UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA ANIMAL

DEFESA DE DISSERTAÇÃO

EFEITO DO PLASMA RICO EM PLAQUETAS PRÉ OU PÓS-INSEMINAÇÃO ARTIFICIAL SOBRE A RESPOSTA INFLAMATÓRIA E ÍNDICE DE FERTILIDADE EM ÉGUAS SUSCEPTÍVEIS A ENDOMETRITE PERSISTENTE PÓS-COBERTURA

Lorenzo Garrido Teixeira Martini Segabinazzi

Botucatu - SP
Setembro/2016

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Orientador: Prof. Dr. Marco Antonio Alvarenga

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LISTA DE ABREVIATURAS E SÍMBOLOS

cm	Centímetros
COX-2	Cicloxigenase-2
CTM	Células tronco mesenquimais
EGF	Fator de crescimento epidermal
EPMB	Extrato de parede de *mycobacterium*
EPPC	Endometrite persistente pós-cobertura
FC	Fatores de crescimento
FGF	Fator de crescimento fibroblastico
HGF	Fator de crescimento hepatocitário
IA	Inseminação artificial
Ig	Imunoglobulina
IGF-1	Fator de crescimento semelhante a insulina 1
IL-1	Interleucina-1
IL-6	Interleucina-6
IL-8	Interleucina-8
iNOS	Óxido nítrico sintase induzida
LPS	Lipopolissacarídeo
LPX_4	Lipoxina A4
LTB_4	Leucotrienos
MMP-3	Metaloproteinase-3
NF-κB	Fator nuclear kappa-B
PAMPs	Padrões moleculares associados a patógenos
PDEGF	Fator de crescimento epidermal derivado de plaquetas
PDGF	Fator de crescimento derivado de plaquetas
PGE	Prostaglandina E
PGF2α	Prostaglandina 2α
PMNs	Polimorfonucleares
PRP	Plasma rico em plaquetas
RANTES	Reguladores da ativação de células T expressadas e secretadas
TGF-β	Fator de transformação de crescimento beta
TLRs	Receptores Toll-like

TNF-α	Fator de necrose tumoral alfa
VEGF	Fator de crescimento vascular endotelial

RESUMO

SEGABINAZZI, L.G. EFEITO DO PLASMA RICO EM PLAQUETAS PRÉ OU PÓS-INSEMINAÇÃO ARTIFICIAL SOBRE A RESPOSTA INFLAMATÓRIA E ÍNDICE DE FERTILIDADE EM ÉGUAS SUSCEPTÍVEIS A ENDOMETRITE PERSISTENTE PÓS-COBERTURA. Botucatu – SP. 2016, p.67. Dissertação (Mestrado) – Faculdade de Medicina Veterinária e Zootecnia, Campus Botucatu, Universidade Estadual Paulista.

A endometrite persistente pós-cobertura (EPPC) é a principal causa de redução da fertilidade nas éguas, causando impactos importantes dentro do mercado do cavalo. Os tratamentos comumente utilizados para EPPC visam apenas minimizar os fatores predisponentes a sua instalação, não atuando diretamente no processo inflamatório. Com o intuito de reduzir a resposta inflamatória, estudos recentes têm demonstrado um aumento da fertilidade de animais acometidos por EPPC, quando se utiliza agentes imunomoduladores. O PRP é modulador da resposta inflamatória que está sendo largamente utilizado na medicina veterinária. Este concentrado de plaquetas contém diversos fatores de crescimento que atuam diretamente nos mediadores inflamatórios, reduzindo o processo e promovendo reparação tecidual. O PRP é benéfico no tratamento de inflamações tendíneas e osteoarticulares, modulando a inflamação e acelerando a regeneração do tecido lesionado. Mais recentemente alguns pesquisadores demonstraram o efeito benéfico do PRP em tratamentos intrauterinos de éguas. Desta forma o presente estudo tem por objetivo revisar os aspectos relacionados a EPPC assim como ao PRP e seu mecanismo de ação.

Palavras-chave: PRP, inflamação uterina, imunomodulação, equino.

ABSTRACT

SEGABINAZZI, L.G. EFFECT OF PLATELET RICH-PLASMA PRE OR POST-ARTIFICIAL INSEMINATION ON THE INFLAMMATORY REACTION AND FERTLITY RATES IN SUSCEPTIBLE MARES TO PERSISTENT BREEDING-INDUCED ENDOMETRITIS. Botucatu – SP. 2016, p.67. Dissertação (Mestrado) – Faculdade de Medicina Veterinária e Zootecnia, Campus Botucatu, Universidade Estadual Paulista.

The persistent breeding-induced endometritis (PBIE) is the main cause of decrease fertility in the horses, thereby causing significant impact in the horse's market. The treatments commonly used for PBIE view only minimize the predisposing factors and do not act directly in the inflammatory process. Aiming to reduce the inflammatory response, recent studies have shown an increase in fertility of animals with PBIE when used immunomodulatory agents. A modulator of the inflammatory response that has been largely used in veterinary medicine is the platelet-rich plasma (PRP). This platelet concentrate contains many growth factors which act directly on inflammatory mediators, reducing process and promoting tissue repair. Several studies have shown that PRP is beneficial in the treatment of osteoarticular and tendon inflammations, modulating inflammation and accelerates the regeneration of injured tissue. More recently some researchers have demonstrated the beneficial effect of PRP in intrauterine treatment of mares. Thus, the present study aimed to do a literature review on the aspects related to PBIE, as well as the PRP and its mechanism of action.

Keywords: PRP, uterine inflammation, immunomodulators, equine.

CAPÍTULO 1

1. INTRODUÇÃO

O Brasil possui o maior rebanho equino da América Latina e o quarto mundial (GUERRA, 2010). Somando equinos, muares e asininos são cerca de 8 milhões de cabeças, movimentando R$7,3 bilhões/ano. O rebanho ainda envolve mais de 30 segmentos na cadeia distribuídos entre insumos, criação e destinação final e compõe a base do chamado Complexo do Agronegócio Cavalo, responsável pela geração de 3,2 milhões de empregos diretos e indiretos (BRASIL, 2014). O Brasil juntamente a Argentina são os países que mais geram potros utilizando a transferência de embriões (IETS, 2011).

O interesse por parte dos proprietários em obter um maior número de descendentes dos animais mais valiosos, torna este um desafio para os profissionais que atuam em programas reprodutivos de equinos. Isto acontece principalmente devido a histórica seleção sofrida pela espécie, já que diferentemente dos animais de produção, na maioria das raças equinas não houve seleção genética relativa ao desempenho reprodutivo, levando a uma variação considerável sobre os índices de fertilidade destes animais.

Outro fator que afeta os índices reprodutivos na espécie é o fato das fêmeas equinas obter seu maior valor econômico quando a sua progênie se destaca, o que ocorre normalmente quando os animais já apresentam idade avançada, juntamente a um declínio da capacidade reprodutiva e dos índices de fertilidade. Contudo, o alto valor da prole estimula o uso destas fêmeas em programas de reprodução assistida mesmo com baixos índices de fertilidade (CARNEVALE, 2008). Estima-se que 30% das fêmeas equinas em programas de reprodução assistida têm mais de 18 anos de idade (ALVARENGA; CARMO, 2009).

Neste contexto, a endometrite persistente pós-cobertura (EPPC) se torna uma enfermidade com impactos econômicos importantes em programas reprodutivos de equinos, sendo considerada a principal causa de subfertilidade nesta espécie e a terceira enfermidade que mais acomete os equinos (TROEDSSON, 1999). As éguas mais afetadas são as fêmeas multíparas com mais de 14 anos de idade (LEBLANC, 2003). Estes animais comumente possuem a anatomia uterina fora dos seus padrões naturais, principalmente

devido ao relaxamento dos ligamentos uterinos, que juntamente com alterações cervicais e excessivas manipulações, ocasionam dificuldades na limpeza uterina, predispondo ao aparecimento da EPPC.

Animais susceptíveis a EPPC podem apresentar líquido no interior do útero mesmo antes da cobertura ou inseminação artificial (IA) (BUCCA et al., 2008), porém a maioria das éguas não apresenta esta reação até serem cobertas. Este acúmulo de líquido torna o útero mais vulnerável às infecções, prejudicando a implantação embrionária (LEFRANC & ALLEN, 2008). Assim os animais susceptíveis a EPPC além do acúmulo de líquido intrauterino, apresentam alta contagem de PMNs, edema uterino exacerbado, útero distendido e flácido (MOREL, 2003), além de histórico de repetição de cios (BUCCA et al., 2008).

