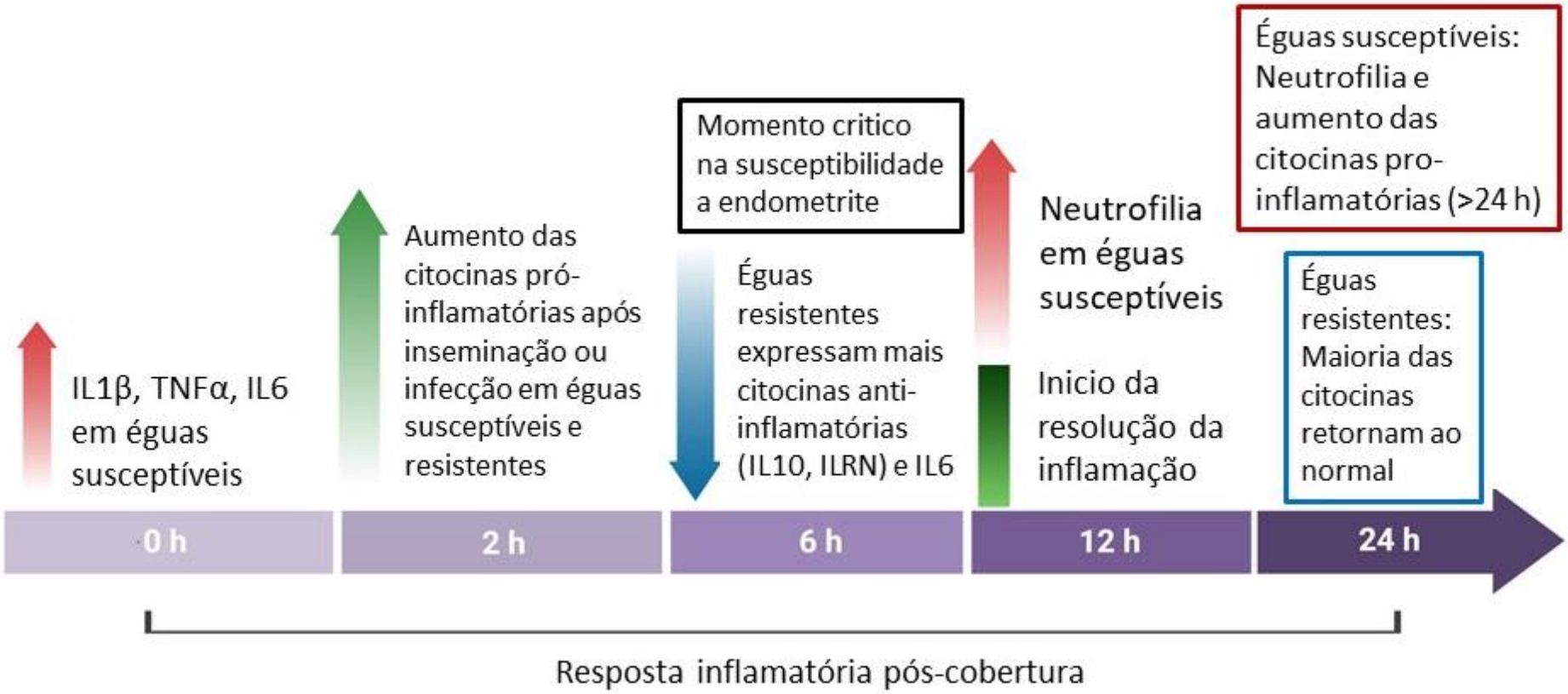

Figura 3. Visão geral da dinâmica de citocinas endometriais em éguas resistentes e susceptíveis à endometrite imediatamente após a cobertura (0 horas) à 24 horas pós-cobertura. (Adaptado de Canisso, Segabinazzi & Fedorka, 2020).

Enquanto a IL6 exerce inicialmente uma resposta pró-inflamatória, sua função é considerada pleiotrópica por ser capaz de ativar diversos receptores e vias com função anti-inflamatória após o processo de inflamação. Um estudo demonstrou que a secreção destas citocinas anti-inflamatórias encontra-se defasado em éguas susceptíveis (WOODWARD et al., 2013b). Éguas susceptíveis á endometrite apresentam uma expressão significantemente mais baixa destas citocinas, indicando uma falha no desenvolvimento desta resposta (FUMUSO et al., 2006). Os autores criaram a hipótese de que esta falha no estabelecimento da resposta anti-inflamatória prolonga a inflamação endometrial em éguas susceptíveis à EPPC.

Além da produção de citocinas anti-inflamatórias de forma defasada, éguas susceptíveis também foram descritas por apresentarem falhas na contratilidade miometrial. Um estudo relatou que éguas susceptíveis à EPPC apresentaram uma resposta miometrial diferente quando submetidas à desafios com bactérias em comparação à éguas resistentes, incluindo alterações de frequência, duração e intensidade das contrações (TROEDSSON et al., 1993). Curiosamente, a contratilidade miometrial é fortemente conectada dentro do sistema imune e particularmente na liberação de óxido nítrico (NO) por citocinas. Produzido através do estímulo da óxido-nítrico-sintase, o NO é uma molécula de sinalização difundida, cálcio independente, que induz o relaxamento do músculo liso (GRISCAVAGE; WILK; IGNARRO, 1996; LIU et al., 1996). Curiosamente, mediadores pró-inflamatórios como IL1 e IFNα, conduzem a um aumento na transcrição desta molécula (GRISCAVAGE; WILK; IGNARRO, 1996). Essa redução da atividade da musculatura lisa é capaz de interferir na limpeza uterina de éguas susceptíveis à EPPC (ALGHAMDI et al., 2005).

Diversos estudos têm investigado a relação do NO com a susceptibilidade à endometrite equina. Pesquisas conduzidas com *explantes* endometriais indicaram uma resposta dose-dependente em estimulação ao NO, embora uma resposta diminuída seja observada em amostras com qualidade endometrial inferior (KHAN et al., 2017). Em éguas susceptíveis, tanto a expressão de NO e atividade estão reguladas (ALGHAMDI et al., 2005; WOODWARD et al., 2013a; KHAN et al., 2018). O prolongado e determinado aumento de citocinas pró-inflamatórias, tal como IL1β, pode ser a causa deste aumento na atividade de NO, conduzindo ao relaxamento do músculo liso e diminuição da atividade miometrial, todos os quais contribuem ao estabelecimento da patofisiologia da EPPC.

O estágio do ciclo estral (alta *vs.* baixa concentração de progesterona) também interfere na resposta imune uterina em éguas (EVANS et al., 1986). Durante o período de dominância da progesterona, o útero equino encontra-se altamente susceptível à infecções, bem como, com a dominância do estrógeno, o útero é mais apto à realizar a limpeza após infecções (EVANS et al., 1986). Por exemplo, ocorre a expressão de soro amilóide A (SAA) e o aumento da IL10 3 horas após a inoculação bacteriana no diestro, mas não em estro (BOWDISH; DAVIDSON; HANCOCK, 2006; NASH et al., 2010).

Curiosamente, matriz metaloproteinases (MMP) tipo 2 e 9 são significativamente estimuladas 5 horas após a inoculação de *Streptococcus zooepidemicus* tanto no estro quanto no diestro (ODDSDÓTTIR et al., 2008). Matriz metaloproteinases estão envolvidas no remodelamento da matriz extracelular (ECM) (CHAKRABORTI et al., 2003) e moduladas por inibidores teciduais de MMPs (TIMPs), que também foram identificados no endométrio de éguas e mulheres (CURRY; OSTEEN, 2003; ODDSDÓTTIR et al., 2008). Sugeriu-se que o equilíbrio entre TIMPs e MMPs exerce um papel no desenvolvimento de fibrose endometrial e degeneração em fêmeas equinas (ODDSDÓTTIR et al., 2008), mas, ainda existem controvérsias (REBORDÃO et al., 2014a). Sabe-se que éguas susceptíveis à EPPC comumente desenvolvem fibrose endometrial e outras alterações degenerativas como modificação da deposição de colágeno (ALPOIM-MOREIRA et al., 2019).

A patogênese e etiologia da fibrose endometrial em éguas ainda é pouco compreendida. Se sabe que tanto MMPs quanto TIMPs regulam a deposição de colágeno e outros componentes da ECM associados com a fibrose (RAWDANOWICZ et al., 1994; SINGER et al., 1999; BRAUNDMEIER; NOWAK, 2006; HOFFMANN et al., 2009). Ainda, componentes NET (ex., myeloperoxidase, elastase e catepsina G) também estimulam a produção de colágeno tipo 1 e 3 e fator transformador de crescimento β1 (AMARAL et al., 2018; REBORDÃO et al., 2018; SZÓSTEK-MIODUCHOWSKA et al., 2019a). Além disso, a expressão endometrial de IL1β e IL6 é estimulada durante o processo de fibrose endometrial em éguas (SZÓSTEK et al., 2013). Estudos in vitro têm demonstrado os efeitos pró-fibróticos da IL1β no endométrio e em outros tecidos (VESEY et al., 2002; BRAUNDMEIER; NOWAK, 2006; XIAO et al., 2008). Um estudo recente realizado in vitro sugeriu que IL1β e IL6 modulam a produção de ECM, MMP e TIMP nas células endometriais equinas e podem ser importantes reguladores da patogênese da fibrose nestes indivíduos (SZÓSTEK-MIODUCHOWSKA et al., 2019b).

Aparentemente, existe uma associação entre a inflamação e o desenvolvimento de fibrose endometrial, em que IL1β e IL6 aumentam a expressão dos componentes ECM, como MMPs (SZÓSTEK-MIODUCHOWSKA et al., 2019b).

Além disso, as células epiteliais endometriais produzem peptídeos antimicrobianos (ex., defensinas, elafina, catelicidina, lactoferrina e lisozima), permitindo a degradação de microrganismos e espermatozoides de forma inespecífica (WIESNER; VILCINSKAS, 2010). Associada às atividades antimicrobianas, estas proteínas afetam a indução de citocinas, quimiotaxia e proliferação celular, além de modularem tanto a resposta imune inata quando a adquirida (BOWDISH; DAVIDSON; HANCOCK, 2006). Peptídeos antimicrobianos são modulados pela presença de bactérias, estágio do ciclo estral e inflamação. Estes incluem fatores que desestabilizam a parede bacteriana, como defensinas, lisozimas e fosfolipase A2 secretada (LINDE et al., 2008; NEVALAINEN; GRAHAM; SCOTT, 2008), e essa que inibe as enzimas bacterianas, como as inibidoras de leucoprotease secretora, que também são conhecidas como peptídeo antimicrobiano do neutrófilo equino (COUTO et al., 1992; COUTO; HARWIG; LEHRER, 1993; TOMEE et al., 1998). Muitas destas proteínas antimicrobianas encontram-se aumentadas no endométrio da população de éguas susceptíveis, incluindo inibidores secretórios de leucoproteases, defensina equina beta, lactoferrina e lisozima (KOLM et al., 2006; MARTH et al., 2018). Diversas destas proteínas também são produzidas dentro dos grânulos dos neutrófilos. Não se sabe se este aumento é devido a um aumento no mecanismo de defesa dentro das glândulas endometriais de éguas susceptíveis ou pela neutrofilia aumentada causada pelo prolongamento da resposta inflamatória.

Proteínas de fase aguda também estão sendo investigadas por seu envolvimento e capacidade diagnóstica na endometrite, assim como marcadoras sistêmicas da inflamação em equinos (NUNOKAWA et al., 1993; HULTÉN et al., 1999, 2010). Soro amilóide A, haptoglobina e fibrinogênio estão todos sendo investigados (TUPPITS et al., 2014). O endométrio histologicamente normal de éguas demonstrou um aumento na expressão de mRNA de SAA em níveis moderados (BERG et al., 2011). Informações conflitantes existem a respeito da detecção sistêmica de SAA em resposta à endometrite. Em um estudo observou-se um aumento circulante de SAA e fibrinogênio entre 3 à 12 horas após a inoculação de *Escherichia coli*, sendo este aumento correlacionado ao aumento da expressão endometrial de SAA (CHRISTOFFERSEN et al., 2010). Em contraponto,

outro estudo não encontrou alterações na concentração de SAA após a cobertura com sêmen congelado (NASH et al., 2010). Se essas diferenças foram causadas pelo tipo de desafios utilizados ou em decorrência das pequenas amostras analisadas, ainda deve ser determinado. Deve-se ressaltar que não há diferenças na concentração circulante de SAA em éguas susceptíveis e resistentes (CHRISTOFFERSEN et al., 2012b).

3. Diagnóstico

O diagnóstico da endometrite envolve uma abordagem multimodal compreendida por um histórico clínico detalhada. Ultrassonografia, cultura uterina, citologia, e biópsia são as ferramentas mais empregadas no diagnóstico de endometrite em éguas (NIELSEN, 2005) (Tabela 2). Éguas susceptíveis à EPPC podem apresentar um histórico de acúmulo de fluído intrauterino antes e após a cobertura (Figura 4), perdas embrionárias recorrentes, retorno precoce ao estro, falhas em ficar prenha após um bom manejo reprodutivo, e presença de descargas vulvares.

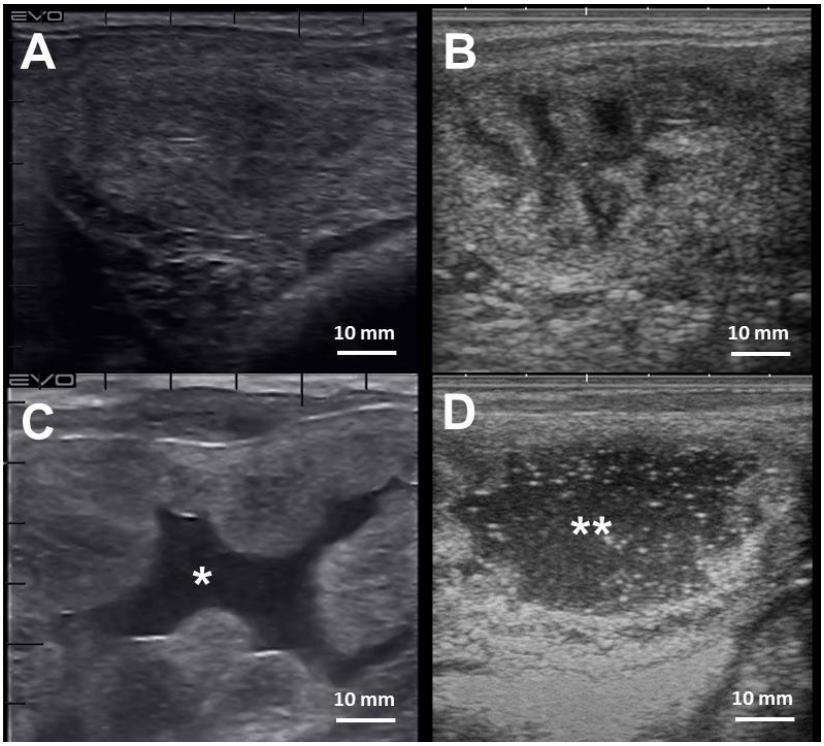

Figura 4. Corte transversal de imagens ultrassonográficas dos cornos uterinos em éguas: (A) Uma imagem do útero equino sem edema endometrial ou acúmulo de líquido intraluminal, comumente observada no diestro; (B) O corno uterino de uma égua em estro, caracterizado pela presença de edema dos vasos linfáticos em torno da submucosa endometrial dando o aspecto de "laranja cortada". (C) Edema exacerbado endometrial com extravasamento e acúmulo de líquido intraluminal (*) em uma égua com endometrite, e (D) extenso acúmulo de fluído hipercogênico (**) em égua com endometrite. Barras de escala de 10 mm (A-D). (Canisso, Segabinazzi *&* Fedorka, 2020).

A citologia endometrial pode ser utilizada para ter acesso ao tipo e proporção das células inflamatórias em relação às células epiteliais endometriais presentes no lúmen uterino (Figura 5). Além disso, a citologia pode ocasionalmente detectar a presença de colônias bacterianas, hifas, fungos e cristais de urina (FERRIS; BOHN; MCCUE, 2015). As amostras de citologia endometrial podem ser obtidas através de um swab com ponta de algodão simples ou dupla, escova citológica ou lavado uterino de baixo volume (COCCHIA et al., 2012; BOHN; FERRIS; MCCUE, 2014; FERRIS; BOHN; MCCUE, 2015). A escova citológica e o lavado uterino de baixo volume permitem uma melhor coleta de amostras para o diagnóstico quando comparados ao swab com ponta de algodão (COCCHIA et al., 2012; WALTER et al., 2012) (Tabela 2). Além disso, as lâminas obtidas com escova citológica apresentam mais células (endometriais e PMNs) quando comparadas às realizadas com swab contendo ponta de algodão (WALTER et al., 2012). Em um estudo realizado, observou-se que os esfregaços obtidos através da escova citológica têm associação positiva entre o número de PMNs e o número de colônias de *Streptococcus β-haemolítico* (COCCHIA et al., 2012), mas não quando *Escherichia coli* foi a primeira bactéria isolada (CHRISTOFFERSEN et al., 2015a). Após a colheita, as amostras endometriais devem ser fixadas e coradas com corantes tipo Romanovsky para avaliação. As amostras podem ser avaliadas tanto com aumento de 400x quanto 1000x e quantificado quanto ao número de neutrófilos em cada 100 células epiteliais (EC) (Figura 5). As seguintes categorias podem ser utilizadas para definir a inflamação endometrial: normal (sem células brancas (WBC) ou raras WBC/ 100 EC), inflamação leve (1-2 WBC/EC), moderada inflamação (3-5 WBC/EC) e inflamação severa (>5 WBC/EC) (FERRIS; BOHN; MCCUE, 2015). Como a endometrite não-infecciosa vai permanecer estimulando a quimiotaxia dos neutrófilos no lúmen uterino, as citologias uterinas não podem ser utilizadas como método único de diagnóstico de endometrite infecciosa. Assim, amostras para cultura endometrial devem sempre ser coletadas e interpretadas em conjunto com o resultado citológico.

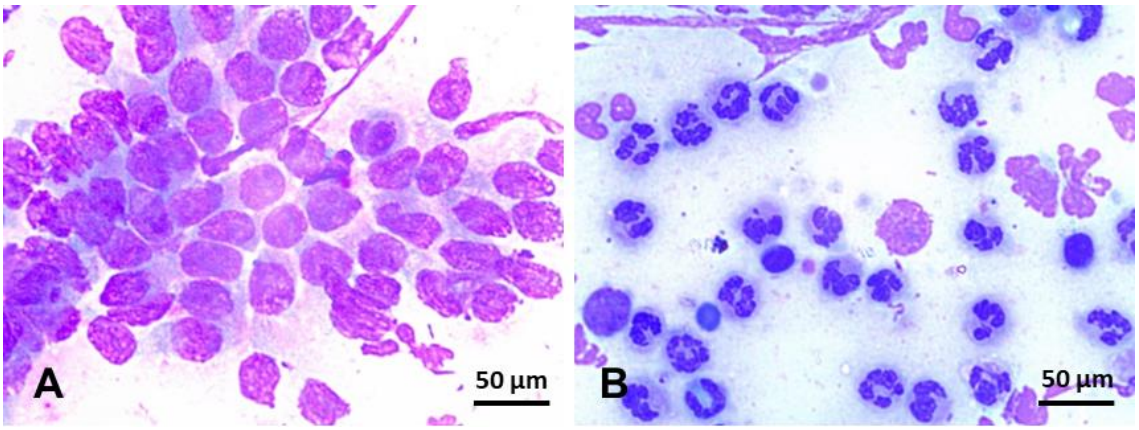

Figura 5. Citologia endometrial de éguas coradas com corantes Romanowsky (x100). (A) Citologia endometrial negativa, nesta lâmina pode-se observar a ausência de células inflamatórias. (B) Citologia endometrial positiva, pode-se observar poucas células epiteliais endometriais, entretanto, observa-se um grande número de neutrófilos hipersegmentados nesta amostra. Barras de escala 50 µm (A e B). (Adaptado de Canisso, Segabinazzi & Fedorka, 2020).

Tabela 2. Resumo dos métodos comuns utilizados no diagnóstico de endometrite em éguas.

Técnicas	Abordagens e aplicações	Limitações
Ultrassonografia	Utilizada como ferramenta de triagem para detectar a presença, qualidade e aparência de IUF, que pode ser sugestivo de endometrite.	Nem todas éguas com endometrite, principalmente as que apresentam endometrite crônica, acumulam IUF. A quantidade e ecogenicidade do fluído pode ser utilizada para predizer a necessidade de outras técnicas para diagnóstico ou abordagens terapêuticas.
Swab com ponta de algodão	Rápido e com baixo custo para a coleta das amostras, tanto para cultura quanto citologia. Os resultados em combinação com a citologia podem ser utilizados para decisão da abordagem terapêutica.	Apenas um pequeno segmento do útero é analisado, e assim, infecções localizadas, onde não existe uma infecção difusa podem ser subdiagnosticadas. Em comparação com a escova citológica, existe uma menor recuperação celular, tornando a avaliação mais difícil.
Escova citológica	Rápido e com baixo custo para a coleta de amostras para cultura e citologia, entretanto, é mais utilizada para citologia.	Apenas um pequeno segment do útero é amostrado, e assim, diagnósticos de infecções locais podem ser perdidos. Bactérias e biofilme não podem ser detectados.
Lavagem uterina com baixo volume	Entra em contato com toda superfície uterina, possibilitando a análise de material para cultura e citologia. Esta técnica é mais utilizada nos casos de endometrite crônica. O fluído recuperado pode ser centrifugado ou permitido decantar para realizar a citologia.	Existe um risco de contaminação com microrganismos comensais do trato reprodutivo caudal. Exige pelo menos um clínico com treinamento e um assistente. Uma quantidade excessiva de fluído pode superdiluir a amostra e causar um falso-negativo, podendo alterar a avaliação citológica. Éguas com útero penduloso podem apresentar uma recuperação de fluído defasada.
Biópsia endometrial	Esta abordagem é principalmente utilizada para avaliação histológica. A biópsia endometrial é uma abordagem sensível e específica para diagnóstico de endometrite em éguas por avaliação histológica e cultura da biópsia. Particularmente utilizada para infecções endometriais profundas. Os resultados podem servir de guia para determinação das estratégias terapêuticas a serem abordadas.	É um procedimento pequeno, mas invasivo. Exige uma pessoa experiente para análise da amostra histológica e realização da cultura.

Adaptado de Canisso, Segabinazzi & Fedorka (2020). IUF: fluído intrauterino.

A cultura endometrial deve ser sempre coletada antes de qualquer procedimento uterino ou vaginal para que seja identificados potenciais contaminantes. A amostra pode ser coletada através de swab com ponta de algodão, lavado de baixo volume e biópsia

(BAIN, 1966; BALL et al., 1988; NIELSEN, 2005; NIELSEN et al., 2010; MØLLER NIELSEN et al., 2012). A cultura endometrial realizada com swab de algodão é a menos sensível (0,34) em comparação com a cultura utilizando biópsia (0,82) (NIELSEN, 2005) ou lavado de baixo volume (0,75) (CHRISTOFFERSEN et al., 2015a). Após a coleta, a amostra deve ser processada ou armazenada em meio de transporte (Ames ou Steward) e ser mantida refrigerada até chegar ao laboratório ou semeado sob o meio de cultura da forma correta. Além disso, pode-se identificar os microorganismos através de testes bioquímicos ou proteínas biológicas ou mapeamento genético. O crescimento bacteriano pode ser grosseiramente estimado através da contagem do número de colônias por placas, sendo sem crescimento (sem colônias), crescimento muito leve (≤ 2 colônias na primeira semeadura), leve (3-5 colônias na primeira semeadura), moderada (>5 na segunda semeadura); e severa (>5 na terceira semeadura) (CHRISTOFFERSEN et al., 2012a; FERRIS, 2016). Após o isolamento bacteriano ou fúngico, deve-se realizar um teste de sensibilidade aos antimicrobianos para que seja determinado o fármaco mais adequado para o tratamento da infecção. Além disso, o PCR está ganhando popularidade na prática clínica para identificação de bactérias e fungos em amostras endometriais. Os resultados podem ser avaliados em 6 horas, enquanto a cultura final e resultados de sensibilidade são demorados (FERRIS et al., 2013).