Os tratamentos comumente utilizados para EPPC podem ser considerados tratamento de suporte, auxiliando na limpeza e manutenção de um ambiente uterino adequado, visto que estes não atuam diretamente na causa da inflamação.

Na tentativa de atuar diretamente sobre os mediadores da inflamação, estudos (FUMUSO et al., 2003; DELL'AQUA Jr. et al., 2006; BUCCA et al., 2008; PAPA et al., 2008; FIORATTI et al., 2013; REGHINI, 2013; METCALF, 2012; METCALF, 2014;) utilizando agentes imunomoduladores tem demonstrado redução do processo inflamatório e um aumento da fertilidade de animais acometidos pela EPPC.

Um modulador da resposta inflamatória que vem sendo largamente utilizado tanto na medicina veterinária como humana é o plasma rico em plaquetas (PRP). Este concentrado de plaquetas contém diversos fatores de crescimento (FC) importantes na reparação tecidual, devido à sua ação mitogênica, quimiotática, neovascular e anti-inflamatória (GONSHOR, 2002; KEVY & JACOBSON, 2004; MAZZOCCA et al., 2013; KIM et al., 2014). Em trabalhos recentes, nos quais a infusão intrauterina de PRP foi utilizada, pode-se observar a redução da reação inflamatória pós-cobertura (REGHINI, 2013) assim como o aumento das taxas de prenhez (METCALF, 2014). O presente estudo tem por objetivo revisar os aspectos relacionados a modulação da resposta inflamatória pelo PRP, assim como os possíveis benefícios desta terapia em fêmeas equinas com EPPC.

2. REVISÃO DE LITERATURA

2.1. Endometrite persistente pós-cobertura (EPPC)

Após a cobertura/IA ocorre no útero uma resposta inflamatória fisiológica, com o objetivo de remover espermatozoides restantes, assim como debris celulares, plasma seminal e outros contaminantes (KOTILAINEN et. al, 1994; TROEDSSON, 1994), a fim de proporcionar um ambiente adequado para o desenvolvimento embrionário (LEBLANC et a., 1998). Em éguas normais, este processo é debelado entre 36 e 48 horas após o contato da célula espermática com o endométrio, entretanto, quando os mecanismos naturais de defesa uterina falham, este processo permanece e a EPPC se instala, tornando o ambiente uterino incompatível com o estabelecimento da gestação (ASBURY, 1986; LEBLANC et al., 1998; LEBLANC, 2003; WATSON, 2000).

Desta forma as éguas podem ser susceptíveis ou resistentes a EPPC. As susceptíveis possuem uma incapacidade dos mecanismos de defesa uterino para debelar o processo inflamatório pós-cobertura naturalmente (BRITO & BARTH, 2003). Segundo Troedsson (1999) a EPPC é a principal causa da redução de fertilidade em éguas, e é considerada a terceira afecção clínica mais comum em equinos depois da cólica e das afecções do trato respiratório.

A célula espermática é o principal fator predisponente para o início da reação inflamatória no útero. Pela quimiotaxia, promovem uma intensa migração de polimorfonucleares (PMNs) para o lúmen uterino (TROEDSSON et al. 1993; TROEDSSON et al., 2001). Quanto maior a concentração de espermatozoides depositados, mais intensa é a resposta inflamatória, assim como a migração de PMNs para o lúmen uterino entre 2 e 4 horas após a IA. No entanto, uma redução no número de PMNs 24 horas após a IA é observada em éguas normais, independentemente da quantidade de espermatozoides depositados (FIALA et al., 2007). Este fato demonstra o início da resolução do processo inflamatório pós-cobertura, que geralmente encerra-se após 48 horas (KATILA, 1995).

Com o início da reação inflamatória, causada pelo sêmen, ocorre a síntese de prostaglandina F2α (PGF2α) (NASH et al., 2010), importante metabólito na promoção das contrações miometriais, que inicialmente tem papel importante no transporte dos espermatozoides até a papila tubárica e mais tardiamente na limpeza uterina, pela eliminação dos espermatozoides restantes, plasma seminal, debris celulares e outros contaminantes através da cervix (TROEDSSON, 1997; TROEDSSON et al., 1998; TROEDSSON et al.,2001). Contudo, em éguas susceptíveis, esse *clearence* uterino é falho, devido a deficiências dos mecanismos naturais de defesa, ocasionando um acúmulo de líquido no interior do útero, instalando-se assim o quadro de EPPC (LEBLANC, 2003) e promovendo um ambiente desfavorável ao desenvolvimento embrionário (CARD, 2005). Além disso, esta inflamação persistente pode levar a luteólise prematura, já que o endométrio lesado induz a produção de PGF2α, levando a consequente perda embrionária (ENGLAND, 2005).

Um dos fatores relacionados a instalação da EPPC é a idade avançada das éguas, já que estas comumente são animais multíparos, que já passaram por diversas manipulações durante procedimentos de reprodução assistida, além de apresentar diversas alterações anatômicas, como conformação perineal alterada (Figura 1), relaxamento dos ligamentos uterino, alterações cervicais e deficiência da contratilidade miometrial (LEBLANC, 2003; CARD, 2005). Outro fator que pode predispor a EPPC independente da idade da fêmea é a incompetência cervical. Mesmo éguas virgens com idade avançada podem apresentar EPPC, que é caracterizada como “síndrome da égua velha virgem” (PYCOCK, 2000), e pode ocorrer também em éguas virgens e jovens que apresentam fechamento cervical precoce (MALSCHITZKY et al., 2006) dificultando dessa forma a limpeza uterina. No entanto este quadro tende a se normalizar após o primeiro parto (PYCOCK, 2000).

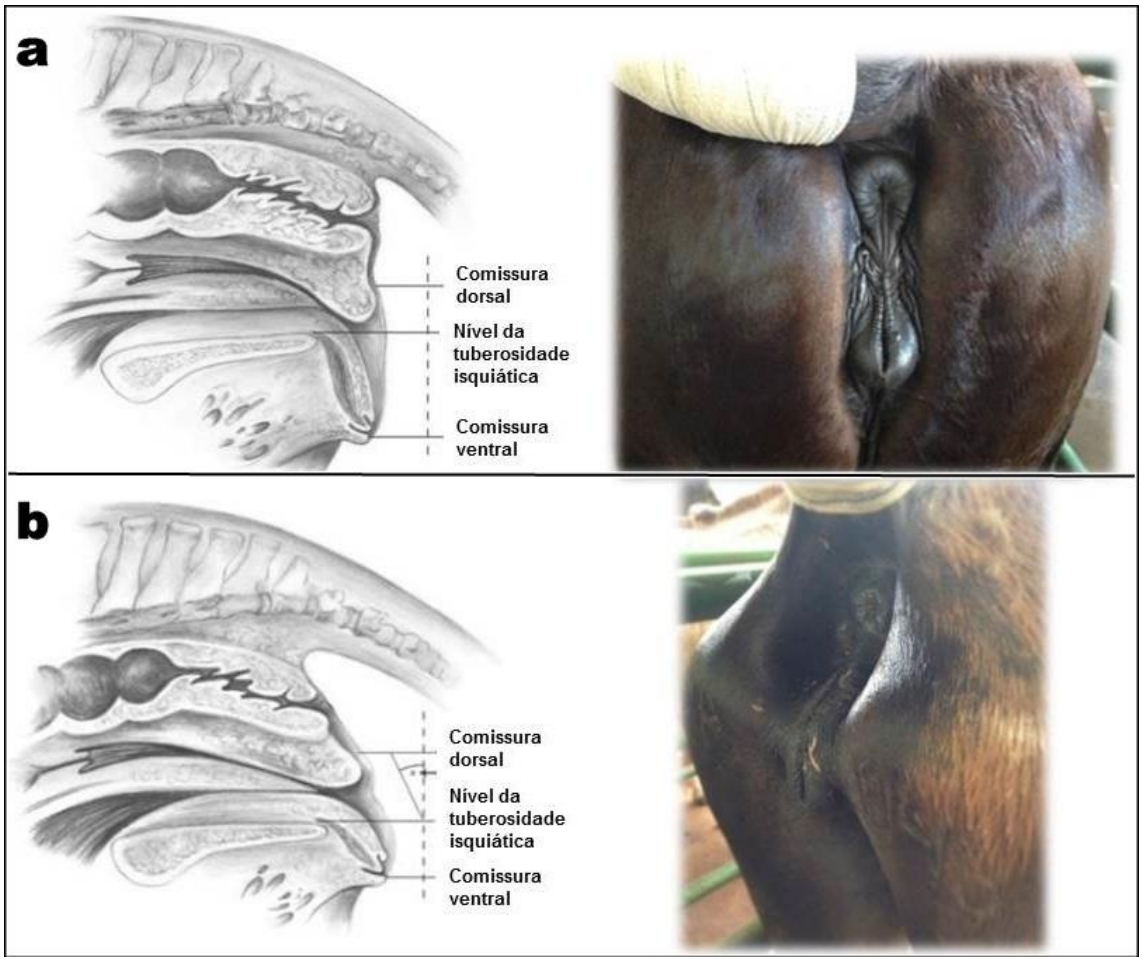

FIGURA 1. Anatomia da genitália externa de éguas com conformação perineal normal (a) e éguas com conformação perineal alterada (b) (adaptado de DESCANIO, 2011).