A biópsia endometrial também pode ser utilizada como ferramenta diagnóstica de endometrite, assim como para prognosticar a capacidade de éguas levarem um potro a termo (KENNEY, 1975; DOIG; MCKNIGHT; MILLER, 1981; RICKETTS; ALONSO, 1991; MØLLER NIELSEN et al., 2012). Um sistema de classificação foi criado por Kenney e Doig, onde o endométrio é avaliado pela distribuição glandular, presença de células inflamatórias, lacunas linfáticas e fibrose, sendo classificada na escala de I-III (KENNEY; DOIG, 1986) (Figura 6). Estes aspectos associados à degeneração tecidual são sinais predisponentes à endometrite, sendo o conjunto encontrado no resultado da biópsia endometrial combinado com o histórico clínico da égua capaz de predizer a fertilidade do animal (KENNEY, 1975; RICKETTS; ALONSO, 1991). Além disso, como mencionado anteriormente, a cultura de amostras de biópsia aumenta a sensibilidade (0,82) quando comparada com a cultura realizada com swab com ponta de algodão (0,34) (MØLLER NIELSEN et al., 2012). Recentemente foi proposto que a expressão gênica das biópsias endometriais, como de defensina- β, lisozima e inibidor secretório de leucoprotease, pode ser utilizado como teste diagnóstico para identificar éguas

susceptíveis à EPPC com sensibilidade de 78-94% (MARTH et al., 2018), entretanto, estes genes ainda devem ser testados na prática clínica.

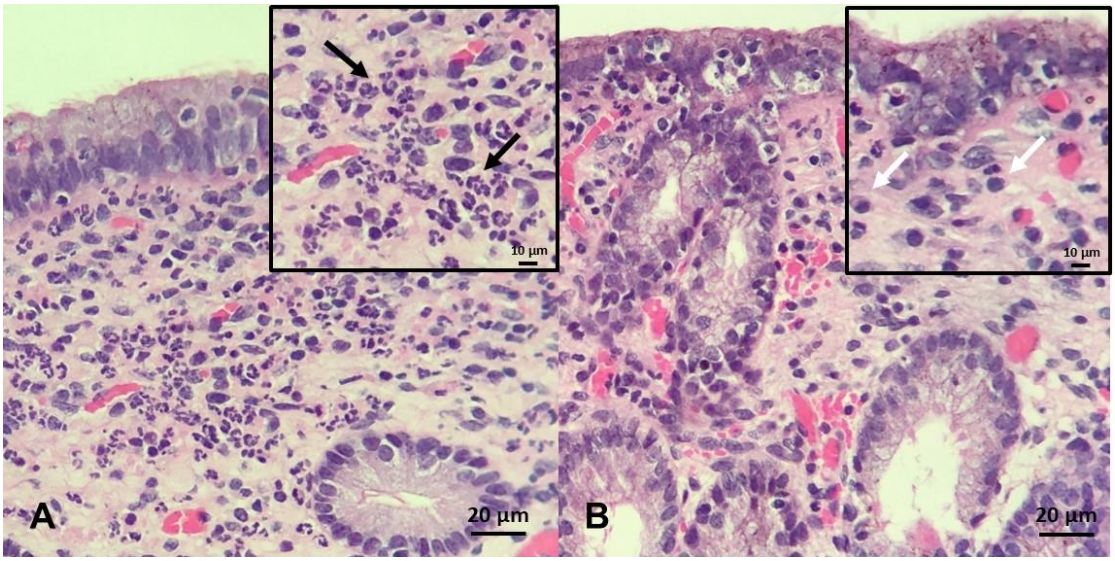

Figura 6. Biópsia endometrial corada com H&E (aumento de 20x e no quadrado 40x): (A) A égua com inflamação aguda, endometrite persistente pós cobertura caracterizada por um infiltrado massivo de neutrófilos no stratum compactum e spongiosum; setas pretas indicam os neutrófilos; (B) Égua com endometrite persistente pós-cobertura crônica caracterizada por infiltrado linfocítico presente no stratum compactum do endométrio; setas brancas indicam os linfócitos. Barras de escala 10 μm no quadrado (A e B) e 20 μm (A e B). (Adaptado de Canisso, Segabinazzi & Fedorka, 2020).

4. Tratamento

Tradicionalmente a endometrite é tratada com lavagens uterinas, agentes ecbólicos, anti-inflamatórios e antimicrobianos (CANISSO; STEWART; COUTINHO DA SILVA, 2016). Recentemente, a falta de respostas às terapias tradicionais e aumento da prevalência de resistência antimicrobiana dos patógenos exigiu que fossem desenvolvidas terapias alternativas para tratar éguas com endometrite crônica (SCOGGIN, 2016). Terapias tradicionais e alternativas são discutidas a seguir.

4.1. Agentes ecbólicos

Agente ecbólicos são uma classe farmacológica de drogas utilizadas para estimular as contrações uterinas e assim, eliminar o fluído acumulado no útero através da cérvix e drenagem linfática (ALLEN, 1991; CADARIO; THATCHER; LEBLANC,

1995; RASCH et al., 1996). A ocitocina é o ecbólico mais utilizado, sendo tipicamente administrada em qualquer momento antes da cobertura (exceto 1 hora após a cobertura ou inseminação) e entre 4 e 72 horas após a ovulação. Acredita-se que o transporte do espermatozoide à tuba uterina é completo 4 horas após a cobertura/inseminação artificial, assim, este é o tempo mínimo de intervalo necessário para que as fêmeas possam receber agentes ecbólicos ou uma lavagem uterina sem que comprometa os índices de prenhez (BRINSKO; VARNER; BLANCHARD, 1991).

Após a ovulação, ocorre o fechamento cervical que previne a eliminação do fluído uterino pela cérvix (TROEDSSON, 1997); contudo, pequenas amostras de líquido podem ser drenadas por via linfática pelos vasos presentes no útero. Doses menores de ocitocina (10-20 UI) induzem rápida contrações uterinas de baixa duração, enquanto doses altas (40UI) podem resultar em tetania, com contrações menos efetivas (RASCH et al., 1996; CADARIO et al., 1999; CAMPBELL; ENGLAND, 2002; MADILL et al., 2002). O efeito da ocitocina exógena é esperado por até 45 minutos, necessitando de doses repetidas (TROEDSSON et al., 1995).

O cloprostenol, análogo da PGF2α, é também um agente ecbólico que é utilizado na prática clínica para o tratamento de acúmulo de líquido intrauterino em éguas (SCOGGIN, 2016). Esta droga induz a atividade miometrial por um tempo prolongado (5 horas) quando comparada à ocitocina (BRENDEMUEHL, 2002; LEBLANC, 2003), beneficiando a limpeza uterina e drenagem linfática em éguas com útero penduloso (LEBLANC, 2003). Entretanto, sinais de cólica (escavar, contrações abdominais, sudorese e taquipneia) são comuns após a administração de PGFα, sendo considerados reações adversas (IRVINE et al., 2002). Além disso, a administração de PGFα 18-24 horas após a ovulação pode comprometer a formação e função do corpo lúteo, resultando em concentrações sistêmicas de progesterona inferiores ao necessário, diminuindo assim, as taxas gestacionais (BRENDEMUEHL, 2002; NIE et al., 2002).

A carbetocina é um análogo da ocitocina, sendo considerada um agente ecbólico mais leve e com ação prolongada quando comparado à ocitocina (SCHRAMME et al., 2008; STECKLER et al., 2012). É considerada um octapeptídeo cíclico com substituição de um grupo amino por um átomo de hidrogênio com uma alteração em uma ligação dissulfeto para uma ligação de tioeter e substituição do grupo hidroxila da tirosina por um grupo metoxil, que evita a metabolização precoce após a administração (SCHRAMME et al., 2008). A carbetocina apresenta 2,5 vezes maior meia-vida (17 minutos) que a

ocitocina (6 minutos) (SCHRAMME et al., 2008; STECKLER et al., 2012). Contudo, até hoje, não existem estudos comparando a habilidade da ocitocina e da carbetocina em promover a contração uterina em éguas.

4.2. Antibióticos

Os antimicrobianos são fármacos necessários para o tratamento de endometrite infecciosa em éguas, contudo, estas drogas também são utilizadas em situações onde não existe uma indicação médica clara, como por exemplo quando aplicado uma única vez, intrauterino, após a cobertura (ZENT; TROEDSSON; XUE, 1998). O uso irracional de antimicrobianos levou ao rápido desenvolvimento de resistência antimicrobiana. Assim, a identificação apropriada do microrganismo, bem como a realização de testes de susceptibilidade aos antimicrobianos, é extremamente importante no tratamento de endometrite e prevenção do desenvolvimento de resistência antimicrobiana.

Pelo fato das principais bactérias isoladas do trato reprodutivo de éguas serem *Streptococcus spp, Escherichia coli, Klebsiella sp, Pseudomonas sp,* e *Staphylococcus sp* (LEBLANC; MAGSIG; STROMBERG, 2007; LEBLANC; CAUSEY, 2009; BELTAIRE; CHEONG; COUTINHO DA SILVA, 2012; WALTER et al., 2012; CANISSO; STEWART; COUTINHO DA SILVA, 2016), os antimicrobianos mais utilizados no tratamento de endometrite incluem betalactâmicos (ceftiofur, ampicilina e penicilina) e aminoglicosídeos (gentamicina e amicacina) (DASCANIO, 2009) (Tabela 3). É interessante que o *Streptococcus zooepidemicus* e a *Escherichia coli*, dois dos microrganismos mais isolados em éguas com endometrite, são descritos por serem altamente resistentes à antimicrobianos comuns (BENKO et al., 2015).

O mecanismo pelo qual a bactéria se torna resistente aos antibióticos têm sido caracterizado como modificações na célula alvo que previne a ligação do antimicrobiano, síntese de enzimas que digerem os agentes antimicrobianos, ativação de vias alternativas que sabotam os mecanismos de ação das drogas, regulação negativa ou eliminação de porinas transmembrana por onde as drogas conseguem acessar as células, e eliminação das drogas por bombas de efluxo (ALEKSHUN; LEVY, 2007; VILA; MARTÍ; SÁNCHEZ-CÉSPEDES, 2007). Além disso, o *Streptococcus zooepidemicus* pode causar uma infecção profundamente dormente (estado vegetativo) nos úteros das éguas, fazendo destas bactérias, resistentes às terapias tradicionais (ALBIHN; BÅVERUD; MAGNUSSON, 2003). Um produto comercial chamado b-Activate (Bojesen and

Petersen Biotech, Copenhagen, Dinamarca) foi descrito por estimular o estágio dormente do Streptococcus para um estágio ativo e proliferativo, tornando a infecção tratável por agentes antimicrobianos (PETERSEN et al., 2015). O custo do produto limita a utilização disseminada na prática clínica.

A utilização de antimicrobianos para o tratamento de endometrite pode ser administrada tanto por via intrauterina quanto sistêmica. Deve-se ressaltar que nem todos antimicrobianos sistêmicos podem ser administrados no útero sem sofrerem alguns ajustes. Um exemplo disso seria a administração de fluoroquinolonas como a enrofloxacina. A formulação sistêmica da enrofloxacina não pode ser utilizada no útero porque seu veículo pode causar necrose endometrial (RODRIGUEZ et al., 2012). Contudo, se preparada com um veículo diferente, a enrofloxacina pode ser seguramente administrada no útero (SCHNOBRICH et al., 2015). Além disso, aminoglicosídeos (gentamicina e amicacina) necessitam da adição de bicarbonato de sódio para contrabalancear seu pH ácido (LEBLANC, 2009; CANISSO; STEWART; COUTINHO DA SILVA, 2016). A infusão de antimicrobianos incompatíveis no útero pode precipitar e deixar resíduos no útero, que poderam ocasionar distúrbios entre as interações embrião-endométrio.

Tabela 3. Antimicrobianos comumente utilizados no tratamento de endometrite infecciosa em éguas.

Classe farmacológica	Terapêutica	Mecanismo de ação	AMR
Aminoglicosídeos (sulfato de amicacina, sulfato de gentamicina e neomicina)	Concentração dependente, bactericida, amplo espectro, G-.	Inibição irreversível da síntese de proteína bacteriana pela ligação com a subunidade 30S do ribossomo bacteriano.	Aminoglicosídeos (sulfato de amicacina, sulfato de gentamicina e neomicina)
Cefalosporinas (β-lactâmicos, terceira geração) (ceftiofur sódico, ácido livre cristalino de ceftiofur)	Tempo dependente, bactericida, amplo especrto, G- e G+.	Inibição da síntese de parede celular por ruptura da camada de peptideoglicanos	Resistência crescente, com base na permeabilidade reduzida por mutação PBP e inativação enzimática por β-lactamase.
Fluoroquinolonas (Enrofloxacina e ciprofloxacina)	Concentração dependente, bactericida, amplo especrto, G- e G+.		Fluoroquinolonas (Enrofloxacina e ciprofloxacina)
Penicilinas de amplo espectro (β-lactamicos) (ampicilina e tiraciclina).	Tempo dependente, bactericida, amplo espectro, G+.	Interferência na síntese de membrana celular bacteriana pela inibição de transpeptidases e enzimas de peptídeoglicanos.	Resistência adquirida em G- por mediação de plasmídio- ou integron-
Penicilinas (β-lactâmicos naturais), (penicilina K, penicilina Na e procaína G)	Tempo dependents, bactericidas, amplo especrto, G+.	Lise de células enfraquecidas pela perda da camada de peptídeoglicanos na membrana através da ligação de PBP's externamente à parede bacteriana.	Mutação de PBP's que reduz a permeabilidade bacteriana, e produção de β-lactamases.
Polimixina (polimixina B)	Concentração dependente, bactericida, amplo espectro, G- (Pseudomonas)	Desorganização da membrana por ligação do PBPs fora da parede bacteriana.	Rara, modificações das LPS na membrana bacteriana e desenvolvimento de uma bomba de efluxo/ sistema potássico.
Sulfonamidas (sulfametoxazol associado a primidina (trimethoprim)	Tempo dependente, bacteriostático, amplo especrto, G- e G+ (Streptococcus spp.)	Interferencias na biosintase do ácido fólico pela competição com PABA pela dihidropteroato sintase.	Mediado via mutações cromossômicas causando hiper produção de PABA ou intensiva dihidropteroato sintase.
Nitroimidazole (e.g., metronidazole)	Concentration-dependent, G+ anaerobes		

Adaptado de Canisso, Segabinazzi & Fedorka (2020). AMR: resistência antimicrobiana; PABA: ácido para-aminobenzóico; PBPs: proteínas de ligação à penicilinas; LPS: lipopolissacarídeos.

Existe um conflito de evidências a respeito dos antimicrobianos mais adequados para administração durante o tratamento de éguas com endometrite. A administração intrauterina de antimicrobianos envolve alterações sistêmicas mínimas na microbiota em outros sistemas corporais, altas concentrações endometriais e menor quantidade de antimicrobiano necessária quando comparada à administração sistêmica (DASCANIO, 2009; LEBLANC, 2009). Deve-se ressaltar que a administração sistêmica de antimicrobianos, particularmente por um período prolongado de tempo, e quando existe a necessidade de alterar as drogas durante o tratamento, podem resultar em diversas alterações indesejadas como diarreia, colite e reações anafiláticas sistêmicas (DASCANIO, 2009). Em contraste, a administração intrauterina pode irritar o endométrio e desenvolver uma endometrite não-infecciosa. Além disso, antibióticos intrauterinos podem levar a absorção inconsistente no tecido, o que pode favorecer o desenvolvimento de resistência antimicrobiana. Provavelmente, infecções profundas e dormentes do endométrio, como as causadas por Streptococcus, a administração de antimicrobianos sistêmicos seja mais eficiente, uma vez que, infusões uterinas podem não resultar em penetração profunda do tecido.

Os tratamentos para endometrite fúngica ainda não estão totalmente elucidados. Existe uma falta de estudos controlados relacionados à endometrite fúngica em éguas e farmacocinética e farmacodinâmica destas drogas no trato reprodutivo. O tratamento inicial deve ser através da correção dos fatores predisponentes (ex., conformação perineal defeituosa, imunossupressão, infusões intrauterinas descontínuas com antibióticos) em combinação com lavagens uterinas e agentes ecbólicos. A eliminação dos fatores predisponentes da endometrite pode, algumas vezes, reestabelecer a fertilidade das éguas (STOUT, 2008; DASCANIO; SCHWEIZER; LEY, 2010). Entretanto, quando necessário, três tipos de agentes antifúngicos (ex., polienos, imidazois e triazois) podem ser utilizados para tratar infecções fúngicas uterinas (STOUT, 2008; BELTAIRE; CHEONG; COUTINHO DA SILVA, 2012). Os polienos são considerados drogas fungicidas, e os azois (imidazol e triazol) são fungostáticos (GIGUÈRE, 2013a). Curiosamente, polienos e azois têm um mecanismo de ação similar, agindo na membrana fúngica através de uma ligação ao ergosterol ou inibindo a síntese de ergosterol (GIGUÈRE, 2013b), respectivamente (Tabela 4). Resultados de estudos realizados in vitro indicaram que os polienos (anfotericina B, natamicina e nistatina) são mais efetivos no tratamento de endometrite fúngica que os azois (cetoconazol, fluconazol e miconazol),

principalmente quando utilizadas em hifas septadas (BELTAIRE; CHEONG; COUTINHO DA SILVA, 2012).

Tabela 4. Fármacos antifúngicos comuns utilizados no tratamento de éguas com endometrite fúngica.

Classe farmacológica	Terapêutica	Mecanismo de ação	AMR
Polienos (anfotericina B, natamicina e nistatina)	Fungicidas e fungostáticos, amplo espectro contra Candida spp, Aspergillus spp, e Mucor spp	Liga-se ao ergosterol presente na membrana, causando ruptura da parede celular	Rara; um único fungo mutante aumente as vias sintéticas para esteróis alternativos que substituem ergosterol na membrana celular.
Imidazois (clotrimazol, cetoconazol e miconazol)	Amplo espectro, atividade contra Candida spp.	Inibição da sintese de ergosterol na membrana celular fúngica pela inibição da enzima 14- α-demetilase, aumentando a permeabilidade de membrana e ruptura celular.	Resistência é encontrada nos fungos filamentosos e após tratamentos prolongados.
Triazóis (fluconazol e itraconazol)	Atividade potente contra Aspergillus.	Bloqueio da enzima C-14-α-desmetilase dependente do citocromo P450 (necessário para a conversão de lanosterol em ergosterol).	Resistência envolve um único ponto de mutação no gene cyp51A, que é codificado pela 14-α-desmetilase.

Adaptado de Canisso, Segabinazzi & Fedorka (2020).

4.3. Lavagem uterina e tratamento de Biofilme

As lavagens uterinas são recomendadas em éguas com excessivo acúmulo de líquido intrauterino (> 2 cm de profundidade) e alta ecogenicidade ultrassonográfica (BRINSKO et al., 2003). Soluções cristalóides como solução de Ringer Lactato (LRS) e solução salina 0,9% são as mais comumente utilizadas nas lavagens uterina em éguas (VANDERWALL; WOODS, 2003). Estudos têm demonstrado que algumas bactérias como *Escherichia coli*, pode utilizar o lactato (presente no LRS) (HUA et al., 2007) e o gluconato (presente na Plasmalyte, uma solução cristalóide pouco utilizada em lavagens uterinas) como substrato para o seu crescimento (EISENBERG; DOBROGOSZ, 1967).

Lavagens uterinas não devem ser utilizadas sozinhas para tratar endometrites infecciosas. Estas soluções podem ser enriquecidas com anti-sépticos (ex., iodo-

povidona, e peróxido de hidrogênio), vinagre para alterar a microbiota uterina nos casos de endometrite fúngica, e aditivos para quebrar o biofilme como mucolíticos (N-acetilcisteína, dimetilsulfóxido, ácido etilenodiaminotetraacético-2-amino-2-hidroximetil-propano-1,3-diol sozinho ou em combinação com Tris; etilenodiaminotetraacetato dissódico desidratado-2-amino-2-hidroximetil-1,3-propanodiol). Apesar da ampla utilização destes produtos no tratamento de endometrite, o seu efeito na microbiota residente no útero é desconhecido.

As lavagens uterinas auxiliam fisicamente na remoção dos microrganismos, debris, células e mediadores infamatórios e células espermáticas mortas do lúmen uterino (BRINSKO et al., 2003; VANDERWALL; WOODS, 2003; KNUTTI et al., 2010). Elas podem ser realizadas a qualquer momento antes da cobertura, ou após 4 horas da cobertura. Quatro horas após a cobertura é considerado o tempo mínimo necessário para os espermatozoides chegarem à tuba uterina, sem interferência na fertilidade (BRINSKO et al., 1990; BRINSKO; VARNER; BLANCHARD, 1991; FIALA et al., 2007).

4.4. Agentes imunomodulatórios

O tratamento para EPPC com anti-inflamatórios não esteroidais (AINEs) ainda é controverso. Os AINES atuam inibindo síntese de prostaglandina-endoperoxidase sintase (tipo 1 e 2) e a cascata do ácido aracdônico como efeito primário. Isso pode diminuir a produção de PGF2α e potencialmente diminuir a atividade miometrial, dificultando a limpeza uterina. Dois estudos onde utilizaram-se fenilbutazona e flunexin meglumine (AINEs não-seletivos COX-2) observaram uma diminuição da limpeza uterina e aumento das reações inflamatórias em éguas que receberam tratamento (LEBLANC, 1997; REILAS et al., 2006). Além disso, a administração de alta (>2x dose recomendada) e continuada dose de AINEs em éguas em estado pré-ovulatório têm demonstrado um aumento dos índices de folículos anovulatórios hemorrágicos (ARMSTRONG, 1981; CUERVO-ARANGO, 2011). Entretanto, quando utilizado as doses recomendadas dos AINEs, não observou-se interferências na ovulação (DONNELLY et al., 2019). Além disso, a combinação de AINEs com ocitocina têm demonstrado benefícios na limpeza uterina (CADARIO; THATCHER; LEBLANC, 1995), reduzindo a infiltração de PMNs, e diminuindo a expressão de COX-2 endometrial (AURICH; ROJER; WALTER, 2010) em éguas susceptíveis.

Outra alternativa é a utilização de AINEs seletivos COX-2, que não agem na via da cicloxigenase-1 (COX-1) (COOK; BLIKSLAGER, 2015). O firocoxib, um AINE seletivo COX-2, tem sido descrito por reduzir a resposta inflamatória pós-cobertura de éguas, com redução da COX-2 no endométrio de éguas tratadas durante o período pré-ovulatório, sem que ocorressem alterações na ovulação (FRISO et al., 2019). Vedaprofeno, outro seletivo inibidor de COX-2, tem sido descrito por afetar positivamente a fertilidade em éguas com EPPC, entretanto, sem que altere o acúmulo de fluído intrauterino e o escore inflamatório do endométrio (ROJER; AURICH, 2010). Com base nestes resultados, AINEs seletivos COX-2 podem ser uma alternativa no tratamento de éguas com EPPC.

Os glicocorticóides são rotineiramente utilizados para modulação da resposta inflamatória pós-cobertura em éguas. A utilização de múltiplas doses de prednisolona antes da ovulação em éguas inseminadas com sêmen congelado aumentou as taxas de fertilidade (DELL'AQUA JR et al., 2006; PAPA et al., 2007). Um estudo posterior demonstrou que a utilização de dexametasona antes da cobertura reduz a inflamação endometrial e aumenta os índices gestacionais em éguas subférteis com histórico de predisposição a EPPC (BUCCA et al., 2008). A nível molecular, a dexametasona afeta a expressão endometrial de citocinas e SAA em éguas susceptíveis após a inoculação intrauterina de *Escherichia coli* (CHRISTOFFERSEN et al., 2012a) ou espermatozoides (RUIJTER-VILLANI; GRAUW; STOUT, 2011; WOODWARD et al., 2015). Uma redução de citocinas pró-inflamatórios IL1β, CXCL8, e SAA e supressão de mediadores inflamatórios, como COX-2, lipo-oxigenase 5, e NO, foram relatadas após a administração de dexametasona, bem como o aumento de moduladores inflamatórios e anti-inflamatórios como IL6, IL10 e IL1RN também foram observados após o tratamento (CHRISTOFFERSEN et al., 2012a). Além disso, a administração de dexametasona alterou na produção de proteínas de fase aguda após desafios bacterianos (ARLAS et al., 2015) e não alterou a função fagocitária dos PMNs (OHMAN et al., 2011), o que poderia predispor as éguas à infecções secundárias. Esta alteração no perfil das proteínas de fase aguda também foi observada com a utilização de prednisolona (WOLF et al., 2012). Um estudo demonstrou que a administração de prednisolona seguida de um desafio bacteriano, aumentou antitripsina e transtiretina além de reduzir IgG (WOLF et al., 2012). Deve-se ressaltar ainda que apesar da terapia com glicocorticóides ser associada com a redução de edema endometrial após a cobertura, repetidas aplicações de dexametasona e

prednisolona podem minimizar a secreção de hormônio luteinizante (LH) levando a falhas na ovulação (FERRIS; MCCUE, 2010). Em um estudo, prolongadas administrações (5 dias) de dexametasona tenderam a reduzir (40%) os índices de ovulação em éguas quando comparado ao placebo (100%), enquanto a administração prolongada de prednisolona não alterou estes resultados (83%) (FERRIS; MCCUE, 2010). Em equinos, a dexametasona pode afetar as funções do hipotálamo (ANDREWS, 1977), hipófise anterior (SCHREIBER; NAKAMURA; ERICKSON, 1982), e ovário (HSUEH; ERICKSON, 1978; SCHREIBER; NAKAMURA; ERICKSON, 1982). Assim, uma baixa dose de única aplicação pode ser recomendada no tratamento de éguas com endometrite pós-cobertura, em vez de aplicações repetidas.

A utilização de extratos bacterianos também foi descrita por alterar a resposta imune do endométrio (FUMUSO et al., 2003, 2006, 2007; ROGAN et al., 2007; ROHRBACH et al., 2007; CHRISTOFFERSEN et al., 2012a). Extrato de parede celular de Mycobacterium phlei (MCWE) é um imunomodulador comercial (Settle, Bioniche Animal Health, Athens GA, EUA) que é utilizado para o tratamento de endometrite equina causada por *Streptococcus zooepidemicus*. Este imunomodulador age através da inibição da resposta imune inata, sendo observada uma diminuição da expressão endometrial de citocinas pró-inflamatórias IL1β, IL6, e TNFα em éguas susceptíveis tanto após a cobertura quanto desafios com bactérias gram-positivas, enquanto ocorreu um aumento da expressão de IL10 anti-inflamatória (FUMUSO et al., 2003). Além disso, éguas tratadas com MCWE também demonstraram uma diminuição do NO (TROEDSSON et al., 1994b). Apesar de a MCWE não ter afetado a expressão de citocinas endometriais após desafios com *Escherichia coli*, observou-se uma pronunciada redução do crescimento bacteriano após o tratamento, além da redução do acúmulo de fluído intrauterino (CHRISTOFFERSEN et al., 2012a). Isso se confirmou no estudo seguinte onde o MCWE foi evidenciado por sua atividade bactericida independentemente da via de administração (venosa ou intrauterina) (ROGAN et al., 2007).

Outro imunoestimulante que foi descrito por aumentar os índices gestacionais em éguas é o *Propionibacterium acnes* (EQStim, Neogen Corp, Lexington KY, USA) (ROHRBACH et al., 2007). Esta terapia induz uma resposta mediadora celular não específica, predominada pela ativação de macrófagos e liberação de citocinas. Em um único estudo investigando este imunoestimulante, éguas com endometrite clínica foram tratadas com *Propionibacterium acnes* por via intravenosa (ROHRBACH et al., 2007).

Repetidas administrações deste tratamento como adjuvante às terapias tradicionais têm aumentado os índices de prenhez e o nascimento de potros em éguas com diagnóstico citológico de endometrite quando comparado ao placebo. Infelizmente, o mecanismo molecular de utilização desta terapia ainda deve ser investigado.

4.5. Lactoferrina

A lactoferrina é uma proteína 80kDa encontrada promiscuosamente em todo corpo, incluindo trato reprodutivo e sistema imune (SUZUKI; LOPEZ; LÖNNERDAL, 2005). Acredita-se que apresenta habilidade bactericida pela sua aptidão em quelar ferro livre (AMMONS; COPIÉ, 2013). A expressão endometrial de lactoferrina varia conforme o estágio do ciclo estral e aumenta durante o estro, indicando sua dependência endócrina (KOLM et al., 2006). A administração de lactoferrina recombinante no momento da cobertura em éguas normais demonstrou diversos resultados, em um estudo sem alterações na expressão de citocinas (FEDORKA et al., 2017a). Em outro estudo, observou-se uma diminuição significativa na IL6 endometrial, além de uma tendência na redução de CXCL8, IL1β, e TNFα (COUTINHO DA SILVA et al., 2017), indicando suas propriedades anti-inflamatórias.

Curiosamente, esta diminuição de TNFα também foi observada em éguas susceptíveis à EPPC quando receberam lactoferrina no momento da cobertura (FEDORKA et al., 2017b). Quando administrada 6 horas após cobertura em éguas susceptíveis, a lactoferrina reduziu a expressão pró-inflamatória de interferon gamma enquanto aumentou a expressão anti-inflamatória de IL1RN (FEDORKA et al., 2018). Este estudo também evidenciou diversas concentrações de lactoferrina (50-500 µg) para diminuir a infiltração de PMNs em éguas susceptíveis, contudo, não afetou a retenção de fluído intrauterino. Os autores sugeriram que a dose recomendada para lactoferrina recombinante humana em infusões intrauterinas deve ser de 1 mL (50 µg/mL) diluído em 10 mL de LRS, que é equivalente à média de concentração observada no ejaculado equino. Em outras espécies, a lactoferrina é administrada por suas propriedades bactericidas e anti-biofilme (AMMONS et al., 2009; AMMONS; WARD; JAMES, 2011), contudo, estas propriedades não foram ainda investigadas em equinos.

4.7. Células tronco

A utilização de células tronco mesenquimais (MSCs) ganhou rápido interesse na medicina humana e veterinária na modulação de processos inflamatórios (TIMMERS et al., 2011; BARRACHINA et al., 2016). Estas células têm a capacidade de se diferenciarem nos mioblastos esqueléticos, parênquima renal, epitélio hepático, epitélio da pele e intestino, além de células neuroectodermais (GROVE; BRUSCIA; KRAUSE, 2004), e células endometriais (DU; TAYLOR, 2007). Elas podem também sinalizar células residuais para suas propriedades anti-apoptóticas, quimiotáticas e imunomoduladoras podendo ser coletadas de diferentes tecidos e com diferentes potenciais.

Injeções (ALVARENGA et al., 2016) ou infusões (MAMBELLI et al., 2014) de MSCs têm sido descritas como possíveis alternativas para o tratamento de fibrose endometrial. Terapias com MSCs podem induzir uma remodelação precoce (7 dias) e também prolongada (60 fias) do endométrio de éguas que apresentam uma endometriose crônica e degenerativa através da modulação de padrões de expressão (citoqueratina, vimentina, actina de musculo liso α e laminina) associado ao desenvolvimento de fibrose patológica no endométrio equino, bem como a proliferação de células epiteliais glandulares (MAMBELLI et al., 2014). Um relatório demonstrou que a administração de MSC diminui os números de neutrófilos e aumenta a expressão anti-inflamatória de ILRN no endométrio de éguas normais (FERRIS; FRISBIE; MCCUE, 2014). Curiosamente, enquanto um estudo demonstrou que MSCs também atenuaram os marcadores de inflamação uterina após o tratamento, as células eram incapazes de penetrar o endométrio e incapazes de permanecerem no lúmen uterino (RINK et al., 2018). Entretanto, outro estudo relatou que a infusão intrauterina de MSCs efetivamente enxertou as MSCs no espaço periglandular (MAMBELLI et al., 2013). Além disso, sabe-se que as MSCs podem estimular as citocinas anti-inflamatórias (IL2, IL4, IL10 e fator de crescimento fibrobástico básico) e diminuir a produção de citocinas pró-inflamatórias (TNFα, IL1β e IL17) em modelos experimentais (BAI et al., 2009; MAO et al., 2010; ZHAO et al., 2015). Utilizando ratos como um modelo experimental, alguns autores indicaram uma regeneração das células endometriais ou um efeito protetivo contra células danificadas do endométrio pela alta expressão de citoqueratina e vimentina, utilizando terapia intravenosa de MSCs (ZHAO et al., 2015). Além disso, neste estudo, a terapia intrauterina com MSCs foi eficiente na estimulação de marcadores de receptividade endometrial (integrina agb3 e fator inibitório de leucemia) (ZHAO et al., 2015), que são reguladores

da função endometrial e tem papel importante na implantação embrionária (ACHACHE; REVEL, 2006). Até o presente momento, nenhum estudo avaliou a eficácia das MSCs em éguas susceptíveis à EPPC, assim, novas pesquisas se fazem necessárias. Além disso, sabe-se que primatas e roedores sofrem decidualização (alterações morfológicas e funcionais no endométrio para preparação da gestação), e equinos não; assim, achados nestas espécies não necessariamente podem simular os achados em equinos.

5. Plasma rico em plaquetas

O plasma rico em plaquetas (PRP) é o plasma com uma concentração elevada de plaquetas (3-9 vezes) que se tornou uma popular terapia alternativa na medicina humana e veterinária. O PRP vem sendo utilizado na prática clínica equina para tratar articulações, bursas e alterações de tecido mole (tendinites, tenosinovites, e feridas de pele) (CARMONA et al., 2007; ARGÜELLES et al., 2008; GEORG et al., 2010; PEREIRA et al., 2019). Um estudo realizado nos anos 80 sugeriu que o tratamento de éguas com plasma sanguíneo diminuiu a resposta inflamatória de éguas com endometrite (ASBURY, 1984). O PRP possui todos os diferentes elementos presentes no plasma sanguíneo, com uma alta concentração plaquetária. Recentemente, a infusão intrauterina com PRP foi observada por reduzir a resposta inflamatória pós-cobertura e aumentar os índices gestacionais de éguas (METCALF; SCOGGIN; TROEDSSON, 2012; METCALF, 2014; REGHINI et al., 2016; SEGABINAZZI et al., 2017; PASCH; SCHMIDT; KING, 2020). Além disso, a combinação de plasma autólogo à terapia antimicrobiana foi reportada por melhorar as taxas de prenhez em éguas lactantes e inférteis (PASCOE, 1995).

As plaquetas ou trombócitos, são fragmentos citoplasmáticos derivados dos megacariócitos, que são as maiores células presentes na medula óssea (HARTWIG; ITALIANO, 2003). O citoplasma das plaquetas é subdividido no cromômero, onde os grânulos se acumulam e o hialômero, onde encontra-se a região agranular rica em proteínas do citoesqueleto (BOSWELL et al., 2012). Grânulos plaquetários contém diversas proteínas como fibrinogênio, fatores de crescimento (fator transformador de crescimento β e fator endotelial vascular de crescimento), citocinas (CXCL8 e TNFα), e peptídeos antimicrobianos (fator plaquetário 6, RANTES, peptídeo ativador de tecido conjuntivo 3, proteína básica de plaquetas, timosina beta-4, fibrinopeptídeo A e B) (PAVLOVIC et al., 2016; BOS-MIKICH et al., 2019).

O mecanismo biológico do PRP na resposta inflamatória ainda não está bem elucidado. Contudo, alguns estudos (WOODELL-MAY et al., 2011; WU et al., 2011; KIM et al., 2014; SUNDMAN et al., 2014) têm demonstrado uma ação anti-inflamatória do PRP devido sua habilidade em suprimir a expressão de COX-2, metalloproteinase-3 (MMP-3), TNFα, IL1 e moléculas de adesão vascular (MAZZOCCA et al., 2013). Além disso, os grânulos plaquetários contém peptídeos antimicrobianos (RANTES, fator plaquetário 4 e timosina beta-4), e estes peptídeos podem contribuir para a atividade bactericida conhecida do PRP contra *Staphylococcus aureus, Escherichia coli*, e *Klebsiella pneumoniae* (TROWBRIDGE et al., 2005; BIELECKI et al., 2007; MOOJEN et al., 2008; YUAN; ZHANG; ZENG, 2008; CIESLIK-BIELECKA et al., 2009; ÁLVAREZ et al., 2011; ANITUA et al., 2012; BURNOUF et al., 2013). Todas estas bactérias são conhecidas por causarem endometrite em éguas (CANISSO; STEWART; COUTINHO DA SILVA, 2016; CANISSO; SEGABINAZZI; FEDORKA, 2020).

Para o tratamento de endometrite em éguas, um estudo determinou que a administração de PRP no momento da cobertura diminuía a resposta inflamatória uterina em éguas com endometrite crônica, apesar de não ter afetado a produção de NO (REGHINI et al., 2016). Este concorda com outro estudo que reportou que o PRP diminuiu a expressão endometrial de COX-2, diminuindo o número de PMN no lúmen uterino e aumentando os índices gestacionais (SEGABINAZZI et al., 2017). Também foi demonstrado por agir como um tratamento anti-inflamatório em éguas susceptíveis, levando a diminuição endometrial da expressão de IL1β, IL6, e CXCL8 (METCALF; SCOGGIN; TROEDSSON, 2012; METCALF, 2014; REGHINI et al., 2016; SEGABINAZZI et al., 2017).

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Hipótese

Nossa principal hipótese é que a administração de PRP a éguas suscetíveis a EPPC modula a resposta inflamatória pós-cobertura. Especificamente, a terapia com PRP reduz a duração e a intensidade da resposta inflamatória pós-cobertura, elevando a taxas de recuperação embrionária de éguas suscetíveis a EPPC.

Hipótese 1. As infusões intrauterinas de PRP em éguas suscetíveis a EPPC melhoraram os parâmetros clínicos (por exemplo, fluido intrauterino pós-cobertura, inflamação endometrial reduzida e aumento da fertilidade) em comparação com infusões de plasma pobre em plaquetas (PPP) ou solução salina.

Hipótese 2. A infusão de PRP em éguas suscetíveis à EPPC melhora o ambiente uterino ao diminuir as citocinas pró-inflamatórias e ao mesmo tempo aumentar as citocinas anti-inflamatórias, além de alterar o perfil das proteínas secretadas no útero.

Objetivo

O objetivo deste estudo foi comparar três métodos de preparação de PRP para infusão intrauterina em éguas e avaliar os efeitos do PRP e PPP em comparação com os ciclos atribuídos ao controle na microbiologia uterina, inflamação endometrial, acúmulo de fluido intrauterino, concentrações de progesterona e taxas de recuperação embrionária em éguas suscetíveis a EPPC

Objetivo 1. Comparar a citologia uterina, a cinética do acúmulo de fluido intrauterino pós-cobertura e a recuperação embrionária em éguas resistentes e suscetíveis a EPPC tratadas com PRP, PPP ou solução salina em um design cruzado randomizado.

Objetivo 2. Comparar a composição proteica do fluido uterino de éguas tratadas com PRP, PPP e solução salina.

Objetivo 3. Comparar diferentes métodos para obtenção do PRP.

Capítulo 2

ARTIGO I – Three manual noncommercial methods to prepare platelet-rich plasma with potential application in mare reproductive practice

Artigo redigido nas normas da Animals, ISSN 2076-2615, fator de impacto 2,323, ranqueada como A1 pelo QUALIS – CAPES de 2019.

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Simple Summary: Platelet-rich plasma (PRP) is a popular therapy in human and veterinary medicine, which is also gaining popularity to manage subfertility in mares. This study aimed to compare three manual non-commercial methods to prepare PRP and its cooling. The three methods concentrated platelets and did not alter viability. Method-1 (i.e., involving double centrifugation) resulted in the greatest platelet concentrations, while method-3 (sedimentation) resulted in the lowest platelet concentration and greater contamination with white blood cells (WBC). Cooling increased platelet agglutination over time across methods and affected platelet viability in PRP obtained with method-3. It remains to be determined whether the different methods and cooling would affect PRP's clinical efficacy.

Abstract: In lieu of PRP's increasing popularity in reproduction, this study aimed to compare three methods to produce PRP and to cool with potential application in mare reproductive practice. Eighteen clinically healthy mares had blood collected via venipuncture in a blood transfusion bag (method-1), blood tubes (method-2), and a syringe (method-3). Method-1, samples were double centrifuged. Method-2 involved one centrifugation, and Method-3 the syringe was kept in an upright-position to sediment for 4h. After processing with three methods, PRP and platelet-poor- plasma (PPP) were extracted and assessed for red (RBC) and white blood cell counts (WBC), platelet counts, and viability. In a subset of mares (n=6), samples were processed with the three methods, and PRP was evaluated at 6 and 24h post-cooling at 5°C. Method-1 resulted in the greatest and method-3 in the fewest platelet concentrations ($P<0.05$), and the latter also had greater contamination with WBC than the others ($P<0.001$). Platelet viability was similar across treatments ($P>0.05$). Cooling for 24h did not affect platelet counts in all methods ($P>0.05$). However, platelet viability was reduced after cooling PRP produced by method-3 ($P=0.04$), and agglutination increased over time in all methods ($P<0.001$). The three methods increased (1.8-5.6-fold) platelet concentration in PRP compared to whole blood without compromising platelet viability. In conclusion, all three methods concentrated platelets and did not affect their viability. It remains to be determined whether the different methods and cooling would affect PRP's clinical efficacy.

Keywords: platelet concentrate; horse; endometritis; blood by-product; platelet viability

1. Introduction

Platelet-rich plasma (PRP) is a biological by-product derived from whole blood (WB) after removal of red blood cells (RBC) and complete or partial removal of white blood cells (WBC) to concentrate platelets in a small volume of plasma. It is traditionally used in human and veterinary medicine to treat orthopedic and dermatologic diseases [1,2]. Recently, PRP also gained popularity to mitigate persistent-breeding induced endometritis (PBIE) [3,4] and improve fertility in mares with chronic degenerative endometritis [5] and barren mares [6–8]. The immunomodulatory properties of PRP have been attributed to growth factors (e.g., hepatocyte growth factor) and cytokines (e.g., transforming growth factor β, CXCL8, and IL1β) present in the platelets [9,10]. In addition, the platelet granules contain antimicrobial peptides (e.g., RANTES and platelet factor 4) that may be beneficial against uterine bacterial infections [11–13]. It turns out that the latter feature could be helping improve the fertility of mares susceptible to PBIE. Worth noting, PBIE is the third most common clinical problem seen in equine practice and the most common cause of subfertility in mares [14].

Automatized (e.g., AngelTM Cytomedix, Inc. Gaithersburg, MD, USA; and Restigen PRP®, Owl Manor, Warsaw, IN, USA) and manual protocols have been described to prepare PRP for usage in clinical practice [15–19]. The first approach offers the advantage of being standardized and less human dependent; however, the high cost of acquiring the machines and supplies can discourage small practices or solo practitioners to routinely use PRP in clinical practice. Conversely, manual methods require no specialized equipment and can be less costly and affordable alternatives to prepare PRP.

The majority of manual protocols used in equine practice involve one or two centrifugations to concentrate platelets in a small volume of plasma (e.g., 2-5 mL) for

injection in tendons or intra-articular treatment [15,20]. However, presumably for intra-uterine therapy, a higher volume (e.g., 10-60 mL) is needed to reach the endometrium's entire surface [14]. In addition, PRP is often used immediately after its preparation at room temperature [21]. However, multiple intrauterine infusions of PRP have been proposed to maximize its efficacy [4], and the ability to cool down PRP for subsequent infusion the next day would facilitate its use in clinical practice. In human medicine, PRP is often stored up to seven days at room temperature (22 °C) [22]; however, this temperature can increase the risk of bacterial growth [23].

Therefore, this study's objectives were to determine and compare three manual non-commercial methods to prepare and cool equine PRP with potential application in mare reproductive practice. Specific endpoints assessed included cell composition, and platelet counts, viability, and agglutination. The hypotheses of the present study were that the methods tested herein produce PRP with platelet concentration (2-9-fold in comparison to WB) with high viability, and cooling PRP has no detrimental effects on platelet viability.

2. Materials and Methods

2.1. Animals

The study was carried out from November 2019 to November 2020 at the College of Veterinary Medicine, University of Illinois Urbana-Champaign. Eighteen light breed mares, with ages between 5 and 16 years old (9.4 ± 3.0 years-old) and weights 400 and 600 kg, which had been using as embryo donor mares in the principal investigator’s laboratory for at least two years, were enrolled in the study. The mares belonged to the teaching/research herd of the University of Illinois Urbana-Champaign. All mares were deemed clinically healthy based on full physical examination and complete blood cell

count immediately before enrollment in the study (Table 1). The mares were housed in individual stalls, fed with 5 kg of mixed alfalfa-grass hay three times a day, with free access to water and trace minerals. All mares were vaccinated and dewormed regularly, following the American Association of Equine Practitioners guidelines (www.aaep.org/guidelines). Mares had feet-trimmed, and teeth floated following the Ag-guide for Agricultural animals following USDA recommendations. All animals were kept at the University of Illinois campus.

Table 1. Mare signalment and platelet and blood cell counting immediately pre-enrolment.

Mare	**Age**	**Breed**	**Weight**	**Blood counts**		
				Platelets ($\times 10^3$/µL)	**WBC ($\times 10^3$/µL)**	**RBC ($\times 10^6$/µL)**
1	5	Quarter Horse	480	120	7.0	7.2
2	6	Quarter Horse	470	116	6.6	6.5
3	9	Thoroughbred	540	125	5.0	6.4
4	8	Arabian	400	150	5.2	7.6
5	12	Paint Horse	520	152	6.0	7.8
6	6	Paint Horse	500	168	5.2	8.1
7	9	Standardbred	500	130	5.5	8.1
8	8	Standardbred	580	124	6.9	9.3
9	12	Standardbred	600	120	6.0	6.8
10	8	Quarter Horse	450	135	5.5	7.2
11	13	Thoroughbred	500	102	5.0	6.2
12	7	Arabian	400	118	6.5	6.7
Subset (n=6)						
13	16	Quarter Horse	480	172	5.1	6.1
14	14	Quarter Horse	520	112	5.6	7.5
15	10	Standardbred	580	106	5.4	6.8
16	11	Arabian	430	100	5.3	6.5
17	7	Saddlebred	510	138	5.9	6.1
18	8	Arabian	400	124	6.1	6.3

The subset of mares used to harvest PRP for cooling.

2.2. Experimental Design

All mares had blood samples sequentially collected in a blood collection bag (Method 1), blood collection tubes (Method 2), and a 60-mL syringe (Method 3). After collection, an aliquot of WB from each method was submitted to the University of Illinois Clinical Pathology Laboratory for a complete blood cell count with an automated machine (Sysmex XT-2000iV, Kobe, Japan). In addition, platelets from WB were stained and had viability assessed as described below. The WB obtained with each method was processed separately to harvest PRP and PPP as diagrammatically depicted below (Figure 1). In addition, a subset of mares (n=6) was randomly selected and had PRP harvested and stored for 24 h in a refrigerator at 5 °C. At 6 and 24 h post-cooled-storage, PRP was assessed for platelet counts, viability, and agglutination as described below. No cryoprotectants were added during cooling.