2.2. Mecanismos de defesa uterina

2.2.1. Barreiras físicas

A vulva, a prega vestíbulo-vaginal e a cérvix compõem as barreiras físicas que impedem a contaminação do útero por microorganismos externos (PASCOE, 1979; HINRICHS et al., 1988; LEBLANC et al., 1995). O comprometimento de alguma destas barreiras, pode predispor as éguas a infecções uterinas (TROEDSSON, 1999). Porém durante a cobertura/IA, estas barreiras são ultrapassadas e o sêmen depositado diretamente no interior do útero, induzindo uma resposta inflamatória aguda do endométrio (TROEDSSON, 1997).

2.2.2. Imunoglobulinas

As principais imunoglobulinas (Ig) secretadas localmente pelo trato genital são a IgG e a IgA (TROEDSSON, 1999). Essas imunoglobulinas (principalmente a IgG) atuam como opsoninas, juntamente ao componente C3b do sistema complemento, auxiliando as células inflamatórias, em especial os neutrófilos, na fagocitose de microorganismos invasores (BRITO & BARTH, 2003). O sistema complemento atua diretamente na mediação da inflamação uterina, promovendo um aumento da permeabilidade vascular, opsonização e quimiotaxia das células inflamatórias e lise dos contaminantes (TROEDSSON et al., 2008). No entanto, esses mecanismos não aparentam ser a causa da susceptibilidade a EPPC em éguas, já que as concentrações de imunoglobulinas não diferem entre éguas resistentes e susceptíveis (TROEDSSON, 1999; DELL'AQUA Jr. et al., 2006).

2.2.3. Inflamação local e mediadores inflamatórios

A inflamação uterina é mediada predominantemente pela resposta imune inata, o que possibilita que a cobertura por um mesmo macho gere prenhez por diversas vezes (LYLE, 2011).

Esta resposta imune é mediada por diversos mecanismos celulares e químicos de resposta rápida (TIZARD, 2008), que se iniciam a partir do reconhecimento de antígenos. O principal fator predisponente para o início da reação inflamatória no útero é o contato dos espermatozoides com o endométrio (TROEDSSON et al., 1993; TROEDSSON et al., 2001). A partir desse contato, os PAMPs (padrões moleculares associados a patógenos) são reconhecidos pelos receptores tipo Toll (TLRs) das células endometriais e das células sentinelas (TAKEDA & AKIRA, 2004; TIZARD, 2008), principalmente macrófagos presentes no endométrio (MELO et al., 2008).

A expressão de TLRs pelo endométrio após o contato com o sêmen ainda é controversa (CUERVO-ARANGO et al., 2008; EATON et al., 2010; NASH et al., 2010). No entanto, sabe-se que essa expressão aumenta principalmente dos TLR-4 e TLR-2, após a inoculação de E*scherichia coli* no útero de éguas resistentes (MARTH et al., 2015). Isso pode explicar o fato de a

expressão de TLRs após o contato do sêmen com o útero ainda ter resultados divergentes, já que o TLR-4 reconhece principalmente os lipopolissacarídeos (LPS) produzidos por bactérias gram negativas (CHOW et al., 1999; HOSHINO et al., 1999), sendo variável a contaminação do sêmen utilizado em cada estudo.

A ativação dos TLRs é a chave para o início da reação inflamatória (TAKEDA & AKIRA, 2004), o qual estimula a síntese do NF-κB (fator nuclear kappa-B) que ativa os genes para a codificação de citocinas pró-inflamatórias, quimiocinas e da cicloxigenase-2 (COX-2) (TIZARD, 2008; GIRLING & HEDGER, 2007), que agem como sinalizadores entre células modulando a resposta da fase aguda da inflamação (BARAÑAO, 1997; FUMUSO et al., 2007). Inicialmente as citocinas são sintetizadas como pró-moléculas e devem ser ativadas pela enzima caspase-1, que já foi descrita no endométrio de éguas submetidas a infecção experimental por *Escherichia coli* (MARTH et al., 2015) e é também sintetizada pelo estímulo do NF-κB (Figura 2) (TIZARD, 2008). Já a COX-2 atua na modulação da cascata do ácido araquidônico e as quimiocinas são proteínas com importante papel no controle da migração celular (TIZARD, 2008).

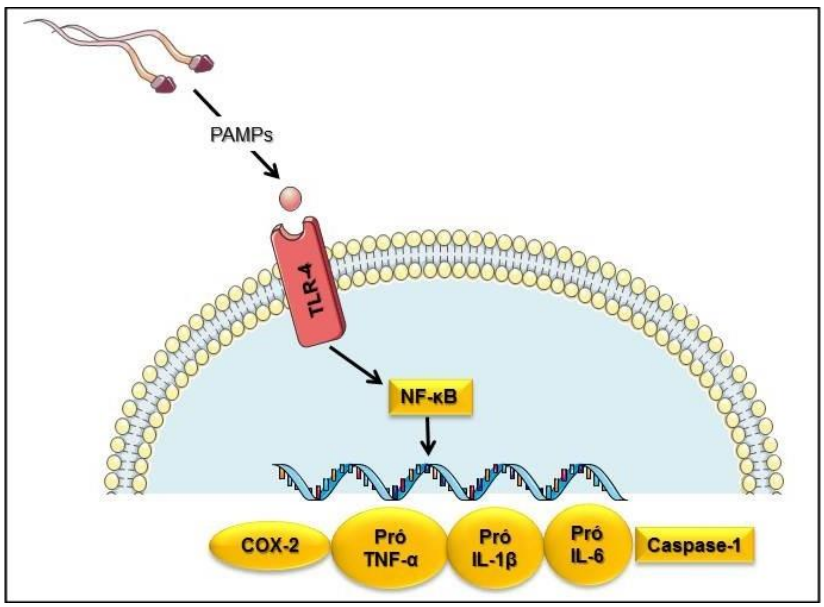

FIGURA 2. Reconhecimento dos PAMPs (Padrões moleculares associados a patógenos) de antígenos pelos receptores Toll-like das células sentinelas e desencadeamento da transcrição genica de fatores pró-inflamatórios pelo NF-κB (Fator nuclear kappa-B).

O ácido araquidônico é sintetizado pela atuação das enzimas fosfolipases sobre os fosfolipídeos liberados pelos tecidos lesionados. Sob a ação das cicloxigenases, especialmente a COX-2 em situações de inflamação, ocorre a síntese de prostaglandinas, principalmente a prostaglandina E (PGE) e PGF2α (BOERBOOM et al., 2004) e sobre a influência da enzima lipoxigenase sintetiza os leucotrienos (LTB_4) (Figura 3) (TIZER, 2008). O aumento da expressão de COX-2 ocorre no endométrio após o contato de plasma seminal ou diluente (PALM et al., 2008), assim como como um aumento local na concentração de PGF2 α no útero de éguas normais,16 horas após a IA (NASH et al., 2010).

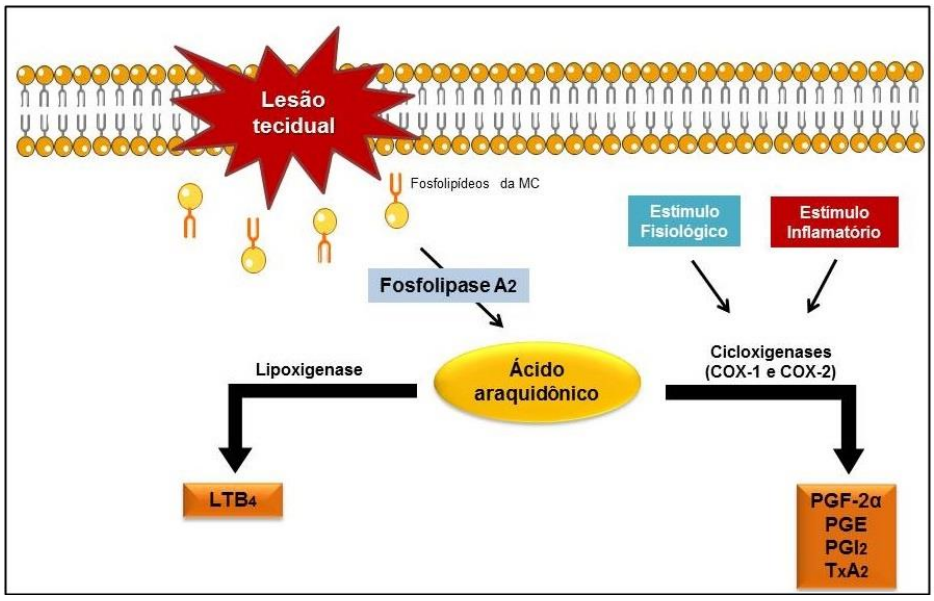

FIGURA 3. Cascata do ácido araquidônico.

A partir da síntese de quimiocinas, prostaglandinas e citocinas, principalmente interleucina-1 (IL-1), interleucina-6 (IL-6) e fator de necrose tumoral-α (TNF-α), ocorre a ativação das células do endotélio vascular, uma constrição das arteríolas e dilatação das vênulas no local afetado, aumentando assim a permeabilidade vascular e extravasamento de exsudato para o interstício, ocasionando edema local (COLLINS et al., 1999). A expressão endometrial dessas interleucinas (IL-1β, IL-6 e TNF-α) é maior em éguas susceptíveis do que em éguas resistentes, mesmo antes do contato com o antígeno (FUMUSO et al., 2003), assim como a expressão da interleucina-8 (IL-8), citocina que atua na quimioatração de PMNs (FUMUSO et al., 2006;

FUMUSO et al., 2007). O aumento dessas citocinas pró-inflamatórias, também ocorre após o contato do plasma seminal ou diluente com o útero desses animais (PALM et al., 2008).

Com as alterações no endotélio vascular iniciam-se as respostas celulares. As células do endotélio vascular passam a expressar P-selectina pelo estímulo inflamatório, que se liga a L-selectina presente na superfície dos neutrófilos, fazendo com que estes diminuam sua velocidade na corrente sanguínea e passem a rolar sobre a superfície do endotélio vascular. Em um segundo momento, os neutrófilos expressam integrinas que se ligam a moléculas de adesão nas células endoteliais até a parada completa e aderência a parede dos vasos, antes da migração para o sítio da inflamação (Figura 4) (TIZARD, 2008).

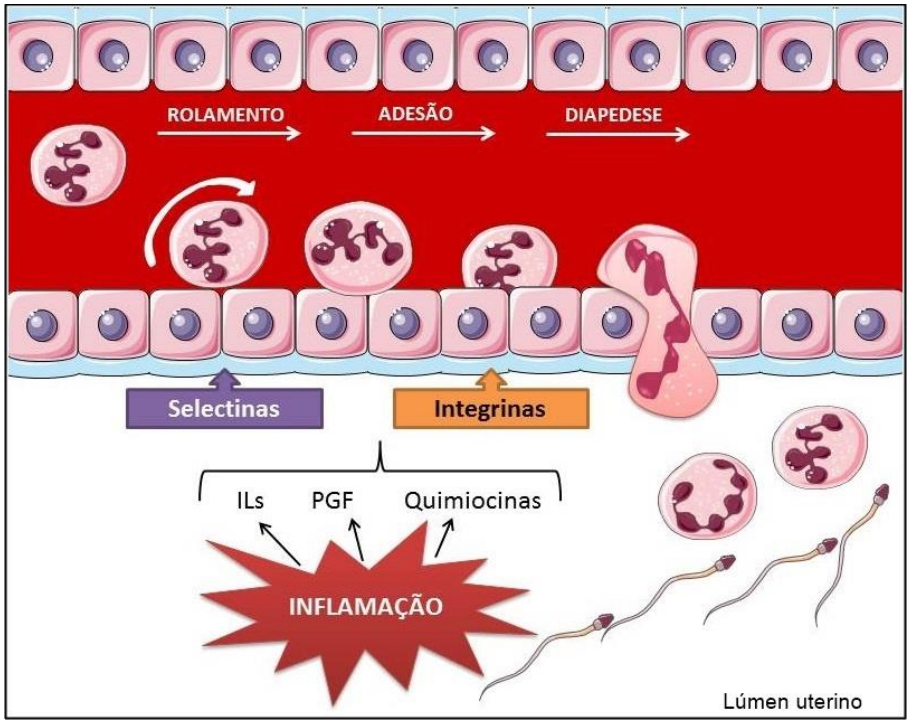

FIGURA 4. Migração de neutrófilos dos vasos sanguíneos para o sítio da inflamação.

Os neutrófilos são a primeira linha de defesa do organismo e a mais importante célula na defesa uterina (ASBURY, 1986; GALINDO et al., 2003). Essas células já se encontram no útero cerca de 30 minutos após o contato do sêmen com o endométrio (KATILA, 1995) e apresentam o pico da reação inflamatória entre seis e 12 horas, sendo eliminada até 48 horas em éguas com

um sistema de defesa funcional (TROEDSSON, 1994; LEBLANC, 2003; TROEDSSON, 1999). Os leucócitos não são apenas responsáveis pela defesa, essas células também secretam citocinas e mediadores quimiotáticos (FUMUSO et al., 2007), que embora em pequena quantidade, quando há uma invasão maciça dessas células para o útero, a contribuição total pode ser significativa (TIZARD, 2008).

Essa inflamação quando exacerbada, causa danos nos tecidos, dessa forma necessitando de mecanismos para o encerramento do processo. Algumas moléculas atuam bloqueando a produção dos mediadores pró-inflamatórios, inibindo seus receptores ou causando morte celular. A interleucina-10 (IL-10), interleucina-1Ra (IL-1Ra) e a IL-6 em uma fase mais avançada, tem importante papel no término da resposta inflamatória (KAPLANSKI et al., 2003; CHRISTOFFERSEN et al., 2012; WOODWARD et al., 2013). Segundo Fumuso et al. (2006) este mecanismo pode ser falho em éguas susceptíveis, já que a expressão da IL-10 é menor nesses animais.

2.2.4. Contrações miometriais

As contrações miometriais, inicialmente tem importante papel no transporte espermático até a tuba uterina e posteriormente na limpeza do útero, eliminando os produtos resultantes da inflamação pós-cobertura (TROEDSSON, 1999). Essas contrações iniciam aproximadamente 30 minutos após o contato do sêmen com o útero, auxiliando no transporte das células espermáticas até o local de fertilização, onde em cerca de 4 horas após a cobertura/IA, já há um número considerável de espermatozoides (BRINSKO et al., 1990; BRINSKO et al., 1991). O restante dos espermatozoides, plasma seminal e debris celulares resultantes do processo inflamatório, são eliminados (TROEDSSON, 1997; TROEDSSON et al., 1998; CAMPBELL & ENGLAND, 2006).