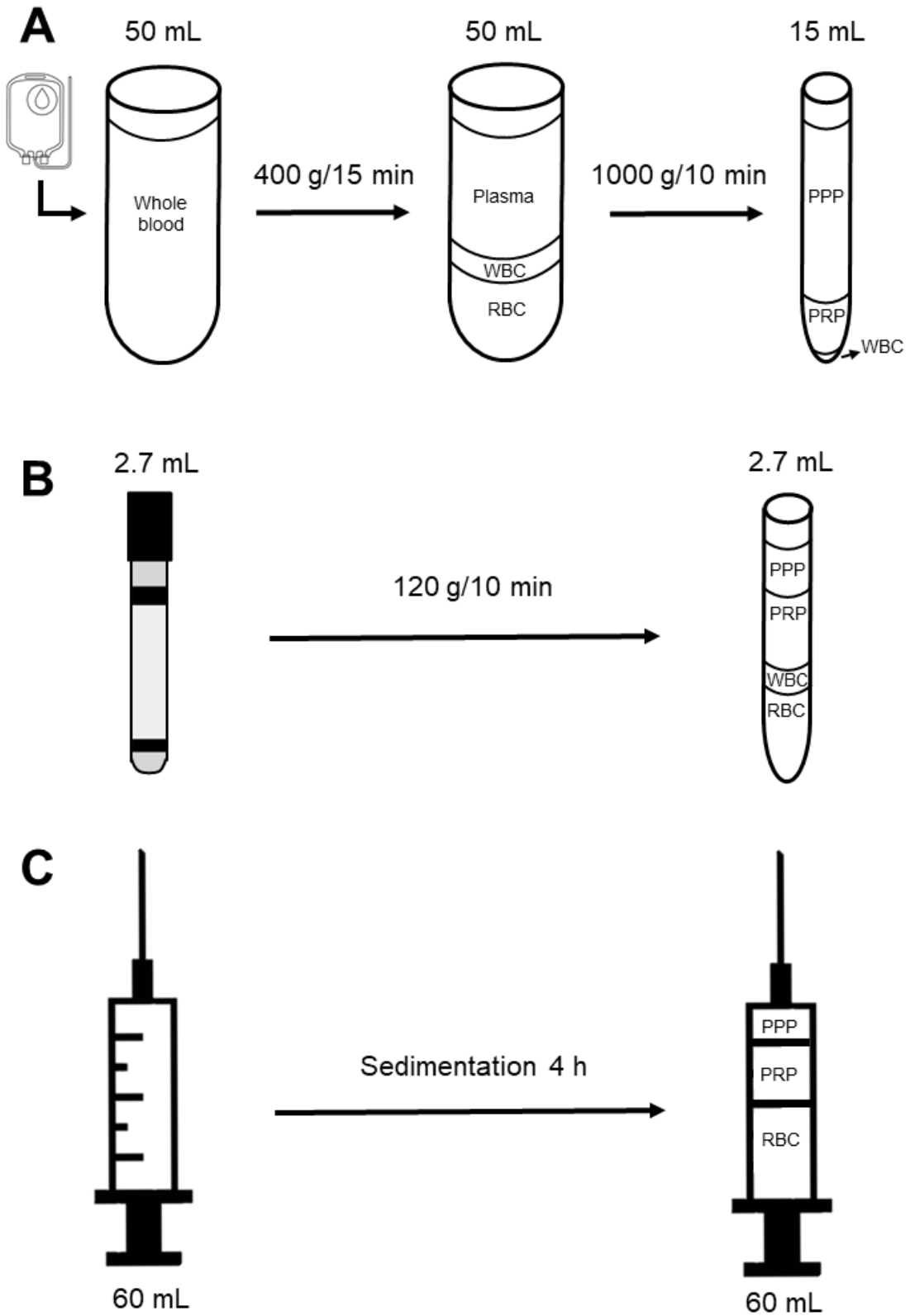

Figure 1. Diagram of three methods to prepare platelet-rich plasma (PRP) for intrauterine infusions in mares. (A) Method 1, blood was collected in a 150 mL blood transfusion bag and processed by 2-step centrifugation; (B) Method 2, blood was collected in 2.7 mL vacutainer tubes and processed by one-step centrifugation; (C) Method 3, blood was collected in a 60 mL syringe and PRP produced by sedimentation. Abbreviations: PPP, platelet-poor plasma; WBC, white blood cells; RBC, red blood cells.

110 **2.2.1. Method 1**

111 Blood was collected from each animal through a puncture of the jugular vein using
112 an 18G needle, into a 150 mL blood transfusion bag (Fresenius Kabi AG, Bad Homburg,
113 Germany), containing 21 mL citrate-phosphate-dextrose solution with adenine as an
114 anticoagulant (CPD-A, Santa Cruz Biotechnology, Inc., USA) according to modified
115 techniques previously described [4,15]. One hundred milliliters of WB were split into two
116 50-mL tubes and were centrifuged at 400 × g for 15 min. The plasma was transferred into
117 15-mL conical tubes and centrifuged at 1000 × g for 10 min. Both centrifugations were
118 performed at room temperature, with deceleration force off. After the second
119 centrifugation, 2.5 mL of plasma at the bottom of each tube was preserved and deemed
120 as PRP, while the supernatant above the 2.5 mL was considered as PPP. The
121 concentrations of platelets, WBC, and RBC were determined in WB, plasma after the first
122 centrifugation, PPP and PRP samples at University of Illinois Clinical Pathology
123 Laboratory.

124 **2.2.2. Method 2**

125 Both PRP and PPP were obtained after single centrifugation as described
126 elsewhere [3]. Briefly, 32.4 mL (12 tubes) of WB was collected from each animal through
127 a puncture of the jugular vein into 2.7 mL blood collection tubes containing 3.2% sodium
128 citrate (Vacutainer, Labor Import, USA). Blood tubes were immediately centrifuged at
129 120 × g for 10 min at room temperature. After centrifugation, the top third layer of plasma
130 was deemed as PPP, while the remaining portion was considered as PRP.

131 **2.2.3. Method 3**

132 Blood was collected through a puncture of the jugular vein using an 18G needle
133 into a 60 mL syringe prefilled with 7 mL of CPD-A as an anticoagulant. Right after

collection, each syringe was wrapped in aluminum foil and placed in an upright position for 4 h. Subsequently, a 21G butterfly catheter was connected to the syringe, and PPP and PRP were recovered by applying steady pressure to the syringe's plunger in an upright position (Supplementary file: Video 1). The top 10 mL of plasma was considered as PPP, and the remaining plasma until the sedimented blood cells was deemed as PRP. Buffy coat was not visible in this method.

2.3. Cell Counts

Whole blood samples collected in 0.1% sodium citrate anticoagulant were submitted to the University of Illinois Clinical Pathology Laboratory for automated quantification of platelet, WBC, and RBC counts. The Sysmex XT-2000iV analyzer (Sysmex)3 reported platelet counts obtained both by DC sheath flow (PLT-I) and fluorescence flow cytometry (PLT-O). Manual blood smear evaluation with platelet estimation was performed for each sample by a board-certified clinical pathologist. If the PLT-O and PLT-I measurements were discordant, then the value closer to the manual platelet estimate was used for statistical analysis.

During the manual counting, the presence and quantity of platelet clumping were graded as score 0, absence of platelet clumps; score 1, small platelet clumps identified in the hemocytometer chamber, but not within the squares used to obtain the platelet count; score 2, small platelet clumps (~2-5 platelets/clumps) occurring in low numbers throughout the chamber and within the squares used for counting; score 3, presence of low numbers of larger platelet clumps (~5-20 platelets/clump), or high numbers of small platelet clumps; score 4, presence of many large platelet clumps, which would invalidate a manual platelet estimate. In addition, manual RBC, WBC, and platelet counts were performed on all PRP and PPP samples. The same board-certified clinical pathologist performed all the assessments.

For RBC and WBC counts, a 1:100 dilution of PRP or PPP was made using 0.9% NaCl solution. Samples were mixed thoroughly and loaded into a hemocytometer. Once the chambers were loaded, the sample was allowed to sediment for 10 min in a humidified chamber. The average number obtained from counting both chambers was used for the statistical analysis. If the counts obtained from each side of the hemocytometer differed by >10%, the counts were rejected, and the procedure was repeated. For platelet counting, a 1:100 dilution of PRP or PPP was made using 1% ammonium oxalate solution; samples were mixed thoroughly and allowed to rest for at least 5 min before loading the hemocytometer. Hemocytometer preparation and cell counting were performed as described above, and platelet clumping was noted, if present. The criteria for platelet clumping assessed on the hemocytometer were those used for the blood smear.

2.4. Platelet Viability

Platelet viability was assessed using a full-spectrum detector-based (filter-less) Cytek Aurora Flow Cytometer (Cytek Biosciences Inc, Fremont, CA, USA) adapted from protocols published elsewhere [24,25]. Immunolabeling of CD41/61, a platelet-specific antigen, was carried out with a primary (#MA5-28370 Invitrogen, Life Technologies Corporation, OR, USA) and a secondary antibody conjugated with a fluorochrome (#P-852, Invitrogen, Life Technologies Corporation, OR, USA). In addition, the association with zombie green (#423112 Biolegend, CA, USA), a dye that binds to cytoplasmic amines, was used to assess the plasma membrane integrity of platelets (onwards referred to as platelet viability). The working solution of Zombie Green was prepared with the dilution in PBS at a 1:100 ratio. Briefly, an aliquot of PRP or PPP was diluted 1:20 in Tyrode's media (134 mM NaCl, 12mM $NaHCO_3$, 2.9 mM KCl, 0.34 mM Na_2HPO_4, 1 mM $MgCl_2$, 10 mM HEPES; pH 7.4) deprived from calcium chloride. Thereafter, the

sample was incubated with Zombie Green working solution (1:1) and anti-CD41/61 monoclonal mouse antibody (1:200) for 30 min at room temperature in the dark.

Samples were washed (1500 × g, 15 min), resuspended in Tyrode's media, and further incubated with polyclonal goat anti-mouse IgG (1:100) for 30 min at room temperature in the dark. Samples were washed (1500 × g, 15 min), resuspended in 200 µL of the same Tyrode's media, and immediately analyzed. This panel identified three different populations (1) viable platelets (CD41/61+ with low zombie green intensity), (2) non-viable (damaged) platelets (CD41/61+ with high zombie green intensity), and (3) debris (CD41/61 negative with high and low zombie green intensity). The sample acquisition by Cytek Aurora flowcytometric analyzer was concluded when at least 1,000,000 fluorescent gated events or 150 µL of the sample were assessed. Single stain controls were used for compensation and unmixing the signals using SpectroFlow® software. Heat-treated platelets (75 °C, 15 min) served as a positive control for non-viable platelets. Data were analyzed with the software FlowJo (FlowJo, V10.6.2, BD Biosciences, NJ, USA); the percentage of events in each population was calculated, and manual compensation was applied as needed.

2.5. Enrichment Factor and Platelet Recovery and Loss

The enrichment factor was calculated for platelets, WBC, and RBC before and after centrifugation for PPP and PRP across methods and after the first centrifugation in method 1. The value obtained in WB was considered the absolute value, and an increase or decrease in platelets or blood cells was used to attain this factor. For instance, if a sample had 100 units of an analyte in WB, and the plasma sample had 160 units, the enrichment factor was deemed as 1.6. Conversely, if the plasma sample had 40 units, the enrichment factor was deemed 0.4. Platelet recovery was calculated by using WB total platelet counts. The amount of platelet recovered in PRP and losses in PPP were

calculated and used for comparisons across methods. Plasma recovery (i.e., the ratio of the PRP volume to the WB volume harvested from the mares) was calculated by using WB total volume as the absolute value, and the final volume of PRP was obtained after processing by each method.

2.6. Data Analyses

The volume of PRP and PPP were normalized to 20 mL for comparisons across methods; specifically, the concentrations (number/µL) of platelets, WBC, and RBC were assessed; therefore, these results were multiplied by 1000 to obtain the concentration per mL, and then by 20 to obtain final amounts. This approach was adopted due to the heterogenous volume of WB harvested for each method and consequent variable concentration. The 20 mL volume was chosen to be a typical volume used for intrauterine infusion in mare studies [3,5]. The sample size was determined based on a parallel study involving the use of PRP in mares. Previous studies comparing methods to prepare PRP typically used 6-8 horses [15–19], while here, eighteen horses were used for most endpoints and six horses for the cooling endpoints. Data analyses were carried out with GraphPad Prism 8.0.1. (GraphPad Prism 8.0.1., GraphPad Software, San Diego, California, USA). The Gaussian distribution curves (platelets, WBC, RBC, enrichment factor, plasma recovery, platelet clumps, and viability) were evaluated using the Shapiro-Wilk normality test. Platelet, WBC, and RBC concentrations, platelet enrichment factor, plasma recovery, and platelet viability were evaluated by ANOVA followed by Tukey's as a post hoc test. Platelet clumps were evaluated by Friedman's test, followed by Dunn's multiple comparisons. Significance was set at $P \leq 0.05$ for all tests. The degree of linear correlation between platelet counting in the WB and PRP for each method was tested using Pearson's coefficient correlation. Strong coefficient of correlation was defined as $r > 0.7$, and moderate $0.5 \leq r \leq 0.7$, and weak correlation when $r < 0.5$.

3. Results

The platelet, WBC, and RBC concentrations of the eighteen mares were not different in the WB across methods (P>0.10) (Table 2). All three methods concentrated platelets at least 1.8 to 5.6-fold in comparison to WB. In addition, WBC and RBC concentrations were reduced 2.9-415-fold and 46.6-321-fold, respectively, in comparison to WB (P<0.0001) (Table 3). The platelet concentration was the greatest in PRP obtained with method 1, followed by methods 2 and 3 (Table 2, P<0.001).

Platelet enrichment was the greatest (5.6) in PRP prepared with method 1, followed by methods 2 (2.5) and 3 (1.8) (Table 2) (P<0.0001). The enrichment factor was 1.6 for platelet concentration when compared with WB after first centrifugation in method 1. In addition, plasma obtained from the first centrifugation in method 1 had a similar platelet concentration compared to PRP obtained with method 3 (P = 0.27); however, lower than PRP obtained by methods 1 (P<0.0001) and 2 (P<0.0001) (Table 2). Concentrations of platelets in PPP were lower than PRP across methods (P<0.0001) (Table 2). In addition, concentrations of platelets in PPP were lower than in WB for methods 1 and 2 (P<0.0007), but not different in method 3 (P=0.99) (Table 2).

Platelet viability was similar in fresh WB and respective plasma samples (P=0.97) (Table 2). Representative images of flow cytometric analyses of platelet viability in PRP are depicted in Figure 2. Strong and positive correlations were found between the number of platelets in the WB and the number of platelets recovered in PRP prepared by methods 2 (r=0.75) and 3 (r=0.73). However, a moderate correlation (r=0.53) was observed between platelet concentration in WB and PRP produced by method 1. There were no changes in the occurrence of platelet clumps in WB and respective plasma samples across methods (P >0.05, Figure 3).

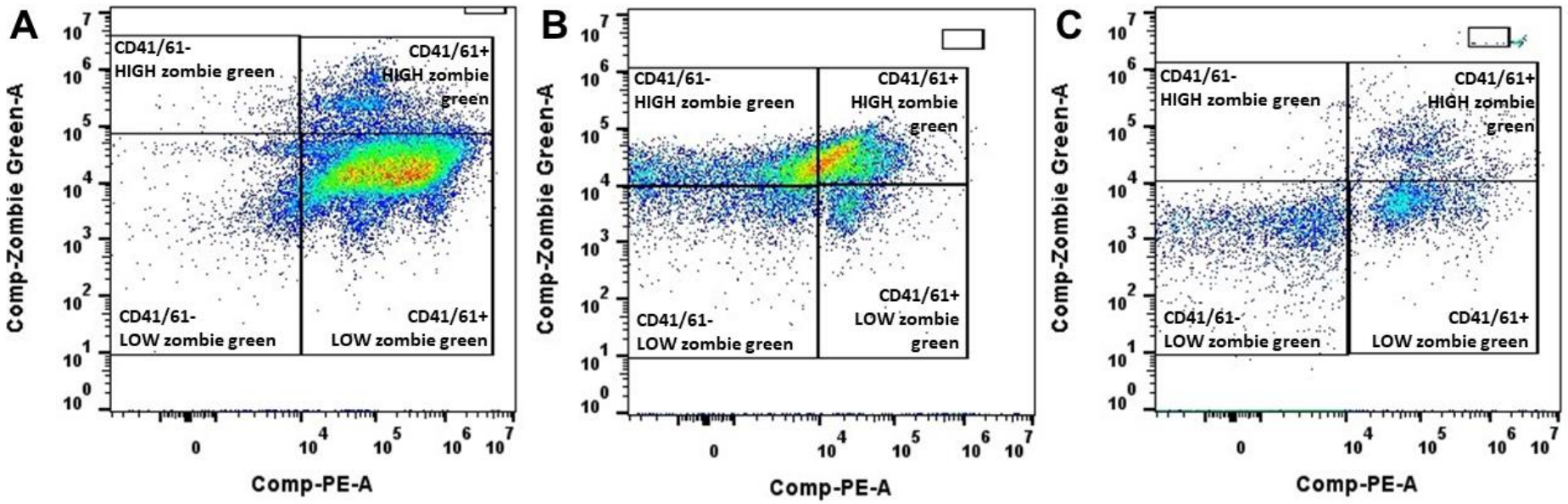

Figure 2. Representative density plot for flow cytometric analyses performed on platelet-rich obtained by Method 1 (A), Method 2 (B) and Method 3 (C). Platelets were identified with a primary (mouse monoclonal antibody anti CD41/61) and secondary antibody anti-mouse IgG conjugated with a fluorochrome (PE, R-phycoerythrin, X-axis) and their membrane integrity was assessed with Zombie Green (Y-axis). The right quadrants enclosed CD41/61 positive events, presumptively corresponding to platelets, with high (damaged membrane) or low (intact membrane) Zombie Green signal. The left quadrants included CD41/61 negative events, likely debris, with high or low Zombie Green signal. Method 1 consisted of collecting whole blood in a blood transfusion bag and then centrifuge at 400 × g/10 min, and then re-centrifuged at 1000 × g/10 min. Method 2 consisted of one centrifugation at 120 × g/10 min. Method 3 involved the assessment of platelet concentration in PRP-3 and PPP-3.

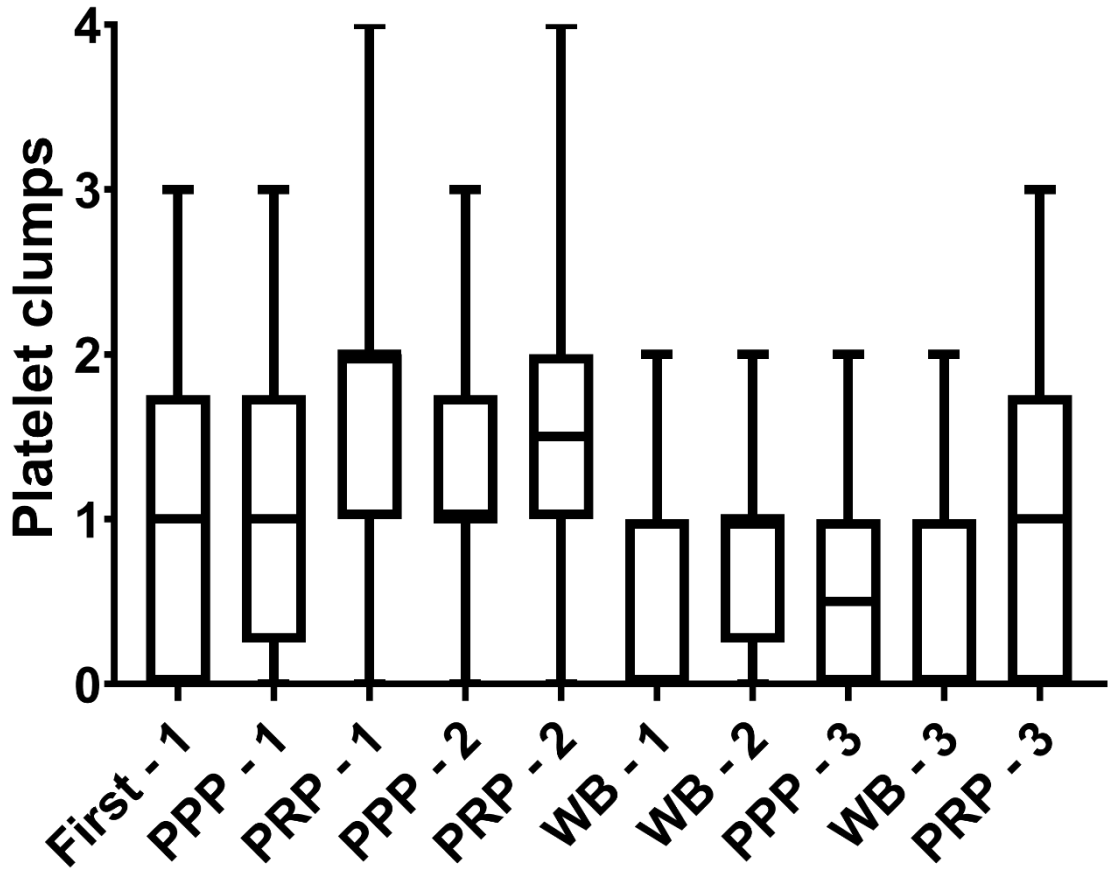

Figure 3. Assessment of platelet harvested, from eighteen mares, by three methods to prepare platelet-rich plasma (PRP) for intrauterine infusions in mares. Method 1 consisted of collecting whole blood (WB) in a blood transfusion bag, centrifugation of WB at 400 × g/10 min (F-C), and centrifugation of plasma at 1000 × g/10 min. Method 2 consisted of one centrifugation at 120 × g/10 min. Method 3 consisted of collecting WB in a syringe and then letting it sediment in an upright position for 4 h. After processing, PRP and platelet-poor plasma (PPP) were obtained across methods. Platelet clumps were assessed across methods. Clumps classification: score 0, absence of platelet clumps; score 1, small platelet clumps identified in the hemocytometer chamber, but not within the squares used to obtain the platelet count; score 2, small platelet clumps (~2-5 platelets/clumps) occurring in low numbers throughout the chamber and within the squares used for counting; score 3, presence of low numbers of larger platelet clumps (~5-20 platelets/clump), or high numbers of small platelet clumps; score 4, presence of many large platelet clumps.

The platelet recoveries in the PRP from methods 1, 2, and 3 were 56%, 81%, and 77%, respectively. In PPP, the platelet losses were 21%, 15%, and 13% using methods 1, 2, and 3, respectively. The recovery factor (i.e., the ratio of the PRP volume to the WB

285 volume) of plasma recovered as PRP was different across methods (P<0.0001; method 1,
286 10.5%; method 2, 33.1%; method 3, 27.2%).

287 The WBC was reduced (P<0.0001) in all PPP and PRP samples, as well as in the
288 plasma after the first centrifugation performed in method 1, when compared with WB
289 (Table 3). However, PRP obtained by method 3 had greater WBC than plasma samples
290 from methods 1 and 2 (P<0.001, Table 3). The WBC was 1.9- and 71.0-fold reduced in
291 the PRP produced by method 1 compared with the PRP from methods 2 and 3, respec-
292 tively. Also, the PRP from method 2 had a 38.1-fold reduction in WBC counts than PRP
293 produced by method 3. The concentration of RBC was also reduced (P<0.0001) in all
294 plasma samples compared with the respective WB samples (Table 3).

Table 2. Assessment of platelet harvested, from eighteen mares, by three methods to prepare platelet-rich plasma (PRP) for intrauterine infusions in mares. Method 1 consisted of collecting whole blood (WB) in a blood transfusion bag, centrifugation of WB at 400 × *g*/10 min (F-C), and centrifugation of plasma at 1000 × *g*/10 min. Method 2 consisted of one centrifugation at 120 × *g*/10 min. Method 3 consisted of collecting WB in a syringe and then letting it sediment in an upright position for 4 h. After processing, PRP and platelet-poor plasma (PPP) were obtained across methods. Platelet concentration, viability, enrichment factor, and the number of clumps were assessed across methods.