Éguas que falham neste mecanismo de limpeza uterina tendem a ser susceptíveis a EPPC (NIKOLAKOPOULOS & WATSON, 1999; TROEDSSON, 1999). Reitzenstein et al. (2002) demonstraram que as contrações miometriais de éguas resistentes tendem a ocorrer em direção à cérvix, facilitando a eliminação do conteúdo acumulado no lúmen uterino, enquanto que em éguas

susceptíveis, as contrações acontecem na direção oposta. Além disso, Troedsson et al. (1993) demonstraram que a atividade miometrial após o contato do endométrio com bactérias, difere entre éguas resistentes e susceptíveis quanto a intensidade, frequência e duração das contrações. Da mesma forma LeBlanc et al. (1998) utilizando cintilografia, demonstrou a deficiência na contratilidade miometrial em éguas susceptíveis, observada pelo maior tempo para a eliminação do radiocolóide em comparação a éguas resistentes. Essas falhas na atividade mioelétrica no útero de éguas susceptíveis podem ser causadas por diferentes fatores, como alterações degenerativas, mudanças vasculares do endométrio, alteração da interação neuromuscular e de resposta hormonal, falha na drenagem linfática, alteração nos padrões de liberação de prostaglandinas e ocitocina e modificação na posição anatômica do trato genital (RIGBY et al., 2001; LEBLANC, 2003).

Falhas na atividade mioelétrica e de limpeza uterina, podem ser a causa principal da instalação da EPPC (TROEDSSON, 1999) e o acúmulo de líquido intrauterino, ainda proporciona um ambiente adequado para o desenvolvimento de microorganismos oportunistas, predispondo a instalação de infecções uterinas (LEBLANC, 2003).

2.3. Terapias aplicadas a EPPC

As terapias normalmente utilizadas para EPPC podem ser consideradas tratamento de suporte, pois não atuam diretamente no processo inflamatório, apenas minimizam os fatores relacionados à sua instalação, na tentativa de promover um ambiente adequado para o desenvolvimento embrionário (LOSINNO; ALVARENGA, 2006). Nos últimos anos, alguns tratamentos utilizando agentes moduladores da resposta inflamatória (FUMUSO et al., 2003; DELL'AQUA Jr., 2006; BUCCA et al., 2008; PAPA et al., 2008; FIORATTI et al., 2013; REGHINI et al., 2016; METCALF, 2012; METCALF, 2014) vêm demonstrando bons resultados no combate a EPPC.

2.3.1. Medicamentos ecbólicos

Os principais medicamentos ecbólicos utilizadas para estimulação de contrações uterinas são a ocitocina e a PGF2α (LEBLANC, 1994; CADARIO et al., 1995). A ocitocina é comumente utilizada em éguas, auxiliando na expulsão do líquido intrauterino. Este fármaco pode ser utilizado até 48 horas após a ovulação, já que após este período, ocorre o fechamento cervical, impedindo a eliminação do líquido através da cérvix (TROEDSSON, 1997). A dose utilizada varia entre 10 e 20 UI, podendo ser administrada por via intramuscular ou intravenosa, ou juntamente ao diluente de sêmen na dose de 30 UI (CAMPBELL & ENGLAND, 2002). Cadario et al. (1999) observaram que doses mais altas de ocitocina por via parenteral, não trazem benefícios no tratamento da EPPC, já que induz a uma tetânia muscular, não ocorrendo a drenagem ou eliminação do fluído do interior do útero.

A prostaglandina, também pode ser utilizada na estimulação das contrações miometriais, sendo o cloprostenol o análogo da PGF2α mais utilizado para este fim (BRITH; BARTH, 2003). Este fármaco induz a uma atividade miometrial mais longa, quando comparado à ocitocina (BRENDEMUEHL, 2002; LEBLANC, 2003), beneficiando a limpeza uterina e a drenagem linfática em éguas com EPPC (LEBLANC, 2003). Porém, o uso de PGF2α nestes casos deve ser cauteloso, pois as PGF2α interferem na luteogênese do CL quando utilizada após a ovulação (BRENDEMUEHL, 2002), resultando em menores níveis de progesterona entre os dias 3 e 5 pós-ovulação (NIE et al., 2002). Deste modo, a utilização de análogos da PGF2α é uma alternativa para éguas que acumulam líquido pré-cobertura (LEBLANC, 2003).

Outro fármaco utilizado com sucesso nos últimos anos é a carbetocina, um análogo da ocitocina que atua promovendo contrações uterinas mais brandas e prolongadas (SCHRAMME et al., 2007; STECKLER et al., 2012; ALVARENGA et al., 2014).

2.3.2. Lavagem uterina

A lavagem uterina é um dos tratamentos comumente utilizado em éguas com EPPC. Esta pode ser realizada, tanto em éguas que acumulam líquido pré-cobertura, com a utilização de ringer com lactato, para a promoção de um ambiente adequado para o recebimento dos espermatozoides (VANDERWALL & WOODS, 2003), como também em tratamentos após a cobertura/IA, com o intuito de remover mecanicamente os restos celulares, debris inflamatórios e outros contaminantes presentes no interior do útero (KNUTTI et al., 2000; LEBLANC, 2003).

Estas lavagens pós-cobertura/IA, podem ser realizadas após quatro horas da introdução do sêmen no trato reprodutivo da fêmea, já que este é o tempo necessário para que uma concentração adequada de espermatozoides tenha chegado até a tuba uterina, não interferindo nos índices de fertilidade (BRINSKO et al., 1990; BRINSKO et al. 1991; FIALA et al., 2007).

Lavagens uterinas são necessárias, principalmente em animais que apresentam coluna de líquido maior que 2 cm visível no exame ultrassonográfico e/ou a presença de líquido com pontos hiperecóicos (BRISKO et al., 2003) (Figura 5). Essas lavagens normalmente são realizadas através de sifonagem, utilizando-se entre 1 a 2 litros de solução fisiológica por lavagem, até que o material retirado seja límpido (LEBLANC, 2003).

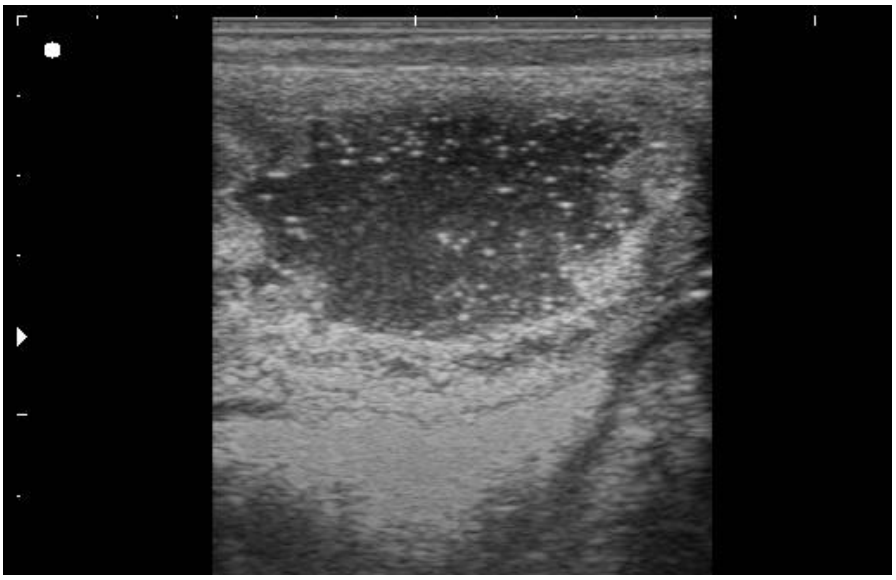

FIGURA 5. Presença de coluna de líquido intrauterino maior que 2 cm com presença de pontos hiperecóicos (Arquivo pessoal).

2.3.3. Anti-inflamatórios não esteroidais

O tratamento da EPPC com anti-inflamatórios não esteroidais (AINES) ainda é controvérsio, pois estes agem diretamente na cascata do ácido araquidônico, inibindo a produção de PGF2α, que é importante na promoção das contrações endometriais responsáveis pela limpeza uterina. Leblanc (1997) e Reilas et al. (2006), utilizando fenilbutazona e flunexin meglumine respectivamente, para o tratamento de EPPC, observaram um atraso na limpeza uterina e um aumento da reação inflamatória em éguas. Além disso, o uso destes fármacos próximo ao momento da ovulação podem causar folículos hemorrágicos anovulatórios (CUERVO-ARANGO, 2011), já que as prostaglandinas também são importantes nesse mecanismo fisiológico (ADAMS, 2003; ARMSTRONG, 1981; MURDOCH et al., 1993).

No entanto, com a associação de vedaprofen com ocitocina, Aurich et al. (2010) observaram um efeito benéfico, com redução no infiltrado de PMNs no útero e na expressão endometrial de COX-2 em éguas susceptíveis a EPPC.

2.3.4. Imunomoduladores

Os tratamentos utilizando agentes moduladores da resposta inflamatória vêm sendo utilizados e estudados nos últimos anos no combate a EPPC, demonstrando eficiente ação na modulação da resposta inflamatória uterina ao sêmen (LEBLANC & CAUSEY, 2009). Já foi demonstrado em alguns estudos (FUMUSO et al., 2003; BUCCA et al. 2008; PAPA et al., 2008, METCALF, 2014) aumento dos índices de fertilidade em éguas com EPPC associado ao tratamento com anti-inflamatórios esteroides ou agentes imunomoduladores.

Diversos moduladores da resposta inflamatória já foram estudados. Um dos primeiros imunomoduladores utilizados na tentativa de reduzir a resposta inflamatória pós-cobertura em éguas, foi o extrato de parede de mycobacterias (EPMB) que se mostrou eficaz na redução da expressão endometrial da IL-1 em éguas susceptíveis (FUMUSO et al., 2003), assim como elevar os níveis da citocina anti-inflamatória IL-10 no endométrio dessas éguas (FUMUSO et al., 2007). Christoffersen et al. (2012), utilizando EPMB, observaram redução do acúmulo de fluído intrauterino em éguas com EPPC, porém não observaram

alteração na expressão das citocinas. Já Woodward et al. (2015) descreveram que a utilização de EPMB causa diminuição na expressão da citocina IL-1β pelo endométrio.

Os anti-inflamatórios esteroidais são outro grupo de medicamentos imunnomoduladores que vem sendo amplamente utilizados na reprodução equina com o intuito de melhorar as condições uterinas de éguas susceptíveis à EPPC. Dell'Aqua Jr. et al. (2006) demonstraram que a utilização de acetato de prednisolona reduz o acúmulo de líquido e ainda aumenta os índices de fertilidade em éguas susceptíveis. Bucca et al. (2008) também observaram um efeito benéfico sobre a fertilidade de éguas susceptíveis quando utilizaram 50 mg de dexametasona antes da cobertura/IA. Esse fármaco ainda pode ser utilizado juntamente ao diluente de sêmen, sem prejuízos à qualidade seminal e auxiliando na redução dos sinais de endometrite pós-cobertura (FIORATTI et al., 2012). A dexametasona ainda promove a redução da expressão de interleucinas pró-inflamatórias, como as IL-1β, IL-6 e IL-8 e aumento das interleucinas anti-inflamatória IL-10 e IL-Ra (CHRISTOFFERSEN et al., 2012; WOODWARD et al., 2015).

Outros tratamentos com agentes imunomodularadores já foram descritos. A utilização de lactoferrina junto ao sêmen reduziu a expressão endometrial de interleucinas pró-inflamatórias (IL-1 e IL-8) (FORSHEY et al., 2011). As células tronco mesenquimais (CTM) promoveram o aumento da expressão da IL-Ra (citocina anti-inflamatória) pelo endométrio de éguas normais (FERRIS et al., 2014). Estes pesquisadores também notaram diminuição da concentração PMNs no lúmen uterino 6 horas após a IA, quando as éguas foram tratadas com meio condicionado autólogo. Rohrbach et al. (2007) observaram um aumento nos índices de prenhez em éguas susceptíveis tratadas com *Propionibacterium acnes*, um estimulante da resposta imune inata.

2.4. Plasma Rico em Plaquetas

O PRP já foi demonstrado ter ação benéfica no útero de éguas susceptíveis a endometrite, reduzindo o acúmulo de fluído intrauterino e do influxo de PMNs para o lúmen uterino (REGHINI et al., 2016), assim como a

expressão de citocinas pró-inflamatórias (IL-1β, IL-6 e IL-8) e da iNOS (enzima induzida pela inflamação) (METCALF et al., 2012), e aumentando os índices de fertilidade em éguas problema (METCALF, 2014).

2.4.1. Plaquetas e fatores de crescimento

As plaquetas são derivadas dos prolongamentos citoplasmático dos megacariócitos, localizados abaixo dos capilares sinusóides da medula que estão em constante contato com a corrente sanguínea (HARTWIG & ITALIANO, 2003). São corpúsculos anucleados, com forma discoide enquanto inativas e que apresentam pseudópodes quando ativadas, possuem tamanho entre 2 e 4 μm de diâmetro (BOUDREAUX, 2010) com um citoesqueleto formado por microtúbulos contrateis contendo actina e miosina, responsáveis pela manutenção do formato discoide das plaquetas, pela estabilização da membrana plasmática enquanto estão inativas (EVERTS et al., 2006). No citoplasma plaquetário existem diversos fatores bioativos, citocinas e proteínas envolvidas na reparação tecidual e coagulação sanguínea (MARX, 2001; JUNQUEIRA & CARNEIRO, 2004).

Os principais componentes encontrados no citoplasma das plaquetas são mitocôndrias, lisossomos, glicogênio, peroxissomos, α-grânulos e grânulos densos (HARRISON & CRAMER, 1993; BLOCKMANS et al., 1995). Nos α-grânulos estão contidos diversos fatores de coagulação e de crescimento importantes na modulação da inflamação, formação de coágulos e síntese de matriz extracelular (SCHNABEL et al., 2006) que são liberados a partir da ativação plaquetária (TEXTOR, 2011), como o fator de crescimento β (TGF-β), fator de crescimento semelhante a insulina 1(IGF-1), fibrinogênio, fator de crescimento derivado das plaquetas (PDGF), fator de crescimento vascular endotelial (VEGF), fator de crescimento fibroblástico (FGF), fator de crescimento epidermal (EGF), fator de crescimento epidermal derivado das plaquetas (PDEGF), fator de crescimento hepatocitário (HGF), tromboplastina plaquetária entre outros (HARRISON; CRAMER, 1993; BENDINELLI et al., 2010; KON et al., 2011).

Esses FC, regulam o metabolismo celular, promovendo reparação tecidual, angiogênese e a inflamação (ANITUA et al., 2004; BOSCH et al.,

2011) pelo aumento da transcrição gênica que elevam a síntese de matriz extracelular (DAHLGREN et al., 2001).

2.4.2. PRP

O PRP é derivado do sangue total e é por definição um volume de plasma que possui uma concentração de plaquetas acima dos níveis normais séricos (MARX, 2001) e diversos FC importantes na reparação tecidual (EL-SHARKAWY et al., 2007; MAIA et al., 2009), devido a sua ação mitogênica, quimiotática, neovascular e anti-inflamatória (GONSHOR, 2002; KEVY; JACOBSON, 2004; KIM et al., 2013, MAZZOCCA et al., 2013).

Para obtenção do PRP o sangue deve ser colhido em tubos contendo citrato de sódio como anticoagulante e pode ser processado por três diferentes técnicas: em tubo, por centrifugação ou por aférese (PRADES et al., 2006). Na medicina veterinária, o método mais utilizado é a centrifugação, pois é um procedimento fácil, de baixo custo e que se obtém uma concentração elevada de plaquetas. Diversas técnicas utilizando uma (CAMARGO et al, 2002; PAGLIOSA & ALVES, 2007) ou duas centrifugações (PAGLIOSA & ALVES, 2007; CARMONA et al., 2007; BARBOSA et al., 2008; MAIA, 2008) já demonstraram resultados semelhantes. Após a centrifugação do sangue, é possível observar a separação de três camadas distintas, onde a mais inferior é formada pelos eritrócitos, no meio ficam os leucócitos, formando a chamada “capa leucocitária” e na camada superior o plasma onde se encontram as plaquetas (FOSTER et al. 2009) (Figura 6). Durante o procedimento de separação do PRP, é importante evitar a fragmentação e ativação plaquetária, pois estes eventos liberam precocemente os FC, comprometendo a atividade biológica do PRP (PIETRZAK & EPPLEY, 2005). Segundo Carmona et al. (2007) e Vendruscolo et al. (2012), centrifugação com elevada força *g* provoca lesão e ativação plaquetárias, promovendo desta forma, baixas concentrações.