		Concentration (×10³/µL)		**Viability (%)**		**Enrichment factor**
		Mean±SD	**range**	**Mean±SD**	**range**	**(%)**
Method 1	**WB**	125.8 ± 27.6****	92-188	94.5 ± 10	60-99	-
	F-C	200.6 ± 60.2***	110-345	-	-	1.59
	PPP	44.2 ± 31.1*****	15-130	85.9 ± 15	57-98	0.35
	PRP	709.4 ± 159.8*	425-960	92.5 ± 8	72-99	5.64
Method 2	**WB**	130.0 ± 18.4****	102-168	87.9 ± 16	50-99	-
	PPP	62.3 ± 23.7*****	40-125	69.6 ± 24	53-99	0.37
	PRP	327.5 ± 64.2**	230-460	87.5 ± 15	55-98	2.52
Method 3	**WB**	129.6 ± 238****	80-172	85.6 ± 15	52-99	-
	PPP	105.9 ± 44.0****	45-180	84.1 ± 17	58-99	0.82
	PRP	239.4 ± 64.5***	180-435	86.8 ± 15	59-99	1.85

Clumps classification: **score 0**, absence of platelet clumps; **score 1**, small platelet clumps identified in the hemocytometer chamber, but not within the squares used to obtain the platelet count; **score 2**, small platelet clumps (~2-5 platelets/clumps) occurring in low numbers throughout the chamber and within the squares used for counting; **score 3**, presence of low numbers of larger platelet clumps (~5-20 platelets/clump), or high numbers of small platelet clumps; **score 4**, presence of many large platelet clumps. Different within columns superscripts (*) indicate differences between groups ($P < 0.05$).

Table 3. White blood cell (WBC) and red blood cell (RBC) counts in whole blood (WB), platelet-rich plasma (PRP), and platelet-poor plasma (PPP) of eighteen mares, having WB harvested and processed by three methods to prepare PRP for intrauterine infusions in mares. Method 1 consisted of collecting whole blood (WB) in a blood transfusion bag, centrifugation of WB at 400 × *g*/10 min (F-C), and centrifugation of plasma at 1000 × *g*/10 min. Method 2 consisted of one centrifugation at 120 × *g*/10 min. Method 3 consisted of collecting WB in a syringe and then letting it sediment in an upright position for 4 h. After processing, PRP and PPP were obtained across methods and WBC and RBC counted.

		White blood cell			**Red blood cell**		
		Concentration (×10³/µL)		**Enrichment factor (%)**	**Concentration (×10⁶/µL)**		**Enrichment factor (%)**
		Mean±SD	range		Mean±SD	range	
Method 1	**WB**	5.9 ± 1.0*	4.4-7.1	-	6.4 ± 8.7*	5.1-7.8	-
	F-C	0.043 ± 0.018**	0.02-0.08	0.007	0.058 ± 0.05**	0.02-0.1	0.009
	PPP	0.014 ± 0.009****	0.01-0.04	0.002	0.020 ± 0.02**	0.01-0.07	0.003
	PRP	0.027 ± 0.017$^{***/****}$	0.01-0.06	0.005	0.042 ± 0.04**	0.03-0.1	0.007
Method 2	**WB**	5.7 ± 1.2*	4.5-7.0	-	7.3 ± 8.9*	6.2-9.3	-
	PPP	0.04 ± 0.03$^{***/****}$	0.01-0.08	0.007	0.069 ± 0.07**	0.06-0.2	0.009
	PRP	0.05 ± 0.04$^{***/****}$	0.01-0.2	0.009	0.083 ± 0.07**	0.02-0.2	0.011
Method 3	**WB**	5.5 ± 0.9*	4.3-7.2	-	6.4 ± 7.4*	4770-7500	-
	PPP	0.8 ± 1.1**	10-3180	0.15	0.13 ± 0.12**	22-400	0.020
	PRP	1.9 ± 1.7**	360-4740	0.35	0.11 ± 0.1**	4-305	0.017

Column with different superscripts (*) indicate differences between groups ($P < 0.05$).

Platelet, WBC, and RBC counts, and platelet viability did not change after cooling-storage (P>0.05; Table 4) of PRP of six mares produced by methods 1 and 2. How-ever, platelet viability was reduced at 24 h of cooled storage in PRP obtained by meth-od 3 (Table 4). In addition, there was an increase in the occurrence of platelet clumps over time in PRP cooled-stored (P<0.05, Figure 4).

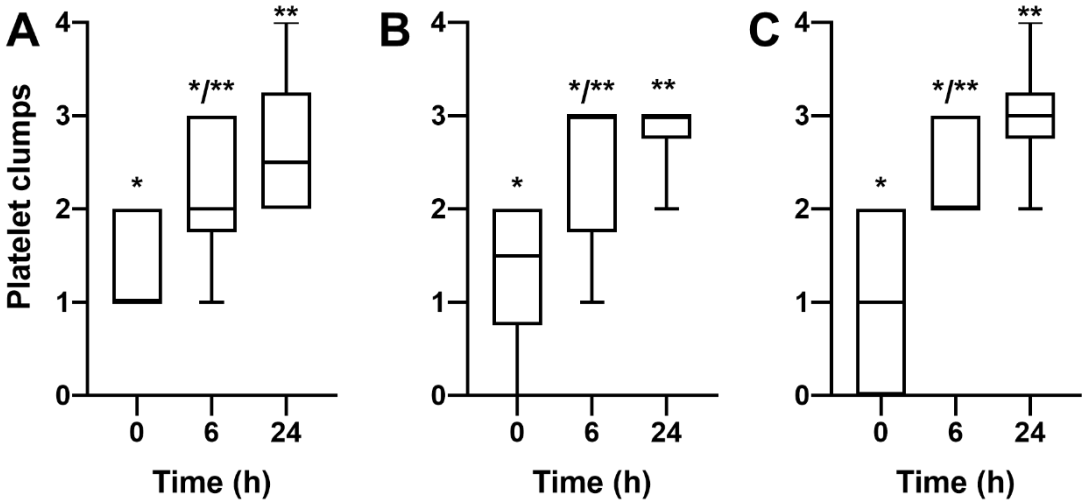

Figure 4. Presence of platelet clumps in platelet-rich plasma (PRP) of six mares, having WB harvested and processed by three methods to prepare PRP for intrauterine infusions in mares and cooled-stored at 5 °C for up to 24h. Method 1 consisted of collecting whole blood (WB) in a blood transfusion bag, centrifugation of WB at 400 × g/10 min (F-C), and centrifugation of plasma at 1000 × g/10 min. Method 2 consisted of one centrifugation at 120 × g/10 min. Method 3 consisted of collecting WB in a syringe and then letting it sediment in an upright position for 4 h. After processing (0h), PRP was obtained across methods and cooled stored at 5 °C for 6 and 24h. Platelet, WBC and RBC counts and platelet viability were assessed in fresh (0h) and cooled-stored PRP at 6 and 24h. Clumps classification: score 0, absence of platelet clumps; score 1, small platelet clumps identified in the hemocytometer chamber, but not within the squares used to obtain the platelet count; score 2, small platelet clumps (~2-5 platelets/clumps) occurring in low numbers throughout the chamber and within the squares used for counting; score 3, presence of low numbers of larger platelet clumps (~5-20 platelets/clump), or high numbers of small platelet clumps; score 4, presence of many large platelet clumps. Different within columns superscripts (*) indicate differences between groups (P < 0.05).

Table 4. Platelet, white blood cell (WBC) and red blood cell (RBC) counts, and platelet viability in platelet-rich plasma (PRP) of six mares, having WB harvested and processed by three methods to prepare PRP for intrauterine infusions in mares and cooled-stored at 5 °C for up to 24h. Method 1 consisted of collecting whole blood (WB) in a blood transfusion bag, centrifugation of WB at 400 × g/10 min (F-C), and centrifugation of plasma at 1000 × g/10 min. Method 2 consisted of one centrifugation at 120 × g/10 min. Method 3 consisted of collecting WB in a syringe and then letting it sediment in an upright position for 4 h. After processing (0h), PRP was obtained across methods and cooled stored at 5 °C for 6 and 24h. Platelet, WBC, and RBC counts and platelet viability were assessed in fresh (0h) and cooled-stored PRP at 6 and 24h.

	Time (h)	Platelet (×10^3/µL)		Platelet viability (%)		WBC (×10^3/µL)		RBC (×10^6/µL)	
		Mean±SD	range	Mean±SD	range	Mean±SD	range	Mean±SD	range
Method 1	**0**	587.8 ± 133	455-825	81.7 ± 9	70-96	2.58 ± 3.2	0.5-5.0	0.04 ± 0.03	0.008-0.10
	6	510.3 ± 102	392-650	55.2 ± 12	39-74	1.25 ± 1.4	0.5-4.0	0.05 ± 0.03	0.02-0.09
	24	495.7 ± 74	412-582	52.3 ± 13	32-72	0.75 ± 0.3	0.5-1.0	0.04 ± 0.02	0.02-0.07
Method 2	**0**	508.7 ± 64	450-625	85.9 ± 9	82-98	2.83 ± 1.6	1.0-5.0	0.20 ± 0.30	0.03-0.73
	6	474.5 ± 102	400-615	61.1 ± 15	36-88	2.83 ± 1.3	2.0-5.0	0.10 ± 0.07	0.04-0.19
	24	448.2 ± 74	375-575	54.6 ± 19	27-83	1.25 ± 0.6	0.5-2.0	0.07 ± 0.05	0.03-0.15
Method 3	**0**	451.2 ± 116	365-610	89.3 ± 6	74-98	47.17 ± 25.9	22.0-85.0	0.12 ± 0.11	0.03-0.31
	6	424.5 ± 94	330-590	60.1 ± 19	36-79	48.17 ± 19.0	22.0-67.0	0.12 ± 0.10	0.03-0.27
	24	368.7 ± 33	320-417	46.9 ± 21	26-78	36.50 ± 26.4	11.0-88.0	0.14 ± 0.13	0.01-0.35

Clumps classification: **score 0**, absence of platelet clumps; **score 1**, small platelet clumps identified in the hemocytometer chamber, but not within the squares used to obtain the platelet count; **score 2**, small platelet clumps (~2-5 platelets/clumps) occurring in low numbers throughout the chamber and within the squares used for counting; **score 3**, presence of low numbers of larger platelet clumps (~5-20 platelets/clump), or high numbers of small platelet clumps; **score 4**, presence of many large platelet clumps. Different within columns superscripts (*) indicate differences between groups ($P < 0.05$).

Discussion

This study was set-forth as the first to compare three manual protocols used to prepare PRP and its cooling. The three methods were selected for having potential application in mare reproductive practice. Previously, blood transfusion bags (450 mL) have been used to prepare PRP for intrauterine infusion [4] and other than mare reproduction [15,17]; here, a smaller bag (150 mL), commonly used for blood collection and transfusion in dogs and cats, was used. Method 2 has been used in clinical practice and in a couple of studies for intrauterine infusion in mares [3,26]. Method 3 has been used in practice but not studied in a controlled study.

Previously reported methods to prepare PRP for intrauterine infusion in mares may involve centrifugation or the use of specialized automated machines to concentrate PRP [3,5–8]. Early studies involving PRP intrauterine infusions in mares applied a specialized blood fractionated machine (AngelTM Cytomedix) [6,7]. The volume of plasma recovered by this technology is much reduced compared with manual methods to the point that the authors of these reports diluted the PRP in PPP to bring the final volume up to 10 mL. The amount of WB needed to prepare PRP varies with the method used. In the two early reports, the authors did not include the amount of blood harvested, platelet concentration, or final counts obtained with the machine [6,7]. In a more recent study, the authors also used a commercially available device (Restigen PRP®) to obtain PRP. For this, 55 mL of WB was harvested in a 60 mL syringe containing 5 mL of anticoagulant from each mare to produce 6 mL of PRP, which was diluted in 9 mL of PPP to produce a final volume of 15 mL for intrauterine infusion [8]; these authors did not provide the platelet concentrations. In another study, using a manual method, 100 milliliters of WB were harvested in vacutainer tubes and then centrifuged at 120 × g for 10 min, then after the first centrifugation, the lower 50% of the plasma was further centrifuged at 240 × g

for 10 min; this protocol yielded 20 mL of PRP (containing ≥ 250×103 of platelets/μL, ~5 ×109 platelets) [5]. In another study, 45 mL of WB was collected in 4.5 mL sodium citrate tubes and centrifuged once at 120 × g for 10 min; the protocol resulted in 20 mL of PRP containing 7±0.3×109 platelets (354±17×103 of platelets/μL) [3]. In the present study, the total platelet obtained ranged from 4.8 ± 0.3 to 14.2 ± 0.9 × 109 when normalized to 20 mL. Therefore, the three protocols investigated herein resulted in platelet concentrations consistent with other studies performing intrauterine infusions with PRP in mares [3,5].

All methods tested herein were sufficient to increase the plasma's platelet concentration (1.8 – 5.6-fold) and reduce RBC and WBC as previously attained by other studies [3,18,19]. Studies suggest that increments of 3 to 9 folds in human platelet concentration compared to the WB is ideal [27]; however, the optimal platelet concentration has yet to be defined for clinical use in horses, including intrauterine infusions in mares [3,15,28].

Method 1 resulted in PRP with platelet concentrations of 2.2- and 3.0-fold higher than methods 2 and 3. This finding could be to the fact that a larger volume of WB was used (3- to 4-fold greater) when compared with other methods, but this is more likely because 2-step centrifugation was used in method 1. The second centrifugation in method 1 applied a much greater g-force than the other methods, yet the platelet viability was not affected in PRP or PPP. Platelet concentration in PRP obtained by methods 2 and 3 was strongly correlated with the platelet concentration in WB, while this was not the case for method 1. These findings suggest that 2-step centrifugation may help standardize platelet recovery in comparison with method 2 that only uses 1-step centrifugation, and method 3 did not employ any imposed force other than the gravitational force.

The volume of WB harvested to produce PRP in this study is equivalent to other studies using PRP in horse joints and tendons [15,28,29] and for intrauterine infusion in mares [6,7]. Tendons have no lumen, and joints have reduced space, thus requiring highly concentrated platelets in a reduced volume of PRP. Conversely, the mare uterus has a large uterine lumen, therefore, presumably requiring a larger volume of PRP. In addition, mares susceptible to PBIE, the population to benefit the most from PRP therapy, are often older, multiparous, and have a large and pendulous uterus [30,31]. Generally, it is believed that intrauterine infusion should have a volume of 10-60 mL to allow the substance being infused to interact with the entire uterine lumen. In the present study, all three methods would meet this presumed ideal volume range.

Of interest, anticoagulants reported for intrauterine infusions in mares include sodium citrate, CPD-A, and acid citrate dextrose solution [3,5–8]. Herein CPD-A was used in methods 1 and 3, and sodium citrate in method 2. There is no data to suggest that either one of the anticoagulants used in the present study is superior to another for intrauterine infusion in mares; however, both were superior to acid citrate dextrose solution in two studies [32,33]. Certainly, in this study, the platelet viability and clumps were not affected by the method used.

While there appears to be no clear advantage of either method over another, some clinicians may feel more comfortable with one method versus another. Interestingly, plasma after the first centrifugation at 400 × g in method 1 yielded 1.6-fold greater platelet concentration than the WB. This finding suggests that PRP may be prepared for intrauterine infusion in mares using 400 × g centrifugation for 10 min in 50 mL conical tubes. This method would minimize the losses associated with the second centrifugation but would increase the volume of fluid infused in the uterus, which can be an issue with

maiden mares with small uteri, while this would certainly not be a problem with multiparous mares.

In all three methods, RBC and WBC were reduced in all plasma samples when compared with WB. Contamination of WBC and RBC in PRP can contribute to platelet aggregation and activation [34]. In addition, removing RBC seems beneficial as their presence in excess can be detrimental to sperm, thus limiting PRP's use pre-breeding [35]. However, controversy exists whether the presence of fresh WBC from WB could be beneficial for the PBIE [36] or other clinical applications [37]. The PRP produced by method 3 had greater WBC concentrations, likely because of the lack of centrifugation to forcefully push blood cells to the bottom and the clinical relevance of this finding remains unclear. There is a scant body of evidence regarding the contribution of WBC in PRP to the healing process and inflammatory properties [38,39]. A beneficial effect has been suggested to be due to their anti-infectious activity [40], and one report has suggested that intrauterine infusion of WBC could eliminate bacterial contamination faster from the uterus of mares susceptible to PBIE [36]. However, it is unknown if including fresh WBC from WB in PRP is beneficial for the treatment of PBIE in mares.

The processing time for the methods tested in the present study was ~120 min for method 1, ~30 min for method 2, and ~240 min for method 3. Although method 3 required more time to be done, this method can be performed in ambulatory conditions, where the clinician may not have a centrifuge available. Four hours of sedimentation was chosen based on a pilot study. The results of the pilot study suggested that 4 hours was the minimal time necessary for RBC sedimentation and to minimize RBC and WBC contamination, and also because four hours is the minimum time that a mare could be flushed after breeding without having compromised pregnancy rates [41] and have the

443 uterus infused with PRP. Therefore, method 3 can be an alternative protocol to prepare
444 PRP in field conditions.

445 Platelets have a short lifespan, and in human medicine, they often are stored at
446 room temperature, which enhances the chance of bacterial contamination [23]. Therefore,
447 cold-storage of PRP has the potential to be an alternative to maintain platelet viability and
448 reduce bacterial growth in PRP [42]. Although in one equine study, there were no platelet
449 contamination or losses in platelet counts in PRP stored for up to seven days at room
450 temperature (22 °C) on a rocker, the technique used for the preservation used a piece of
451 specialized research equipment not available in clinical practice [18]. Also, the authors
452 reported a reduction in platelet function over time [18]. In the present study, platelet
453 counting and viability did not change up to 24 h of cooled-storage at 5 °C in PRP produced
454 by methods 1 and 2. However, there was a reduced number of viable platelets in PRP
455 cooled-stored for 24 h in PRP obtained by method 3. It is unclear why PRP produced by
456 method 3 had lower platelet viability after cooled storage, this could be due to the greater
457 contamination with WBC and RBC in PRP produced with method 3 when compared with
458 methods 1 and 2. The presence of increased WBC in PRP can affect the metabolic activity
459 of platelets, increase lactate production and glucose consumption, which can undergo
460 wastage in the quality of PRP [34]. Other authors reported no changes in platelet counting
461 in equine [43] or human [44] platelet concentrate after cooled-storage, but they did not
462 report the concentration of RBC or WBC in their PRP.

463 Although the number of mares used for harvesting blood and processing PRP was
464 greater than previous studies [15–19], there was a great range in age and breeds. While it
465 is unknown if breed or age affects platelet concentrations, the wide range of mares used
466 herein reflected the efficacy of the methods tested in the present study to produce PRP in
467 wide range population of horses. Also, while only six mares were used to cool down PRP,

we believe that this subset of mares was representative of the whole group of mares. Though it is possible if a large group of mares was used in the cooling segment of the study, different results could have been obtained. Another limitation of the present study is that the clinical efficacy of the methods was not tested in mares.

5. Conclusions

In conclusion, the three manual methods assessed to prepare PRP concentrated platelets. Method 1 (i.e., involving double centrifugation) resulted in the greatest platelet concentrations, while method 3 (sedimentation) resulted in the lowest platelet concentration and greater contamination with WBC. Cooling PRP did not compromise platelet viability for methods 1 and 2. However, cooling PRP prepared with method 3 decreased platelet viability. Also, cooling PRP without cryoprotector increased platelet agglutination overtime and across methods. In vivo studies are warranted to assess the clinical efficacy of PRP obtained with the three methods and to determine the role of WBC and RBC contamination and platelet agglutination during cooling.

Author Contributions: I.F. Canisso contributed to study design, study execution, data analysis, interpretation, and preparation of the manuscript. L.G.T.M. Segabinazzi contributed to study design and execution, data analysis, interpretation, and preparation of the manuscript. G. Podico contributed to study design, study execution, and preparation of the manuscript. M. Rosser and S. Nanjappa contributed to study execution and methods. M. Alvarenga contributed to the study design and preparation of the manuscript. All authors gave their final approval of the manuscript.

Funding: This study was financially supported by São Paulo State Research Foundation (FAPESP grant #2018/02856-3) and the USDA Hatch Animal Health (#ILLU-888-912).

Institutional Review Board Statement: The study protocol was approved by the University of Illinois Institutional Animal Care and Used Committee protocol #19141.

Data Availability Statement: The original contributions presented in the study are included in the article/Supplementary Materials, further inquiries can be directed to the corresponding author/s.

Conflicts of Interest: The authors declare no conflict of interest.

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Capítulo 3

ARTIGO II – Intrauterine blood plasma platelet-therapy mitigates persistent breeding-induced endometritis, reduces uterine infections, and improves embryo recovery in mares

Artigo redigido e publicado (10.3390/antibiotics10050490) na Antibiotics, ISSN 2079-6382, fator de impacto 3,893, ranqueada como A1 pelo QUALIS – CAPES de 2019.

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Abstract: Microorganisms, including pathogenic or opportunistic bacteria and fungi, may gain access to the uterus during breeding and infectious endometritis plays a major role in equine subfertility. This study aimed to assess the post-breeding inflammatory response, endometrial culture, and embryo recovery in mares susceptible to PBIE treated with plasma-rich (PRP) or -poor (PPP) in platelets. Mares (n=12) susceptible to PBIE had three cycles randomly assigned to receive intrauterine infusions of LRS (control), or autologous PRP or PPP pre- (48 and 24h) and post-breeding (6 and 24h). Mares were bred with fresh semen from one stallion. Intrauterine fluid accumulation (IUF) and endometrial neutrophils were assessed every 24h up to 96h post-breeding. Uterine cytokines (Ilβ, IL6, CXCL8 and IL10) were evaluated before (0h), 6 and 24h post-breeding, and endometrial culture three and nine-days after breed. Embryo flushing was performed 8d post-ovulation. Data were analyzed with mixed model, Tukey's post-hoc test, and multivariate regression. PRP treatment reduced endometrial neutrophils, post-breeding IUF and pro-inflammatory cytokines when compared to control-assigned cycles, but not significantly different than PPP. Controls had a significantly higher percentage of positive bacterial cultures (33%) in comparison to PRP-assigned cycles (0%), whereas cycles treated with PPP were not significantly different from the other groups (25%). The PRP-assigned cycles had significantly greater embryo recovery rates (83%) than the control (33%), though not significantly different than PPP (60%). Plasma infusion reduced the duration and intensity of the post-breeding inflammatory response and improved embryo recovery in mares susceptible to PBIE. Platelets incrementally downregulate PBIE and appear to have a dose-dependent antimicrobial property.

Keywords: Endometrium, uterine inflammation, equine, PRP, immunomodulation.

1. Introduction

Endometritis is the third most common disease affecting horses in the United States [1] and is the number one cause of subfertility and poor reproductive efficiency in mares [2]. Mares are classified as susceptible or as resistant to endometritis based on their ability to clear uterine infection/inflammation by 48-72 hours post-breeding [3]. Post-breeding induced endometritis can be caused by infectious (i.e., bacteria and fungus) and by non-infections agents such as sperm [4]. All mares display a physiological and transient uterine inflammatory response; however, the mares deemed susceptible to persistent breeding-induced endometritis (PBIE) have a delayed and prolonged uterine inflammatory response [5]. The persistent endometrial inflammation leads to a hostile uterine environment for the embryo entering the uterus, compromising embryo recovery and pregnancy rates [6,7].

Infectious endometritis plays a major role in equine subfertility; up to 25-60% of mares failing to become pregnant have bacterial uterine infection [8–11]. Microorganisms, including pathogenic or opportunistic bacteria and fungi, may gain access to the uterus during breeding. While the resistant mares respond rapidly to the presence of sperm and microorganisms, inadequate immune response may lead to persistent inflammation and infection in mares susceptible to PBIE [12].

Traditionally, PBIE has been treated with multi-modal therapeutics, such as a combination of uterine lavage, ecbolic agents, anti-inflammatories, and antibiotics [8]. While employing multi-modal therapeutics effectively manages PBIE, some mares, particularly aging broodmares and embryo donors' mares enrolled in embryo transfer programs for multiple years, often fail to respond to traditional therapy for PBIE (8). Also, in numerous stud farms, all mares in the premise are prophylactically infused with broad-

spectrum antibiotics regardless of need; such practice likely contributes to the development of antimicrobial resistance.