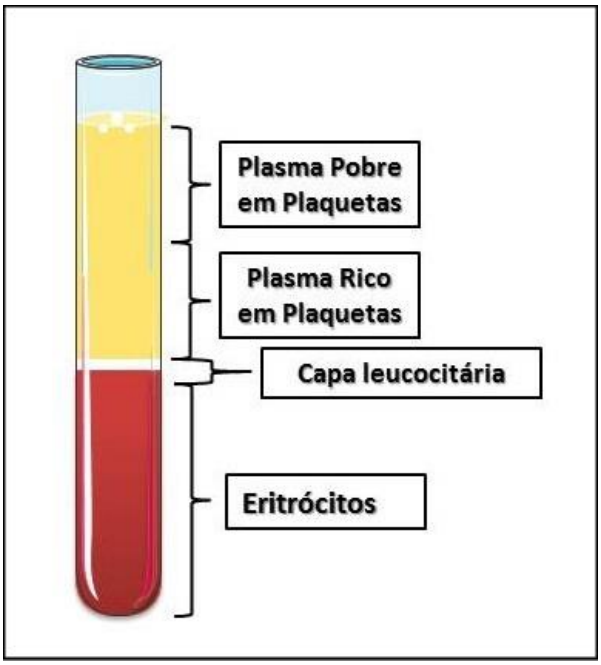

FIGURA 6. Separação das camadas após a centrifugação do sangue para a produção do PRP.

Para a liberação dos FC no local da lesão, deve ocorrer a ativação das plaquetas, com consequente estimulação dos α-grânulos e liberação das moléculas no local lesionado, iniciando assim o recrutamento de outras plaquetas, leucócitos e proteínas plasmáticas que atuarão no processo de reparação tecidual (HARRISON & CRAMER, 1993). Para ativação das plaquetas *in vitro*, podem ser utilizados vários agentes fisiológicos (trombina, serotonina, epinefrina, tromboxano) e farmacológicos (cloreto e cálcio, cálcio ionóforo) (CARMONA, 2006). Segundo Marx (2004) e Pietrzac & Eppley (2005), 70% dos FC são secretados pelos α-grânulos nos primeiros 10 minutos após a ativação plaquetária e cerca de 95% durante a primeira hora (KEVY & JACOBSON, 2001). Porém, mesmo após a liberação maciça dos FC após a ativação, as plaquetas continuam sintetizando essas moléculas por aproximadamente 10 dias, prolongando seus efeitos biológicos (WROBLEWSKI et al., 2010; TEXTOR, 2011). Em outro estudo, McCarrel & Fortier (2009) demonstraram não ser necessária a ativação das plaquetas por agentes exógenos, provocando assim uma liberação mais lenta e prolongada dos FC.

O uso de PRP já se mostrou eficaz no tratamento de enfermidades tendíneas (PRADES et al., 2006; MAIA et al., 2009; RIDERMANN et al., 2010), ligamentares (PRADES et al., 2006), osteoarticulares (CARMONA et al., 2009;

PRADES et al., 2006; FRISBIE et al., 2007), ósseas (LOPEZ et al., 2010) e cutâneas (DE ROSSI et al., 2009) de equinos, diminuindo o tempo de tratamento e melhorando a recuperação do tecido lesado.

2.4.3. Mecanismos de modulação da resposta inflamatória

Os mecanismos biológicos do PRP sobre a resposta inflamatória ainda não são bem elucidados. No entanto alguns estudos já demonstraram a ação anti-inflamatória do PRP na supressão de COX-2, metaloproteinase-3 (MMP-3), TNF-α, IL-1 (WOODALL et al., 2008; WOODELL et al., 2011; WU et al., 2011; SUNDMAN et al., 2013; KIM et al., 2014) e das moléculas de adesão vascular, que são expressas em tecidos inflamados para a migração de células inflamatórias (MAZZOCCA et al., 2013). O aumento dos marcadores de regeneração tecidual também é observado, sugerindo que o PRP atua suprimindo as citocinas pró-inflamatórias (KIM et al., 2014). Essa supressão, sobretudo do TNF-α, é uma das explicações para os benefícios clínicos do PRP, especialmente em tratamentos de lesões musculoesqueléticas (PRADES et al., 2006; FRISBIE et al., 2007; MAIA et al., 2009; RIDERMANN et al., 2010; LOPEZ et al., 2010; KON et al., 2011; LIPPROSS et al., 2011), pois essa citocina é o principal mediador inflamatório e ativador das metaloproteinases nos tecidos (TETLOW et al., 2001; KAPOOR et al., 2011).

Essa supressão dos mediadores inflamatórios ocorre pela capacidade do PRP em suprimir a expressão do NF-κB (BENDINELLI et al., 2010; VAN BUUL et al., 2011), que é o principal regulador do processo inflamatório e responsável pela expressão dos genes pró-inflamatórios (Figura 7) (PERKINS, 2006; GHOSH & HAYDEN, 2008, ULIVI et al., 2008; TIZARD, 2008).

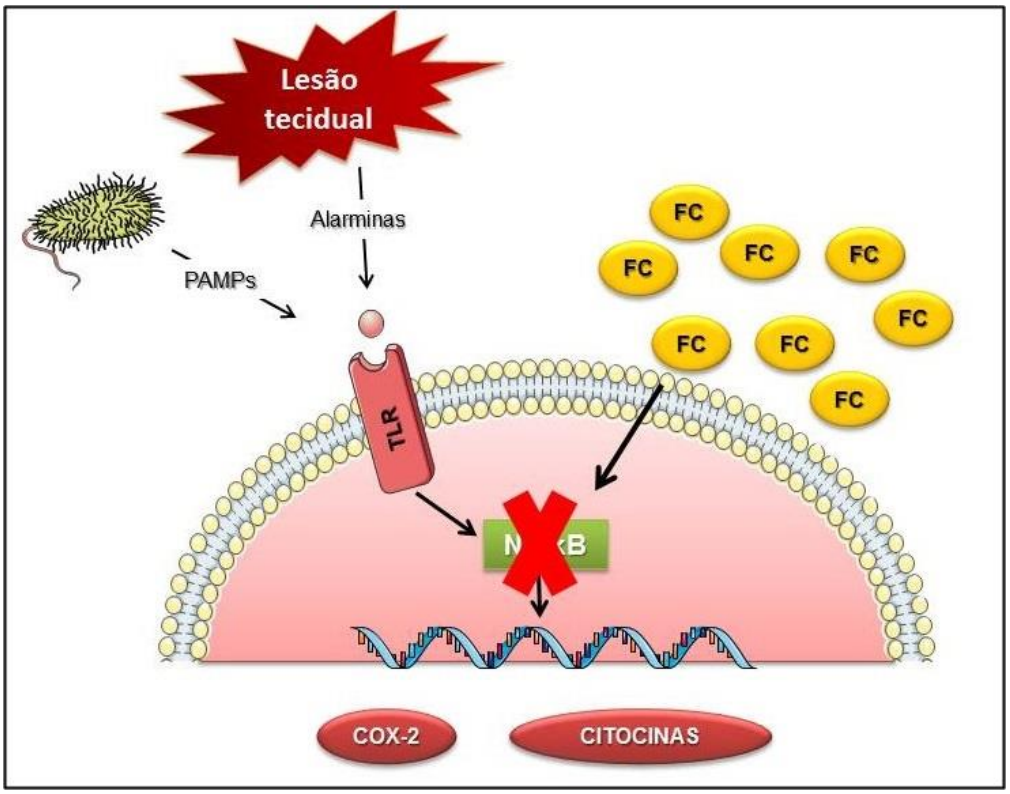

FIGURA 7. Ação dos FC (fatores de crescimento) presentes no PRP sobre a modulação da resposta inflamatória e inibição do NF-κB (fator nuclear kappa-B).

Além disso, o PRP ainda aumenta os níveis de Lipoxinas A4 (LPX_4) e RANTES (reguladores da ativação de células T expressadas e secretadas) (EL-SHARKAWY et al., 2007). As LPX_4 são moléculas lipídicas oriundas do ácido araquidônico e atuam na modulação da reação inflamatória, diminuindo a quimiotração de neutrófilos (BANNENBERG et al., 2005), já os RANTES, uma quimiocina expressa principalmente em células T, mas que também é sintetizada nos α-grânulos plaquetários (HOLME et al., 1998) e pode ter um efeito benéfico no controle da inflamação, pois inibem a liberação de histamina pelos basófilos (ALAM et al., 1992), o que conduz a redução do processo inflamatório e reparação tecidual (EL-SHARKAWY et al., 2007).