In recent years, the lack of response to conventional therapy coupled with the increasing incidence of antibiotic-resistant bacteria led to the development of alternative treatments for mares suffering from PBIE [13]; these has been largely attributed in part to the indiscriminate use of antibiotics in veterinary medicine (e.g., non-select intrauterine infusion in mares). Multidrug-resistant microorganisms pose a major global threat to public health. Autologous platelet-rich plasma (PRP), a whole blood plasma with a high platelet concentration, is becoming a popular nontraditional therapy in human and veterinary medicine as an alternative to circumvent such problems. This byproduct has been used for its anti-inflammatory, regenerative, and antimicrobial properties [14–18].

Platelets, or thrombocytes, are fragments of cytoplasm derived from megakaryocytes, which are large cells present in the bone marrow [19]. The cytoplasm of platelets is subdivided into chromomere, where granules accumulate, and the hyalomere, an agranular region rich in cytoskeletal proteins [15]. Platelet granules contain numerous proteins such as fibrinogen, growth factors (e.g., transforming growth factor β, vascular endothelial growth factor), cytokines (e.g., CXCL8 and TNFα), and antimicrobial peptides (e.g., platelet factor 4, RANTES, connective tissue activating peptide 3, platelet basic protein, thymosin beta-4, fibrinopeptide A and B) [20,21].

In mare reproductive practice, studies showed that intrauterine infusion with PRP could improve pregnancy rates by mitigating the post-breeding inflammatory response [22–26], but PRP has not been critically assessed in embryo donors mares, a group of mares prone to PBIE. Platelets' natural antimicrobial peptides are thought to be responsible for PRP's benefit on septic arthritis in horses [27]; however, PRP's anti-microbial beneficial properties have yet to be evaluated in mares susceptible to PBIE.

The hypotheses of this study are that the administration of PRP to embryo donor mares susceptible to PBIE will result in fewer uterine infections and a lessened post-breeding inflammatory response in comparison with platelet-poor-plasma (PPP) or control (Lactate Ringer's Solution LRS) treatments. Specifically, PRP therapy reduces the duration and intensity of the post-breeding inflammatory response and reduce the chances of post-breeding uterine infection leading to enhanced embryo recovery rates of mares susceptible to PBIE. This study aimed to compare PRP and PPP's effects into control-assigned cycles on uterine microbiology, endometrial inflammation, intrauterine fluid accumulation, progesterone concentration, and endometrial receptor expression and embryo recovery rates in mares susceptible to PBIE.

2. Results

Screening mares for susceptibility to PBIE

After screening twenty two mares, twelve mares were identified as susceptible, and seven as resistant to PBIE [3], three were deemed intermediate. Susceptible mares had an increased intrauterine fluid until 72 h post-sperm challenge ($P < 0.05$), while resistant mares did not ($P > 0.05$) (Supplement Figure 1). In addition, susceptible mares had greater intrauterine fluid accumulation than resistant mares at all time points post-sperm challenge ($P < 0.05$). Similarly, PMNs were more abundant post sperm-challenge ($P < 0.05$) in susceptible mares than resistant mares (Figure 1). There was an effect of time ($P < 0.05$) but no differences between mare groups for edema scores ($P > 0.05$) (Supplement Figure 2). Eight mares were classified as IIB and four mares as III in the susceptible group, whereas in the resistant mares, two were classified as I, and five were classified as IIA.

Platelet-rich or -poor plasma

Mares susceptible to PBIE had platelet concentration in whole blood ranging from 74.6 to 188.0 × 103 platelets per µL (Table 1). There was a moderate correlation (r=0.5) between platelet concentration in the whole blood and the PRP's final platelet concentration. Mean platelet concentration increased 5.2-fold in PRP compared to whole blood count (P = 0.0003, Table 1). There was a reduction in RBC (266-fold) and WBC (248.5-fold) in PRP and PPP in comparison to whole blood (P < 0.0001). Platelet-poor plasma had lower platelet concentration than whole blood (3.3-fold reduction) and PRP (17.3-fold reduction) (P < 0.02, Table 1). Platelet viability was similar for both PRP (97.0±0.7%) and PPP (97.2±0.6%) (P = 0.79, Figure 1). The mean number of platelets infused in the uterus of mares was greater in PRP (24.9±1.2×109 platelets) than PPP (1.4±0.2×109 platelets) (P <0.001).

Table 1. Platelets, red blood cells (RBC) and white blood cells (WBC) in the whole blood (WB), platelet-rich (PRP), and platelet-poor plasma (PPP) obtained from 96 venipunctures from mares susceptible to persistent breeding-induced endometritis. Each mare cycle was assigned in a cross over-designed to either PRP (n=12), PPP (n=10), or control (n=12) group.

	WB	**PRP**	**PPP**
Platelets (10^3/µL)	119.9±30[b]	622.9±144[a]	36.0±25[c]
RBC (10^6/µL)	6.4±0.9[a]	0.02±0.022[b]	0.01±0.011[b]
WBC (10^3/µL)	5.6±1.2[a]	<0.001[b]	<0.001[b]

Mean ± SEM. Different superscripts (abc) denote differences among columns within rows (P < 0.05).

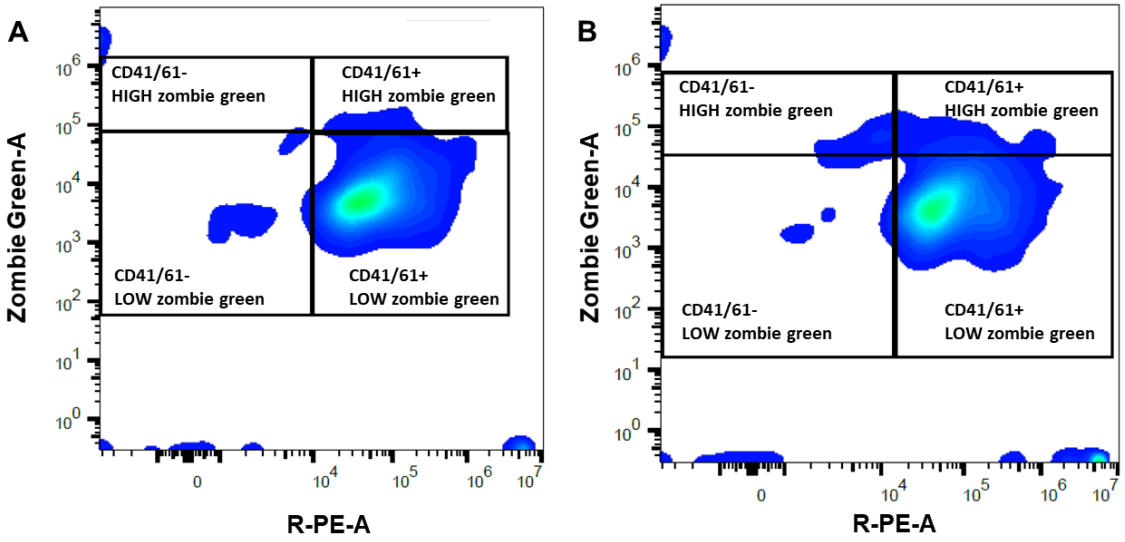


Figure 1. Representative density plot for flow cytometric analyses performed on platelet-rich (A) and -poor (B) plasma obtained from a mare susceptible to persistent-breeding induced endometritis. Platelets were identified with a primary (mouse monoclonal antibody anti CD41/61) and secondary antibody anti-mouse IgG conjugated with a fluorochrome (PE, R-phycoerythrin, X-axis) and their membrane integrity was assessed with Zombie Green (Y-axis). The right quadrants enclosed CD41/61 positive events, presumptively corresponding to platelets, with high (damaged membrane) or low (intact membrane) Zombie Green signal. The left quadrants included CD41/61 negative events, likely debris, with high or low Zombie Green signal.

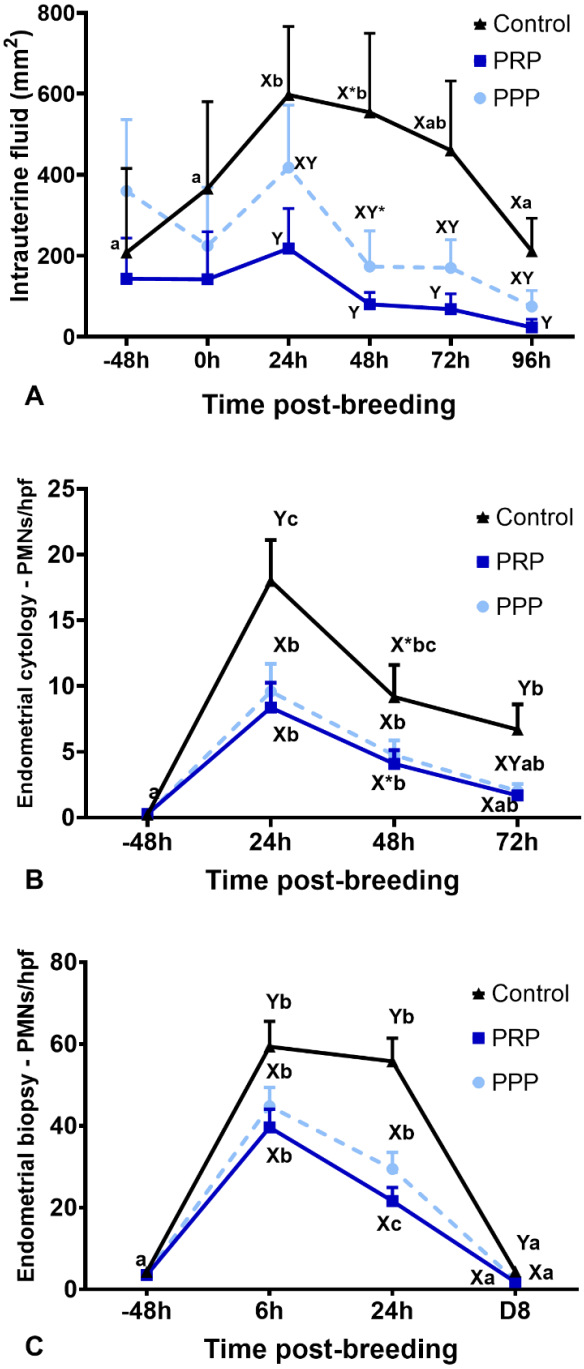
Semen parameters

Twenty-six ejaculates were harvested and used for breeding in the study. Breeding doses and sperm parameters did not differ across cycles during the experiment ($P > 0.05$, Table S1).

Intrauterine fluid accumulation and endometrial edema

There were no differences for intrauterine fluid accumulation before treatment (-48h) and breeding (0 h) in all cycles ($P > 0.05$, Figure 2A). Endometrial edema scores were not different among groups at 0, 24, 48, 72 and 96 h ($P > 0.05$). However, intrauterine fluid accumulation was reduced up to 96 h post-breeding in mares with assigned cycles to receive PRP when compared to mares assigned to the Control cycles ($P < 0.05$). The cycles assigned to PPP were not different than PRP or Control-assigned

cycles ($P > 0.05$) (Figure 2A). However, at 48 h, intrauterine fluid accumulation tended ($P = 0.09$) to be reduced in the PPP assigned cycles when compared to the Control cycles. At 96 h after breeding, mares in the Control cycle had more intrauterine fluid accumulation than mares treated with PRP ($P = 0.043$). Two mares stopped cycling due to seasonality while assigned to PPP cycles; therefore, only ten cycles were fully completed for PPP.



144

145 **Figure 2.** Post-breeding inflammatory response in mares susceptible to persistent
146 breeding induced endometritis. Assessments were carried out from pre- (early onset of
147 estrus) and post-breeding, and on the day of embryo flushing (eight days post-ovulation,
148 D8): (A) intrauterine fluid accumulation pre- and post-breeding; (B) Mean
149 polymorphonuclear cells (PMNs) assessed in 5 high-power fields (hpf) in endometrial
150 cytology, and (C) Mean PMNs counts assessed in 5 hpf in endometrial biopsies. Mares
151 (n=12) had estrous cycles (n=34) assigned to receive four intrauterine infusions with
152 Lactate Ringer's Solution (Control, n=12), platelet-rich (PRP n=12), or -poor plasma
153 (PPP n=10) in a cross-over design. Different superscripts denote the effects of time (^{abc})
154 and differences between groups (^{XY}) ($P < 0.05$). Asterisk (*) denotes tendency ($0.05 < P$
155 < 0.1).

156 **Inflammatory cell counts on endometrial biopsy and cytology**

157 Endometrial cytology revealed that PRP reduced ($P < 0.0001$) the PMNs number
158 at 24 and 72 h, as well as tended ($P = 0.08$) to reduce at 48 h after breeding when compared
159 with similar time points in the control-assigned cycles (Figure 2B). The PPP-assigned
160 cycles did not differ from the other cycle-assignments for the number of PMNs in
161 cytology ($P > 0.05$). However, both PRP and PPP reduced the number of PMNs in
162 endometrial biopsies at 6 ($P = 0.001$) and 24 h ($P < 0.0001$) post-breeding and eight days
163 post-ovulation ($P = 0.0047$, Figure 2C, Figure 3). There were no differences in the number
164 of lymphocytes among groups or time points ($P > 0.05$; Figure 3). Moderate correlation
165 ($r = 0.65$) was observed between PMNs counted in endometrial cytology samples and
166 endometrial biopsy samples.

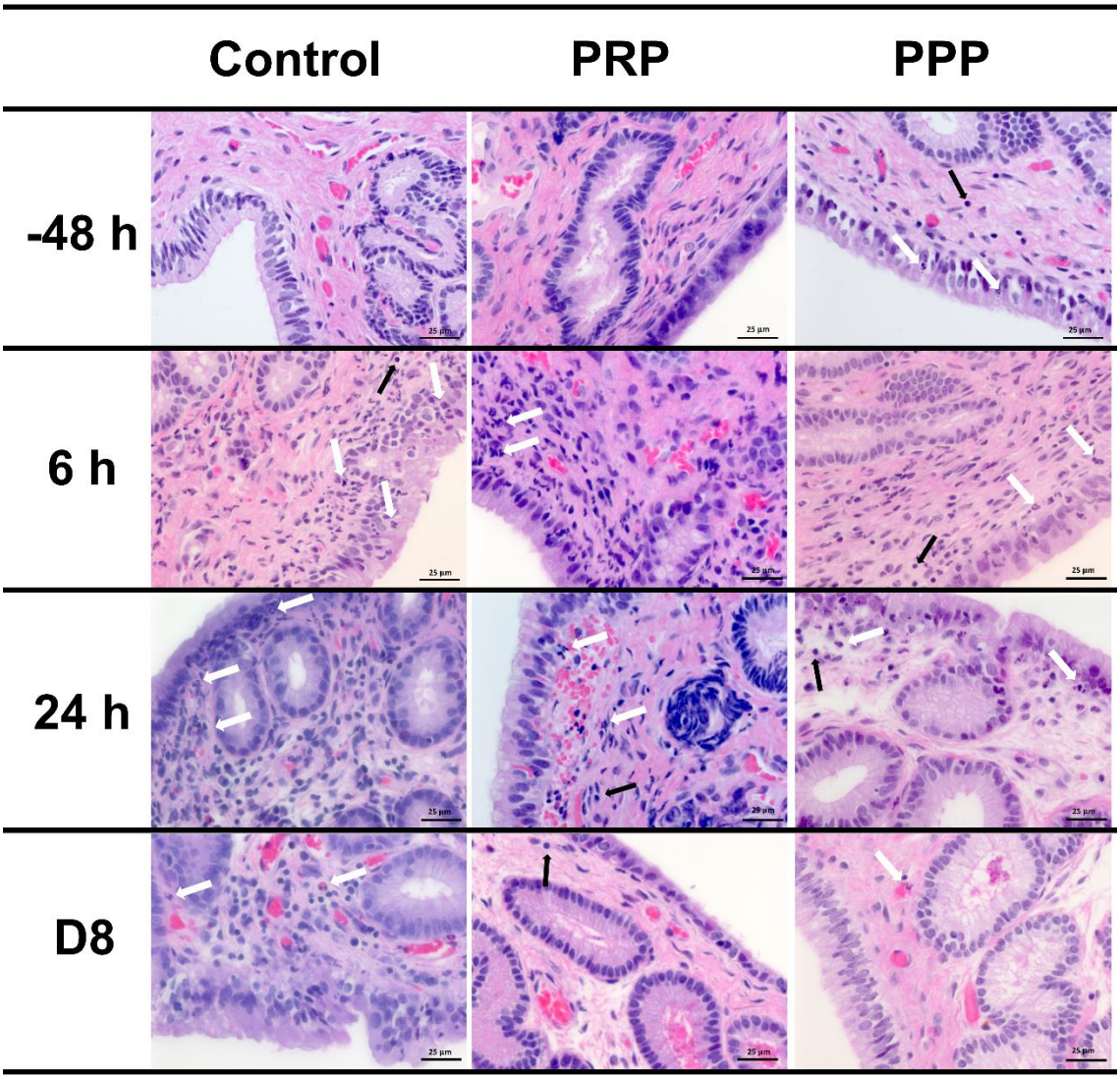


Figure 3. Representative images of H&E-stained endometrial biopsies collected from mares susceptible to persistent breeding-induced endometritis pre- (-48h) and post-breeding (6 and 24h), and on the day of embryo flushing (eight days post-ovulation, D8). Mares were treated with four intrauterine infusions of Lactated-Ringer's solution (Control), platelet-rich (PRP), or -poor plasma (PPP). White arrows indicate neutrophils and black arrows indicate lymphocytes. Magnification ×400.

Cytokine concentrations in uterine fluid

Cytokine concentrations were assessed in a subset of mares. There were no changes in the concentration of the cytokines assessed in the present study among cycles before breeding ($P > 0.05$). Concentrations of IL1β increased 6h post breed in all groups ($P < 0.05$, Figure 4A), and cycles treated with PRP had lower IL1β and IL6 at 24h than control-assigned cycles ($P < 0.05$, Figure 4A, B). CXCL8 increased post-breeding in

mares at the Control-assigned cycle (P <0.05, Figure 4C). Higher concentrations of CXCL8 were noted post-breeding (6 and 24h) in mares assigned to control cycles compared with PRP-assigned cycle (P <0.05, Figure 4C). There were no changes in IL10 concentrations in the uterine fluid across time or treatment (P >0.05, Figure 4D).

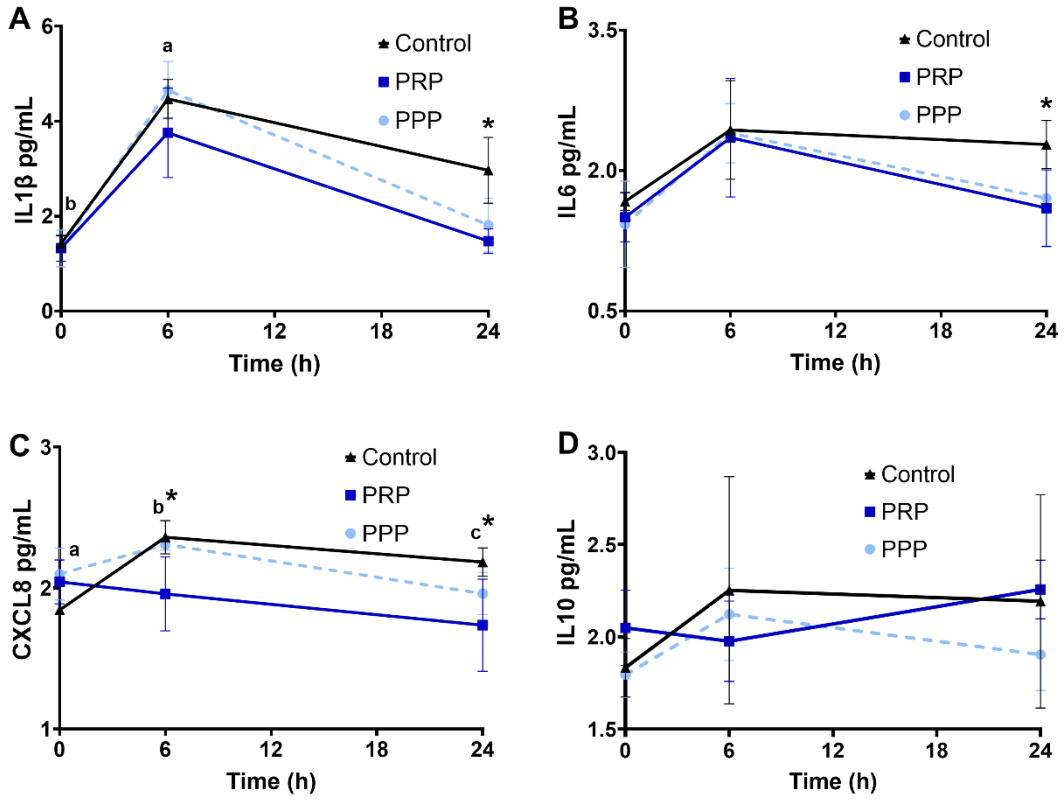


Figure 4. Concentrations (log10) of cytokines (A, IL1β; B, IL6; C, CXCL8; D, IL10), in uterine fluid of mares susceptible to persistent breeding induced endometritis. Assessments were carried out from pre- (0h, early onset of estrus), 6 and 24 h post-breeding. Mares (n=6) had estrous cycles (n=18) assigned to receive four intrauterine infusions with Lactate Ringer's Solution (Control, n=6), platelet-rich (PRP n=6), or -poor plasma (PPP n=6) in a cross-over design. Different superscripts denote the effects of time (^{abc}). Asterisk (*) denotes difference between Control- and PRP-assigned cycles (P < 0.05).

Immunohistochemical evaluation

Positive nuclei to PR were brown-stained, while negative nuclei were blue-stained by hematoxylin counterstain. The PR expression was intensely detected in epithelial cells and glandular epithelium; however, it was rarely detected in the stroma. There were no

changes in PR expression between treatments (P > 0.05; Figure 5). Negative controls showed no immunoreaction.

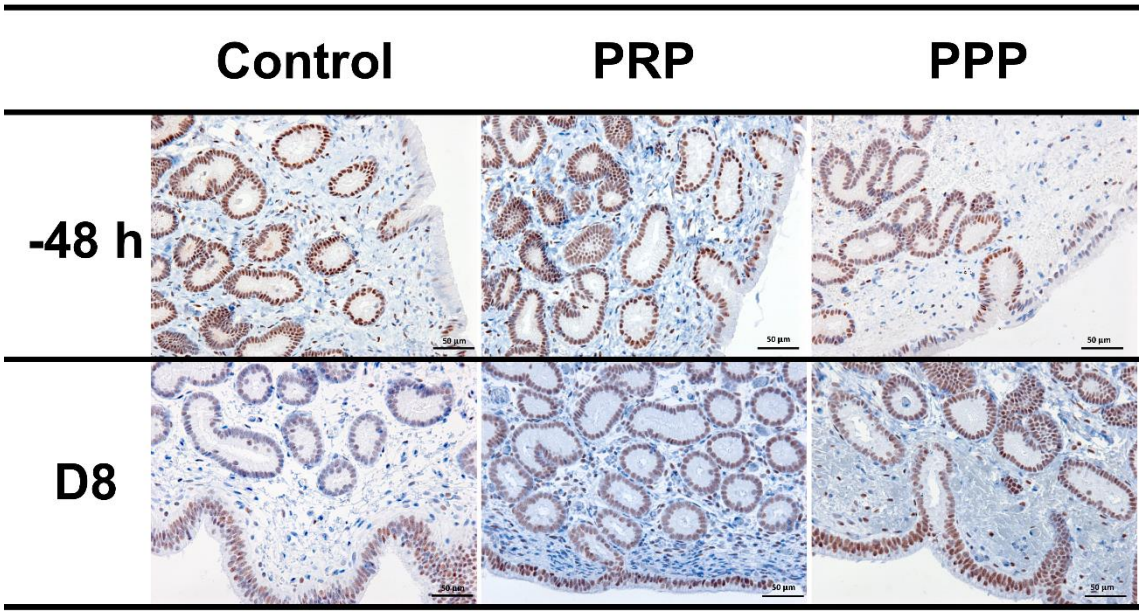


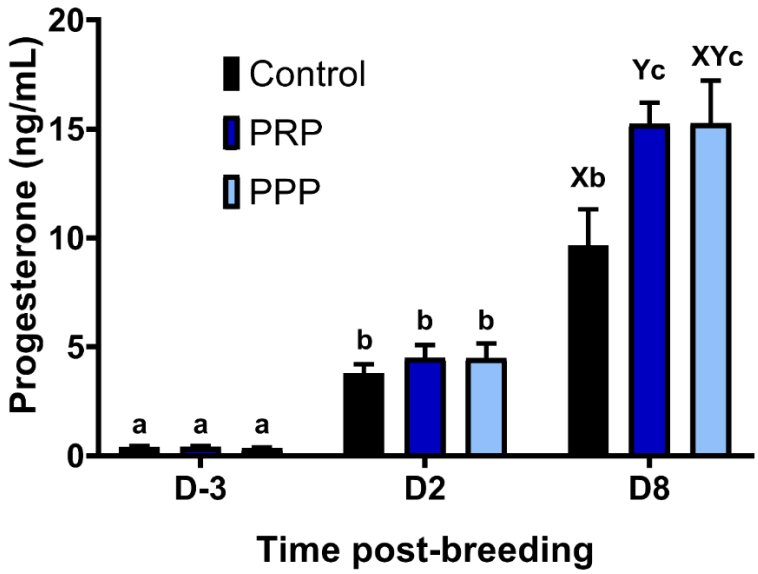
Figure 5. Representative images of immunohistochemical PR expression in uterine biopsies (200 × magnification) from mares susceptible to persistent breeding-induced endometritis pre-breeding (-48h) and on the day of embryo flushing (eight days post-ovulation, D8). Mares were treated with four intrauterine infusions of Lactated-Ringer's solution (Control), platelet-rich (PRP), or -poor plasma (PPP). Positive nuclei to PR were brown-stained, while negative nuclei were blue stained by hematoxylin counterstain.

Endometrial culture and progesterone concentrations

Estrous cycles assigned as Control and PPP tended (P = 0.08) to result in a higher percentage of positive aerobic bacterial cultures at Day-2 than PRP cycles. In contrast, at Day-8, mares assigned as control had a higher percentage of positive bacterial cultures in comparison to PRP-assigned cycles (0%) (P = 0.0373), whereas cycles assigned to PPP were not different from the other groups (P > 0.05, Table 2). The concentrations of progesterone increased over time across groups (P < 0.0001), and PRP assigned cycles had greater progesterone concentrations at Day-8 than in the Control-assigned cycles (P = 0.0376), but not different than PPP assigned-cycles (P > 0.05; Figure 6). There were no differences on progesterone concentrations between mares with positive and negative

219 embryo flushes in the control group (P > 0.05) or all cycles together positive vs. negative

220 embryo flushing (P > 0.05; Figure S3).



221

222 **Figure 6.** Circulating progesterone concentrations pre- and post-ovulation in mares
223 susceptible to persistent breeding induced endometritis. Mares (n=12) had estrous cycles
224 (n=34) assigned to receive intrauterine infusions with Lactate Ringer's Solution (LRS)
225 (Control, n=12), platelet-rich (PRP n=12), or -poor plasma (PPP n=10) in a cross-over
226 design. Assessments were carried out from the early onset of estrus (D-3), 48 h post-
227 ovulation (D2) and on the day of embryo flushing, eight days post-ovulation (D8):
228 Different superscripts denote the effects of time (^{abc}) and differences between groups (^{XY})
229 (P < 0.05).

Table 2. Aerobic culture results of aerobic obtained Day 2 (D2) and Day 8 (D8) post-ovulation in mares susceptible to persistent breeding-induced endometritis. Estrous cycles (n=34) from twelve mares were assigned in a cross over design to receive intrauterine uterine infusions with Lactated Ringer's Solution (Control, n=12), platelet-rich (PRP, n=12) or -poor plasma (PPP, n=10).

	D2			D8		
Mare ID	**Control**	**PRP**	**PPP**	**Control**	**PRP**	**PPP**
1	-	-	-	-	-	-
2	-	-	*Enterococcus spp.*	-	-	-
3	*Streptococcus β-hemolytic*	-	-	*Streptococcus β-hemolytic*	-	-
4	-	-	-	-	-	*Klebsiella pneumoniae*
5	*Klebsiella pneumoniae*	-	-	*Klebsiella pneumoniae*	-	-
6	-	-	-	-	-	-
7	*Streptococcus β-hemolytic*	-	*Streptococcus β-hemolytic*	-	-	-
8	-	-	-	*Escherichia coli*	-	-
9	-	-	*Streptococcus β-hemolytic*	*Escherichia coli*	-	*Streptococcus β-hemolytic*
10	-	-	NP	-	-	NP
11	-	-	NP	*Escherichia coli*	-	NP
12	-	-	-	-	-	-

NP, not performed

Embryo recovery rates

Embryo recovery was greater in PRP-assigned cycles (83%) when compared with Control-assigned cycles (33%) ($P = 0.0361$), whereas PPP-assigned cycles had intermediate embryo recovery rates but not different from the other assigned cycles ($P > 0.05$). Of interest, when treatment was considered as a dependent variable (PRP and PPP), mares had greater fertility rates after plasma therapy ($P = 0.0356$; 72%, 16/22) than the Control-assigned group. The PRP and PPP treatments increased 2.5 and 1.5-fold the embryo recovery rates compared with the control assigned cycles. In addition, when data were adjusted for the number of ovulations per cycle, PRP therapy increased embryo recovery (73.3%) compared with the Control cycle (28.6%) ($P = 0.0268$). In the PPP assigned cycles, the embryo recovery rate was intermediate (53.9%), when embryo recovery rates were adjusted for the number of ovulations per cycle but not different than other cycles-groups ($P > 0.05$).

A total of 20 successful embryo collections were attained. Four embryos were recovered from four mares in the Control-assigned cycles, with two of these mares having double ovulations. Eleven embryos were recovered in PRP-assigned cycles, and seven embryos were recovered from the PPP-assigned cycles. Three mares experienced double ovulations in both plasma-assigned cycles. The overall grade quality, stage of development, and diameter of embryos collected were similar across cycle assignments ($P > 0.05$, Table 3).

Table 3. Embryos obtained from mares susceptible to persistent breeding-induced endometritis. Estrous cycles (n=34) from twelve mares were assigned in a cross over design to receive intrauterine uterine infusions with Lactated Ringer's Solution (Control, n=12), platelet-rich (PRP, n=12) or -poor plasma (PPP, n=10). Embryo flushing was performed eight days after ovulation, with 4L of LRS. All embryos recovered were measured and graded for development (e.g., blastocyst, or expanded blastocyst) and quality [28].

	Control			**PRP**			**PPP**		
Mare ID	Embryo stage	Quality grade	Diameter (µm)	Embryo stage	Quality	Diameter (µm)	Embryo stage	Quality grade	Diameter (µm)
1	-	-		Blastocyst	1	340	Blastocyst	1	260
							Expanded blastocyst	1	1100
2	-	-		-	-		-	-	
3	-	-		Expanded blastocyst	1	1230	Early blastocyst	1	175
4	Expanded blastocyst	1	540	Expanded blastocyst	1	860	Expanded blastocyst	1	840
5	-	-		-	-		-	-	
6	Early blastocyst	2	195	Expanded blastocyst	1	1360	-	-	
7	Blastocyst	1	960	Early blastocyst	2	190	Expanded blastocyst	1	940
8	-	-		Blastocyst	1	340	Expanded blastocyst	1	650
9	-	-		Blastocyst	1	300	-	-	
10	-	-		Blastocyst	1	360	NP	NP	
11	-	-		Early blastocyst	1	165	NP	NP	
12	Blastocyst	1	360	Blastocyst	1	280	Blastocyst	1	320
				Blastocyst	1	360			

Grade 1 embryo with a spherical shape, uniform size of blastomeres, color, and texture, with no visible abnormalities. **Grade 2** embryo with slight irregularities in shape, size of blastomeres, color or texture, and can present some extruded blastomeres. **Grade 3** embryo can have a large percentage of extruded blastomeres, partial collapse of blastocele, or moderate shrinkage of trophoblast from zona pellucida. **Grade 4** degenerated embryo with variable advanced stages irregularities. [28]. NP, not performed.

3. Discussion

The present study was set-forth to assess blood plasma therapy (rich or poor in platelets) in embryo donor mares susceptible to PBIE. Intrauterine treatment with plasma mitigated PBIE in susceptible mares as evidenced by a reduction of intraluminal and endometrial PMNs, uterine inflammatory cytokines, lessened intrauterine fluid accumulation and decreased the number of positive bacterial cultures compared to control-assigned cycles. Ultimately, intrauterine PRP therapy increased the percentage of embryo recovered per flushing and per number of ovulations, likely due to its immunomodulatory and antimicrobial properties of platelets turning a hostile uterine environment into an embryo-friendly uterine environment in embryo donor mares susceptible to PBIE.

It is unclear how PRP's infusions improve endometrial receptivity and fertility in mares. In human, it shown that PRP enhances migration and proliferation of endometrial cells [29], promotes neo-angiogenesis in the endometrium of infertile women [30], and upregulates genes involved in implantation (e.g., prostaglandin-endoperoxide synthase 2 [COX2], tumor protein p53 [TP53], estrogen receptors [ER-α and ER-ß] and progesterone receptor) [31]. It is also possible that the antimicrobial and anti-inflammatory properties of PRP may act synergistically to improve the uterine environment.

Platelet-rich plasma is routinely used in equine clinical practice to treat joints, bursae, and soft tissue injuries (e.g., tendonitis, tenosynovitis, and skin wounds) [32–34]. Interesting recent findings demonstrated potent in vivo antimicrobial properties against bacterial growth in equine synovial fluid [17] and in infected skin wounds of dogs [16]. In mare reproductive practice, autologous PRP administration before breeding to barren mares susceptible to endometritis improved pregnancy rates [24,25]. Also, PRP was shown to downregulate endometrial transcripts for interleukins IL1β, IL6, and CXCL8

and consequently mitigated post-breeding inflammatory response [23,25]. Our findings herein with embryo donor mares corroborate with these previous studies with broodmares, highlighting PRP's benefits for uterine immunity and reproductive performance. However, the mechanisms of action of PRP in the uterus remain to be fully elucidated. It is not well known if this downregulation in inflammatory markers post-breeding in mares treated with PRP is due to the anti-inflammatory or the antimicrobial properties of PRP.

Studies in other body systems (i.e., human chondrocytes) suggested that PRP acts by inhibiting the translocation of nuclear factor-kappa B (NF-kB) to the nucleus [35,36], as hepatocyte growth factor contained in PRP prevents the migration of NF-kB from the cytosol to the nucleus [35]. The downstream effects of NF-kB include activation of pro-inflammatory cytokines, chemokines, and COX-2 that regulate the inflammatory signals [37]. Cytokines and COX-2 act as mediators between cells modulating the acute inflammatory response [38]. Worth noting, PRP was shown to suppress COX-2 in the endometrium of mares susceptible to PBIE [25].

Mares susceptible to PBIE have pronounced expression of pro-inflammatory and reduced anti-inflammatory cytokines to regulate acute inflammation when compared to resistant mares [39,40]. Specifically, the endometrial mRNA abundance of IL1β, IL6, CXCL8, and TNFα are higher in susceptible than resistant mares, even before contact with antigen [39], therefore, justifying the strategy used herein to start treating mares before breeding, but it remains to be determined if treating mares with PRP both before and post-breeding have additional immunomodulatory benefits than treating mares only pre- or post-breeding. Infusion of PRP pre- or post-breeding was reported to result in similar downregulation of the post-breeding inflammatory response in mares [25].

The chemoattraction of PMNs is mainly mediated by CXCL8 [40,41]. One study demonstrated a reduction in transcripts for endometrial CXCL8 in PRP-treated mares [23]. In the present study, a reduction in luminal CXCL8 concentration at 6 and 24h post-breeding was also observed in mares treated with PRP; this can explain the suppression of inflammatory cells observed in the present study. Future studies should be carried to determine the mechanistic interactions between the endometrium of mares susceptible to PBIE and plasma and platelets.

Intrauterine infusion of bacteria upregulates inflammatory cytokines in mares [42–45]. Pathogenic microorganisms may gain access to the uterus during breeding and the contamination of the uterus with bacteria, increase the inflammatory reaction and delays the healing process by causing ongoing inflammation [8,43,45]. Of interest, none of the PRP-assigned cycles had a positive bacterial culture at two- and eight-days post-ovulation, whereas 25% and 42% control-assigned cycles had positive bacterial cultures two days post-ovulation and on the day of embryo flushing, respectively. On the other hand, the PPP-assigned cycles, which had 17 times fewer platelets than PRP, had three (30%) estrous cycles with a positive aerobic culture two- and eight-days post-ovulation. Platelets contain Growth factors and antimicrobial peptides [46,47], which may be responsible for the findings of the present study. Peptides in platelets may explain the dose-dependent antimicrobial activity obtained here and in another study using horse' synovial fluids as a model [17]. The peptides contained in PRP have antimicrobial activity against Staphylococcus aureus, Escherichia coli, and Klebsiella pneumoniae [18,48,49], which are known causes of endometritis in mares [8]. While platelet-derived antimicrobial peptides have not been described in horses, it is reasonable to suggest that horses are like to other species. Interestingly, an early study that the addition of autologous plasma to antibiotic therapy after breeding lactating and barren mares could

improve pregnancy rates per cycle compared with antibiotics alone [50]. Although the apparently greater concentration of platelets in PRP is correlated with increased antimicrobial potential, the plasma components may also play an important role in antimicrobial activity of PRP [46].

Plasma contains the complement system, an element of the immune system essential for humoral defense mechanisms against infectious agents. Bacterial cell lysis and leukocyte recruitment are associated with the activation of the complement cascade, and apparently, the plasma components (complement factors) play an important role for the antimicrobial activity of platelet concentrates [46,51]. In the present study, we demonstrated that both PRP and PPP alleviated the post-breeding endometrial inflammatory response in mares. Our results are consistent with an in-vitro study with human endometrial cells in which PRP and PPP had similar immunomodulatory capabilities [29]. The lack of differences between PRP and PPP for some of the endpoints assessed in the present study could simply mean the number of platelets contained in PPP herein was enough to elicit equivalent immunological properties to PRP. The minimal number of platelets per infusion or the number of infusions has not been determined yet. It is possible that after four infusions with PPP, a minimum number of platelets was achieved in the uteri of mares susceptible to PBIE in the present study.

Bacterial uterine infection induces endometrial inflammation [8,45]. The inflammatory cascade during the peri-ovulatory period and early diestrus may induce excessive production of PGF2α, which can negatively affect the luteal function and progesterone concentrations, and its reduction is thought to affect pregnancy rates in mares [52]. Plasma progesterone concentrations at Day-8 post-ovulation of mares in the control cycle were lower than those treated with PRP or PPP. It is unknown if progesterone concentrations were reduced in the control-assigned cycles due to chronic

360 inflammation or due to the fact that pregnancy rates were greater in the plasma assigned
361 cycles. Worth noting there were no differences in progesterone concentrations between
362 cycles with a positive and negative embryo flushes cycles in the control group or all cycles
363 together positive vs. negative embryo flushes. A previous study showed that mares
364 becoming pregnant had greater progesterone concentrations five days post-ovulation than
365 mares bred but not becoming pregnant [53]. In addition, endometrial infection with
366 pathogenic bacteria (e.g., Streptococcus zooepidemicus) has been reported to decrease
367 progesterone concentrations in the early diestrus of mares [54]. In addition, intrauterine
368 PRP therapy upregulated progesterone receptors in bovine endometrial cells [31], which
369 led the authors to suggest that intrauterine therapy with PRP may improve cow's fertility
370 [31,55]. However, there were no changes in PR expression in the endometrium of mares
371 treated with PRP or PPP in the present study. This could mean simply a difference
372 between species or the small number of mares used herein, or due to the fact that the
373 embryos were collected eight days post ovulation, it is possible that differences in
374 progesterone receptor could have been seen if mares were sampled later in
375 diestrus/pregnancy. Thus, it remains to be determined if PRP therapy modulates the
376 progesterone receptor.

377 Assessment of post-breeding intrauterine fluid accumulation is an essential
378 clinical parameter associated with endometritis in mares [8]. After immune response
379 activation, the pro-inflammatory cytokines, including chemokines (i.e., TNF, IL1, IL6,
380 and CXCL8), are released, and vascular endothelial cell activation occurs. Constriction
381 of arterioles and dilation of venules results from acute inflammation, which increases
382 vascular permeability and exudate leakage to the interstitium, causing edema and
383 intrauterine fluid accumulation [56]. Pre-breeding intrauterine fluid accumulation not
384 associated with breeding is indicative of susceptibility to PBIE [57]. Four mares in the

present study exhibited intrauterine fluid accumulation before insemination; however, all of them had negative aerobic culture and negative endometrial cytology before each treatment. Intrauterine fluid accumulation in these cases originates from endometrial gland secretion and transudation [58], and it is usually associated with the estrus phase with increased endometrial secretion and edema [59]. In the present study as well as previous studies, PRP therapy also showed a reduction in the intrauterine fluid accumulation in barren mares and mares with chronic degenerative endometritis [22,24], which suggests a beneficial effect of this therapy for mares with delayed uterine clearance.

4. Materials and Methods

All experimental protocols conducted in the present study were approved by the Institutional Animal Care and Use Committee of the University of Illinois Urbana-Champaign under protocol # 19141. The study was carried out from May to December 2019 at the University of Illinois Veterinary Teaching Hospital.

Screening mares for susceptibility to PBIE

Twenty-two light breed mares (11.7 ± 1.2, range 5 to 18 years-old) belonging to the University of Illinois were screened for susceptibility to PBIE as previously described [3]. All the mares were previously used as embryo donors in the principal investigator's research program for at least two consecutive breeding seasons.

Eight ejaculates were collected from two Quarter Horse stallions (n=4/each) with a Missouri artificial vagina (Nasco, Fort Atkinson, WI, USA). After collection, raw semen was centrifuged at 600 ×g for 10 min, the supernatant (seminal plasma) was discarded. The sperm pellet was pooled, concentration assessed, packed (Whirl-Pak, Nasco) at 2 billion sperm in 20 mL of PBS, and stored at -20 °C. Two freezing-thawing cycles were performed to obtain killed sperm before each use in the screening test.

Before the screening, each mare should have no signs of pre-existent uterine infections or inflammation as determined by endometrial aerobic bacterial culture, endometrial cytology, and no intra-uterine fluid accumulation detected on transrectal ultrasonographic examination. Mares had transrectal palpation and ultrasonography examination performed every other day until a 30 mm follicle was detected. Thereafter, mares were examined daily until the follicle reached ≥ 35 mm in the presence of endometrial edema score ≥1. Endometrial edema was scored on each transrectal ultrasonographic examination on a scale of 0 (absent) to 4 (max). Ovulation was induced with 500 µg of histrelin acetate (Botupharma Sao Paulo, Brazil), and mares had 2 billion killed sperm infused into the uterus. Endometrial cytology and ultrasound examination of the reproductive tract was performed daily up to 96 h. Endometrial biopsy and aerobic bacterial culture samples were obtained immediately before and 96 h after the sperm challenge. Thereafter, mares were classified as susceptible to PBIE if they had intrauterine fluid accumulation (column ≥ 2 cm), a positive endometrial cytology (≥ 3-5 PMNs/hpf), and/or a positive aerobic culture at 96 h after sperm challenge. In addition, mares classified as susceptible to PBIE had IIB or III scores in the Kenney and Doig classification. [60].

Mares that failed to display intrauterine fluid accumulation had negative endometrial cytology at 48 h and negative aerobic bacterial culture at 96 h after the sperm challenge was deemed resistant to PBIE. To be classified as resistant to PBIE, mares also had endometrium classified as I or IIA [60]. A mare fitting one criterion of susceptibility to PBIE but not another was deemed as intermediate.

Experimental design

Mares screened in the preliminary study classified as susceptible to PBIE (n=12, 14.2 ± 0.8, range 8 to 18 years old) were enrolled. All mares were randomly assigned into

three groups in a crossover design: PRP, PPP, and LRS. After the mare had an estrous cycle assigned in one of the groups, the mare had a washout cycle to minimize the previous estrous cycle's potential carryover effects. Before each treatment cycle, all mares needed to have a negative aerobic bacterial endometrial culture, along with an endometrial cytology free from inflammation during estrus. Otherwise, mares with positive endometrial cytology or aerobic bacterial culture were treated as needed and had an additional washout cycle. During each washout cycle, mares needed to fit similar criteria before being assigned in the next treatment cycle.

For all three groups, each intrauterine infusion (four in each estrous cycle) consisted of 40 mL of LRS, PRP, or PPP. Transrectal palpation and ultrasonography examination were performed three times a week, and prostaglandin F2alpha (dinoprost 5 mg/animal i.m., Lutalyse®, Zoetis, USA) was administered if a CL was present to bring mares back into estrus.

Once a preovulatory follicle was detected (≥33 mm in the presence of endometrial edema score ≥1), endometrial culture, cytology, low volume uterine lavage and biopsy were obtained. In addition, a plasma sample was obtained via venipuncture for assessment of progesterone concentrations with an immunoassay. After sampling, mares were submitted to uterine lavage with 2 L of LRS. Immediately after uterine lavage, mares were enrolled in one of the three groups to receive an intrauterine infusion of 40 mL of one of the treatments described above. Before each intrauterine procedure, the perineum was aseptically prepared using iodine scrub, rinsed with clean water, and dried with a paper towel. In the subsequent day, each mare received histrelin acetate (500 µg, i.m.) for induction of ovulation followed by intrauterine treatment. On the next day, mares were inseminated with ~2 billion of fresh semen. Six hours post-breeding, low volume uterine lavage and an endometrial biopsy were obtained, and the uterus was flushed with 2 L of

LRS and infused with 40 mL of one of the treatments. The next day, transrectal palpation and ultrasonography examination, endometrial cytology, low volume uterine lavage and biopsy were performed. Thereafter, the uterus was flushed with 2 L of LRS and infused with the respective cycle-assignments.

Transrectal palpation and ultrasonography examination was performed daily to confirm ovulation and to assess the endometrial edema and intrauterine fluid accumulation until three days post-ovulation. If intrauterine fluid accumulation was present, the height and the width of the fluid column (mm2) were measured with the ultrasound caliper function at the uterine bifurcation. Oxytocin (20 units, i.m.) was administered twice daily after each intrauterine infusion (6 and 12 h post-treatment) and then morning and afternoon 12 h apart from 48 to 96 h post-breeding. Endometrial cytology was assessed daily until two days after ovulation was confirmed. Mares failing to ovulate by 24 h post-insemination had the estrous cycle discarded, submitted to a washout cycle, and re-assigned back in the same treatment group.

Embryo flushing was performed eight days after ovulation (Day 8), with 4L of LRS. All embryos recovered were measured and graded for development (e.g., blastocyst or expanded blastocyst) and quality [28]. In grade 1, the embryo had a spherical shape, uniform size of blastomeres, color, and texture, with no visible abnormalities. A grade 2 embryo could have slight irregularities in shape, size of blastomeres, color or texture, and could present some extruded blastomeres. Grade 3 embryo could have a large percentage of extruded blastomeres, partial collapse of blastocele, or moderate shrinkage of trophoblast from zona pellucida. Grade 4 embryo were those with varying advanced stages of degeneration and irregularities. Immediately after embryo flushing, each mare had an endometrial biopsy collected. Thereafter, prostaglandin F2alpha (5 mg, dinoprost, i.m.) was administered to each mare to return to estrus.

Preparation of PRP and PPP

Immediately before intrauterine infusion of PRP or PPP, 450 mL of blood was collected from each animal through a venipuncture of the jugular vein using an 18G needle, into a blood transfusion bag (Jorgensen Labs, Loveland, CO, USA) containing 63 mL of citrate-phosphate-dextrose solution with adenine as an anticoagulant. Four hundred milliliters of whole blood were split into eight 50-mL tubes, and the samples were centrifuged at 400 ×g for 15 min. The supernatant was transferred into 15-mL conical tubes and again centrifuged at 1000 ×g for 10 minutes. After the second centrifugation, 2.5 mL of plasma at the bottom of each tube was preserved and used as PRP, while the supernatant was used as PPP. The concentration of platelet, white-blood, and red blood cells were determined in whole blood, PRP, and PPP samples using manual counting with a hemocytometer at the University of Illinois Veterinary Diagnostic Laboratory.

Assessment of platelet viability

Platelet viability was assessed using a full-spectrum detector based (filter-less) Cytek Aurora Flow Cytometer (Cytek Biosciences Inc., Fremont, CA, USA) adapted from protocols published elsewhere [61,62]. Immunolabeling of CD41/61, a platelet-specific antigen, was carried out with a primary (CD41/61, monoclonal antibody CO.35E4, #MA5-28370 Invitrogen, Life Technologies Corporation, OR, USA) and a secondary antibody conjugated with a fluorochrome (goat polyclonal anti-mouse IgG conjugated with R-phycoerythrin; #P-852, Invitrogen, Life Technologies Corporation, OR, USA). In addition, the association with zombie green (#423112 Biolegend, CA, USA), a dye that binds to cytoplasmic amines, was used to assess the plasma membrane integrity of platelets. The working solution of Zombie Green was prepared with the dilution in PBS at a 1:100 ratio. Briefly, an aliquot of PRP or PPP was diluted 1:20 in Tyrode's media (134 mM NaCl, 12mM NaHCO3, 2.9 mM KCl, 0.34 mM Na2HPO4, 1

510 mM MgCl2, 10 mM HEPES; pH 7.4) deprived from calcium chloride. Thereafter, the
511 sample was incubated with Zombie Green working solution (1:1) and anti-CD41/61
512 antibody (1:200) for 30 min at room temperature in the dark.

513 Samples were washed (1500 ×g, 15 min), resuspended in Tyrode's media, and
514 further incubated with goat anti-mouse IgG (1:100) for 30 min at room temperature in the
515 dark. Samples were washed (1500 ×g, 15 min), resuspended in 200 µL of the same
516 Tyrode's media, and immediately analyzed. This panel identified four different
517 populations (1) intact platelets (CD41/61+ with low zombie green intensity), (2) damaged
518 platelets (CD41/61+ with high zombie green intensity), and (3) debris (CD41/61 negative
519 with high or low zombie green intensity). The analysis was concluded when at least
520 1,000,000 fluorescent gated events or 150 µL of the sample were assessed. Single stain
521 controls and compensation were used to unmix the signals. Heat-treated platelets (75 °C,
522 15 min) served as a positive control for damaged platelets. Zombie Green and R-
523 phycoerythrin were excited and detected with a 488 nm and 575 nm fluorescence detector,
524 respectively. Data were analyzed with the software FlowJo (V10.6.2, BD Life Sciences,
525 NJ, USA); the percentage of events in each population was calculated, and manual
526 compensation was applied as needed.

527 **Semen collection and processing**

528 Semen from one fertile Quarter Horse stallion housed at the University of Illinois
529 Urbana-Champaign, IL, USA, was used for all inseminations. Semen was harvested with
530 a Missouri artificial vagina, with the gel-free semen fraction assessed for sperm
531 concentration in a Nucleocounter SP-100 (Nucleocounter SP-100, Chemometek,
532 Denmark) following the manufacturer instructions. Briefly, 50 µL of semen was diluted
533 in 5 mL of lysis buffer (Reagent S100, ChemoMetec, Denmark) and loaded into the
534 cassettes before the assessment.

The sperm motility parameters were assessed using computer-assisted sperm analysis (CASA) using default settings recommended by the manufacturer (Spermvision, Minitube of America, Verona, WI, USA) for equine sperm. The CASA's preset values were: static cell area 14-80 µm2; straightness threshold for progressive motility 90%; average path velocity threshold for static cell <9.5 µm/s; cell intensity 106; light-emitting diode illumination intensity 1800-2550. Each sample was incubated for 10 min at 37 °C before each evaluation. A small aliquot (10 µL) of extended semen was placed on a pre-heated slide with a coverslip for the assessments. The percentage of total percent and progressive sperm motility was recorded for descriptive purposes. Each insemination consisted of ~2 billion sperm.

Aerobic bacterial culture

For aerobic endometrial cultures, the samples were obtained using double-guarded swabs (Jorgensen Labs, Loveland, CO, USA) vaginally inserted into the uterus covered with a sterile sleeve at pre- (48 h) and post-breeding (72 h). In addition, a sterile cotton-tip swab was inserted in the recovered fluid obtained from the cup filter immediately after the embryo flushing and submitted to aerobic culture. After collection, swabs were plated in the chromogenic agar (Spectrum CS Culture System, Vetlab Supply, Inc, FL, USA). Plates were incubated at 37 °C for 48 h. Bacterial growth was identified with a matrix-assisted laser desorption ionization time-of-flight mass spectrophotometer at the University of Illinois Veterinary Diagnostic Laboratory.

Endometrial Cytology

Endometrial exfoliative cytology was performed pre- (48 h) and post-breeding (24, 48, and 72 h). The samples were obtained using a disposable cytobrush (Jorgensen Labs, Loveland, CO, USA). Briefly, the cytobrush was inserted manually through the vagina and cervix into the uterus using a sterile sleeve. After collection, the slides were

prepared and stained by Diff-Quick (Siemens Healthcare Diagnostics Inc., Deerfield, IL, USA). The samples were then microscopically examined under 400× objective, and the number of inflammatory cells in ten higher power fields were counted. An evaluator blinded of the treatment assessed all the samples. Positive endometrial cytology was defined as ≥ 3-5 PMNs per high-power-field.

Low volume lavage and Multiplex Immunoassay

Low volume lavage involves infusion of 120 mL of PBS into the uterus, and then the fluid was retrieved by gentle rectal manipulation of the uterus. The recovered uterine fluid was placed in two 50 mL tubes and centrifuged at 400 × g for 20 minutes at 5 °C, with the supernatant recovered and centrifuged again at 2000 ×g for 20 minutes at 5 °C to remove cellular debris. After the second centrifugation, the supernatant was saved and stored at -80 °C for proteomic analyses.

Uterine fluid interleukins from a subset of mares (n=6) randomly selected were analyzed using an equine-specific multiple sandwich immunoassay based on flowmetric MILLIPLEX MAP® technology in accord with the workflow previously published [63]. The detection level was defined as the signal-to-noise-ratio (limit of detection) divided by the square root of 2 (1.6 pg/mL). Data points below the limit of detection were not considered. Concentration of IL1α, IL1β, IL6, CXCL8, IL17α, IL10, MCP-1 and RANTES in uterine fluid obtained by low volume uterine lavage were measured. Of these, IL-17a, MCP-1 and RANTES were below the limit of detection for all samples and thus were not considered further. IL-1α was detected in only two samples with levels only minimally over the limit of detection and was therefore excluded as well.

Each sample of uterine fluid was measured undiluted, and calibration curves for these plates were prepared in assay buffer, as previously described by Skogstrand et al. [64]. Additionally, samples of interleukins were measured undiluted and standards were

prepared with the serum matrix added to all standards and quality controls, following the guidelines of the manufacturer and as previously described by Fedorka et al. (2019) [65]. The mean of intra- assay coefficients of variation weas 3.2%.

Endometrial biopsy: inflammatory cell count and immunolabelling for progesterone receptors

Biopsy samples were taken at the cranial uterine body using a sterilized alligator jaw biopsy forceps. After collection, biopsies were immediately fixed in 10% neutral buffered formalin and embedded in paraffin for histological evaluation. Tissue was sectioned at 5 µm thickness and stained with hematoxylin and eosin. For each sample, an evaluator blinded of treatment counted the number of PMNs and lymphocytes from five randomly selected high-power fields at 400× magnification. The averages were recorded and compared across groups.

A subset of mares (n=4) was used for immunohistochemistry analyses for progesterone receptors pre-breeding (-48h) and eight days post-ovulation (D8, day of embryo flushing) following manufacturer's recommendations (Vectastain Standard Elite; Vector Laboratories, Inc., Burlingame, CA, USA. For immunohistochemistry, tissue sections (5 µm) were prepared on poly-L-lysine-coated glass slides to detect the presence of progesterone receptors (PR). The immunohistochemical staining of all samples was performed using the avidin-biotin-peroxidase complex procedure with a commercial immunoperoxidase kit (Vectastain Standard Elite; Vector Laboratories, Inc., Burlingame, CA, USA). Tissue sections were immersed in a pre-heated solution at 94 °C of Dewax and HIER Buffer H (Thermo Fischer Scientific, Lab Vision Corporation, Fremont, CA, USA) diluted 1:15 with deionized water for 40 minutes. This solution is designed to simultaneously dewax and perform heat-induced epitope retrieval. Endogenous peroxidase was blocked using 1 % hydrogen peroxide in Tris buffer for 45 min. Sections

were incubated for 18 hours at 4°C with anti-PR (#MA1-12626, Thermo Fischer Scientific, Lab Vision Corporation, Fremont, CA, USA) mouse monoclonal antibodies diluted 1:400. After incubation with the secondary biotinylated anti-mouse immunoglobulin (diluted 1:200; Vector Laboratories, Inc.) for 30 min, the avidin-biotin-peroxidase complex method (Vector Laboratories, Inc.) was performed. Positive staining was visualized with 3.3-diaminobenzidine-4 HCl (Vectastain, Vector Laboratories, SK-4100), and nuclei were counterstained with Mayer's hematoxylin. Diluent negative control sections were produced by the omission of the primary antibody.

Slides were evaluated by a blinded operator at 200× magnification using a microscope (OLYMPUS BX51, Olympus, Tokyo, Japan) coupled with a camera and the software ProgRes C14 PLUS (Jenoptik, Jena, Germany). Five randomly selected areas were evaluated in each section. The percent and the intensity of PR immunoreactivity (brown stained) cells were assessed separately in the surface epithelial cells, glandular epithelium, and stroma. The intensity was scored as 1, weak positive staining; 2, moderate positive staining; 3, intense positive staining.

Progesterone assay

Plasma samples were collected from the jugular vein once a preovulatory follicle was detected (D3; ≥33 mm in the presence of endometrial edema), and 48 h (D2), and eight days after ovulation (D8) for determination of plasma progesterone concentrations. The concentrations of progesterone were assessed with a chemiluminescence platform (Immulite 1000 Siemens Medical Solution USA, Inc.). The intra-assay coefficient of variation was 2.6%, and sensitivity was 0.1 ng/mL.

Statistical analyses

Data analyses were carried out with GraphPad Prism 8.0.1. (GraphPad Software, San Diego, California, USA). Luminex data were log (log10) transformed for

normalization. Data were evaluated with a mixed model and Tukey's post hoc test. The percentage of embryo flush with at least one embryo, the number of embryos per ovulations, and the number of positive bacterial cultures were assessed using multivariate regression analysis. Significance was set at $P \leq 0.05$ for all tests, and a statistically significant tendency was determined with $0.05 < P < 0.1$. All data are presented as mean ± SEM. The degree of linear correlation between PMNs counting in endometrial cytology and biopsy, as well as platelet concentration in the whole blood and the PRP, was tested using Pearson correlation. Strong coefficient of correlation was defined as $r>0.7$, and moderate $0.5 \leq r \leq 0.7$, and weak correlation when $r < 0.5$.

5. Conclusions

In conclusion, the current study suggests that PRP may have antimicrobial properties and intrauterine infusion of PRP reduce the chances of uterine infection after breeding in mares as indicated by the absence of positive bacterial culture in PRP-assigned cycles. In addition, the intrauterine treatment with blood plasma therapy (rich or poor in platelets) mitigates the post-breeding uterine inflammatory response of embryo donor mares susceptible to PBIE. The apparent improved immune response is likely one of the major factors contributing to enhancing embryonic survival and consequent greater embryonic recovery obtained herein. Administration of blood plasma as prepared herein improved plasma progesterone concentrations in mares susceptible to PBIE. Finally, as described herein, plasma infusions can be used as an alternative method to manage embryo donor mares susceptible to PBIE, and PRP may augment antibiotic therapy in broodmare's practice.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: The post-breeding inflammatory response in mares after the sperm challenging. Mares were categorized as susceptible (n=12) and resistant (n=7) to persistent-breeding induced endometritis (PBIE) immediately pre-(0h) and post-sperm challenging (24-96h). (A) Intrauterine fluid accumulation measured at the uterine bifurcation; (B) Mean PMNs counted in endometrial cytology in five high-power fields (hpf). Different superscripts denote the effects of time (abc) and differences between mares within each time point (XY) ($P< 0.05$). Figure S2: Endometrial edema score (median) in mares after the sperm challenging. Mares were categorized as susceptible (n=12) and resistant (n=7) to persistent-breeding induced endometritis (PBIE) immediately pre-(0h) and post-sperm challenging (24-96h). Score 0, No edema; 1, Mild edema; 2, Moderate edema; 3, Evident edema; 4, Exacerbated edema. Different superscripts denote the effects of time (A,B) ($P < 0.05$). Figure S3: Circulating progesterone concentrations pre- and post-ovulation in mares susceptible to persistent breeding-induced endometritis with a positive (Embryo) or negative (N-Embryo) embryo flushing at eight days post-ovulation. Assessments were carried out from the early onset of estrus (D-3), 48 h post-ovulation (D2) and on the day of embryo flushing, eight days post-ovulation (D8). Table S1: Semen parameters for the breeding doses used for breeding mares assigned to (Control), platelet-rich (PRP), or -poor plasma (PPP). All ejaculates (n=26) were obtained from a single fertile stallion.

Author Contributions: L.G.T.M. Segabinazzi contributed to study design and execution, data analysis, interpretation, and preparation of manuscript. I.F. Canisso contributed to study design, study execution, data analysis, interpretation, and preparation of manuscript. G. Podico, L.L. Cunha and G. Novello contributed to study design, study execution and preparation of the manuscript. M. Rosser contributed to study execution.

F.S. Lima and M. Alvarenga contributed to study design and preparation of manuscript. All authors gave their final approval of the manuscript.

Funding: This study was financially supported by São Paulo State Research Foundation (FAPESP grant #2018/02856-3) and the USDA Hatch Animal Health (#ILLU-888-912).

Institutional Review Board Statement: The Institutional Animal Care Unit Committee (protocol #19141) approved all procedures carried out in the present study.

Informed Consent Statement: Not applicable

Data Availability Statement: The original contributions presented in the study are included in the article/Supplementary Materials, further inquiries can be directed to the corresponding author/s.

Acknowledgments: In this section, you can acknowledge any support given which is not covered by the author contribution or funding sections. This may include administrative and technical support, or donations in kind (e.g., materials used for experiments).

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

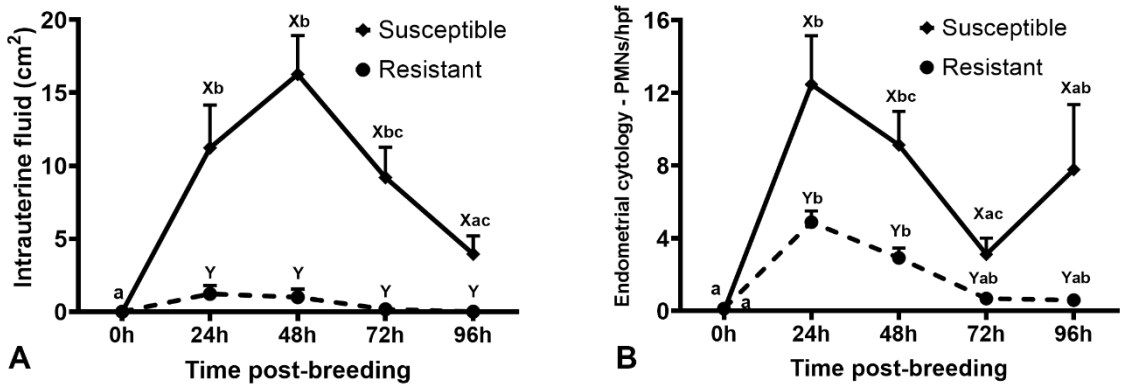

Figure S1. The post-breeding inflammatory response in mares after the sperm challenging. Mares were categorized as susceptible (n=12) and resistant (n=7) to persistent-breeding induced endometritis (PBIE) immediately pre-(0h) and post-sperm challenging (24-96h). (A) Intrauterine fluid accumulation measured at the uterine bifurcation; (B) Mean PMNs counted in endometrial cytology in five high-power fields (hpf). Different superscripts denote the effects of time (^{abc}) and differences between mares within each time point (^{XY}) ($P < 0.05$).

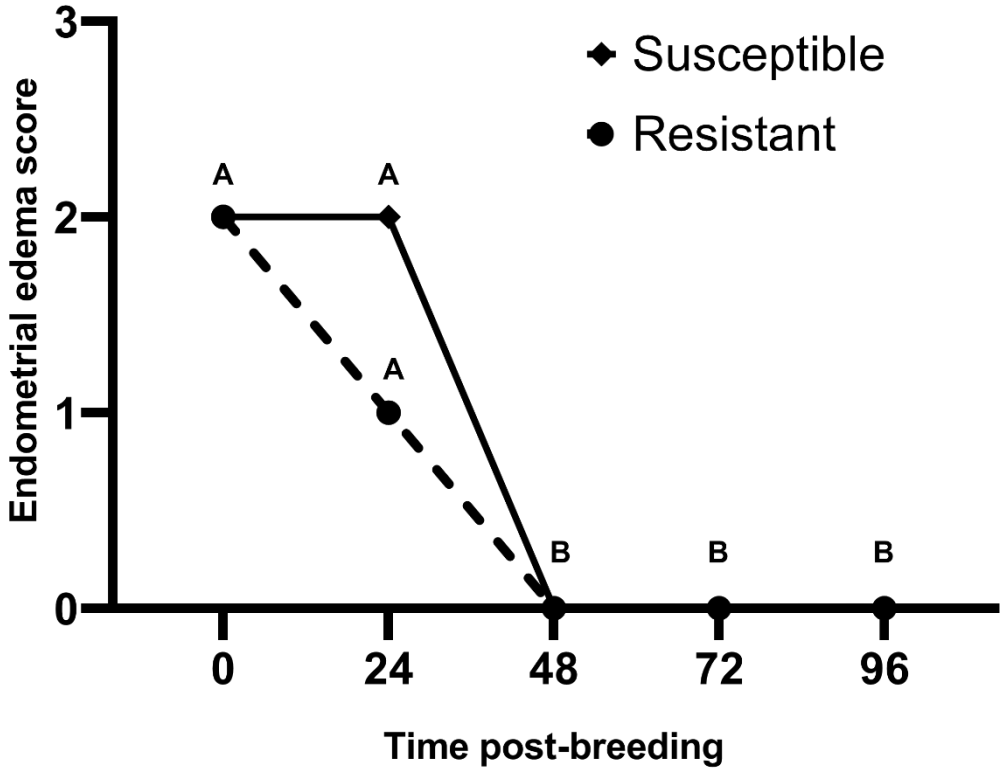

Figure S2. Endometrial edema score (median) in mares after the sperm challenging. Mares were categorized as susceptible (n=12) and resistant (n=7) to persistent-breeding induced endometritis (PBIE) immediately pre-(0h) and post-sperm challenging (24-96h). Score 0, No edema; 1, Mild edema; 2, Moderate edema; 3, Evident edema; 4, Exacerbated edema. Different superscripts denote the effects of time (^{A,B}) ($P < 0.05$).

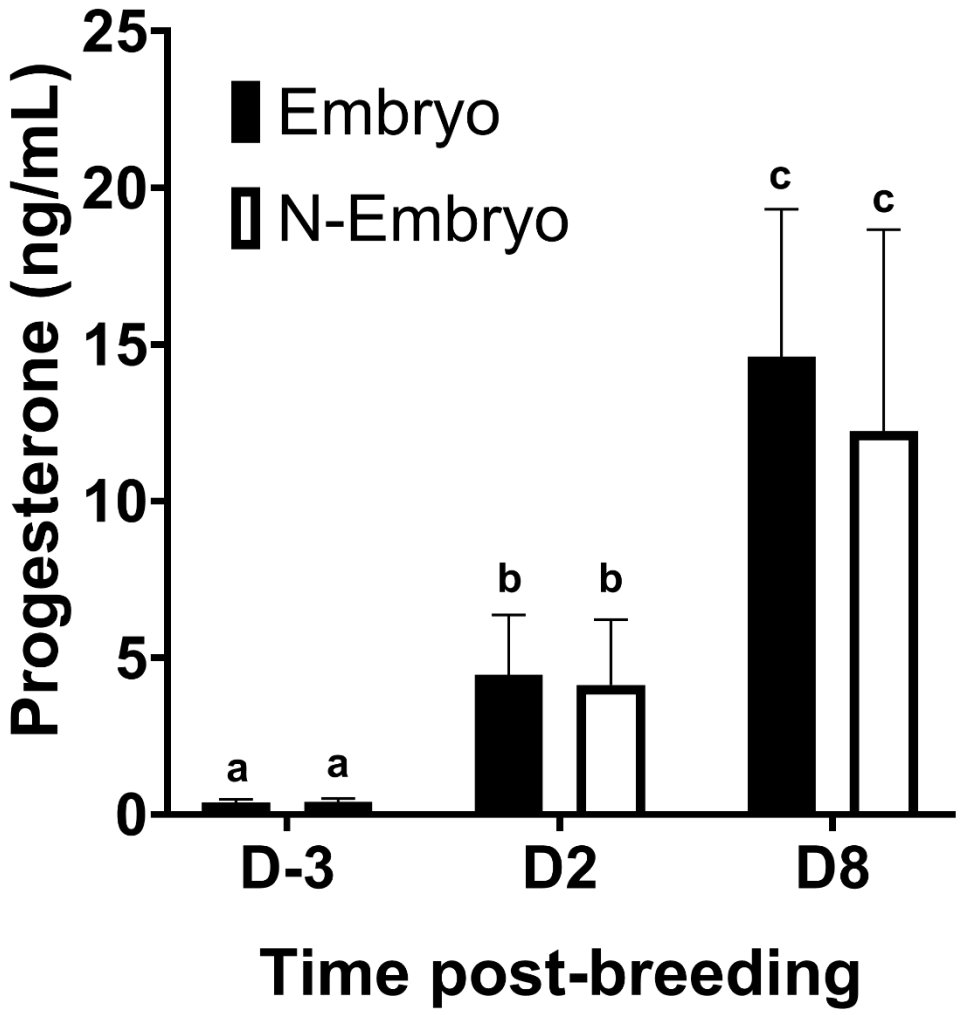

Figure S3. Circulating progesterone concentrations pre- and post-ovulation in mares susceptible to persistent breeding-induced endometritis with a positive (Embryo) or negative (N-Embryo) embryo flushing at eight days post-ovulation. Assessments were carried out from the early onset of estrus (D-3), 48 h post-ovulation (D2) and on the day of embryo flushing, eight days post-ovulation (D8).

Table S1. Semen parameters for the breeding doses used for breeding mares assigned to (Control), platelet-rich (PRP), or -poor plasma (PPP). All ejaculates (n=26) were obtained from a single fertile stallion.

Parameters	**Control**	**PRP**	**PPP**
Volume (mL)	13.3±1.0	15.4±1.3	14.7±0.9
Total sperm concentration ($\times 10^9$)	2.7±0.14	2.5±0.15	2.6±0.9
Total motility (%)	80.5±1.7	80.3±2.1	81.0±1.0
Progressive motility (%)	74.0±2.0	74.4±2.0	75.0±1.4

Data represented as Mean ± SEM