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HIPÓTESE

O plasma rico em plaquetas (PRP) pode modular a resposta inflamatória uterina assim como melhorar os índices de fertilidade em éguas susceptíveis a endometrite persistente pós-cobertura.

OBJETIVOS

- Verificar a eficiência do PRP sobre a resposta inflamatória pós-cobertura em éguas;
- Verificar o melhor momento da aplicação de PRP no útero, para a modulação da resposta inflamatória uterina em éguas susceptíveis a EPPC;
- Verificar a interferência do momento do tratamento com PRP sobre a fertilidade de éguas susceptíveis a EPPC.

CAPÍTULO 2

ARTIGO I

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Uterine clinical findings and fertility rate in susceptible mares treated with PRP at different moments of oestrous cycle.

Abstract

The persistent-breeding induced endometritis (PBIE) is the main cause of decrease fertility in the horses, thereby causing significant impact in the horse's market. A modulator of the inflammatory response that has been largely used in veterinary medicine is the platelet-rich plasma (PRP) that acts directly on inflammatory mediators. Thus, the present study aimed to determine if the time of intrauterine infusion of PRP interferes in the inflammatory response and pregnancy rates in susceptible mares. A total of 13 mares classified as susceptible to PBIE based on presence of intrauterine fluid 48 hours after artificial insemination (AI), more than 20% of polymorphonuclear cells (PMNs) 48 hours after AI and historic of less than 30% of embryo recovery rates were used for this study. The mares were inseminated with fresh semen, randomly assignment in three consecutive cycles in a cross-over study. PRP was prepared by single centrifugation protocol (120 *g*/10min) and just samples with a minimum concentration of 250,000 platelets/μL were used for treatments. The cycles were classified as control cycle (C): no pharmacological interference; PRP Pre (PreAI) 20 mL of PRP was infused 24 hours before AI; PRP Post (PostAI): 20 mL of PRP was infused four hours after AI. Follicular dynamic was monitored daily by transrectal ultrasound and when a >35 mm follicle and endometrial edema at least grade 2 (min. 0 and max. 4) were detected the ovulation induction was performed with deslorelin acetate (1mg, IM). AI was performed 24 hours after ovulation induction with the semen deposition (800 x 10^6 total sperm) in the uterine body. The intrauterine fluid (FLU) was evaluated by ultrasonography, before and 24 hours after AI; the percentage of PMNs in

uterine cytology (CYT) was observed before and 24 hours after AI, using bright-field microscopy; pregnancy diagnosis were performed with ultrassonographic exams 14 days after ovulation. The results were evaluated by Kolmogorov-Smirnov normality test. The ANOVA by Turkey test were used to compare the parametric continuous data and Kruskal-Wallis test followed by Dunns test to evaluate the nonparametric data. Multiple linear regression models and conception rate was evaluated by logistic regression model, considering the pregnancy as the dependent variable, considering a 5% significance level. Both PRP treatments were able on downregulate ($p<0.05$) the PMNs number in CYT after breed comparing with control cycle. The FLU did not differ ($p<0.05$) between cycles, however the conception rates were significantly ($p<0.05$) higher in treated cycle. In conclusion, PRP is benefit to reduce the inflammatory response in PBIE mares independently of the time of treatment, increasing the chances of achieving a pregnancy in this group of mares.

Key-words: Platelet-rich plasma, endometritis, subfertility, uterine inflammation.

1. INTRODUCTION

An acute inflammation is a physiological process that start with the recognition of bacteria or semen by the endometrium (Christoffersen et al., 2010; Fumuso et al., 2007; Fumuso et al., 2003; Kotilainen e al., 1994; Nash at al., 2010) and aim to eliminate the sperm excess and some contamination from the uterus (Troedsson, 1999; Troedsson et al., 1998). After the antigen recognition, inflammatory cell are recruitment (Alghamdi et al., 2005; Rigby et al., 2001) and start release pro-inflammatory mediators, producing vasodilation and consequently leukocytes migration and exudate presence (Collins et al., 1999; Parham, 2009). However, at the same time some anti-inflammatory cytokines are release to control this inflammatory process, preventing tissue damage (Kaplanski at al., 2003; Parham, 2009; Woodward et al., 2013) and restore normal endometrial physiological function (Baumann and Gauldie, 1994). Mares that failure in this process may be characterized as susceptible to persistent breeding-induced endometritis (PBIE) (Hughes and Loy, 1969).

The PBIE is the main cause of subfertility in mares. This disturb is characterized by the exacerbated influx of polymorphonuclear cells (PMNs) into the uterus and inability to eliminate intrauterine fluid after breeding (Bucca et al., 2008; Morel, 2003;

Zent et al., 1998). Failure in the uterine clearence may result in an unfavourable environment to embryo growth (Troedsson et al., 2001), causing a significant economic loss to the mare owner (Neely et al., 1979; Zent et al., 1998). Additionally, these mares have a highest endometrial expression of pro-inflammatory cytokines than resistant mares, even before the sperm contact (Fumuso et al., 2007, 2003), whose may also result an unfavorable environment for receiving the semen.

Susceptible mares to PBIE need intensive monitoring, in order to assist the uterus clearance, minimizing the tissue damage and maintain an adequate uterine condition (Asbury, 1986; Leblanc et al., 1994; Nie, 2003; Pycock and Newcombe, 1996; Rasch et al., 1996). Immunomodulatory treatments, using glucocorticoids (Bucca et al., 2008; Dell'Aqua Jr et al., 2006; Fioratti et al., 2012; Fumuso et al., 2003; Papa et al., 2008) or some biological products (Christoffersen et al., 2012; Ferris et al., 2014; Fumuso et al., 2003) showed good results to modulate or even suppress the uterine inflammatory response. The platelet rich-plasma (PRP) is a biological product that has been used in the treatment of localized inflammation in horse (Arguelle et al., 2008; Carmona et al., 2007; Lopes et al., 2010; Maia et al., 2009; Metcalf et al., 2012; Prades et al., 2006; Reghini et al., 2016) by the immunomodulatory properties (Gonshor, 2002; Kevy and Jacobson, 2004; Kim et al., 2014; Mazzocca et al., 2013) and consists in a blood product that contains an increased concentration of platelets in comparison with the whole blood (Marx, 2001).

The PRP is an inexpensive and accessible source of growth factors, whose are stored in the α-granules of the platelets and are released after platelet activation (Textor et al., 2011). The rationale for PRP uses in inflammatory conditions, consists of release many growth factors such as insulin-like growth factor-I, epidermal growth factor, fibroblast growth factor, transforming factor ß, vascular endothelial growth factor in the injured area, and local increase in lipid molecules (lipoxin A4) (El-Sharkawy et al., 2007), that were important in tissue repair owing to its anti-inflammatory properties (Gonshor, 2002; Kevy and Jacobson, 2004; Kim et al., 2014; Mazzocca et al., 2013; Woodall et al., 2008), increasing levels of chemokines that alter the chemotactic gradient, decreasing the leukocyte migration from the circulation (Alam et al., 1992) and inhibiting of the nuclear factor-κB (NF- κB) (Bendinelli et al., 2010; Van Buul et al., 2011), which is the main regulator of the inflammatory process (Ghosh and Hayden, 2008; Perkins, 2006; Ulivi et al., 2008).

Metcalf et al. (2012) and Reghini et al. (2016) using PRP inside the mares uterus reduced some inflammatory markers, however the treatments were performed in two different times in each study (pre and post AI). Thus, the aim of this study was to evaluate the effect of PRP treatment time (pre or post AI) on uterine inflammation and conception rate in mares susceptible to PBIE.

2. MATERIALS AND METHODS

This study was approved by the Animal Care and Use Committee, São Paulo State University – UNESP Botucatu, SP, Brazil.

2.1. Animals

Thirteen crossbred mares between eight and 22 years old, weighing 350-450 kg, from Department of Animal Reproduction and Veterinary Radiology, São Paulo State University, UNESP Botucatu, SP/Brazil were used in the study. All animals were with good body condition (score: 6.0-8.0) (Henneke et al., 1983), remained in similar handling and pasture and received 10 kg of ryegrass silage daily, water and mineral salt ad libitum. Mares were chose by reproductive history and had all these characteristics: presence of fluid accumulation 48 hours after AI (>10 mm of fluid column); exacerbated neutrophil infiltration (>20%) 48 hours after AI in the uterine cytology; poor embryo recovery rates (<30%).

A breeding soundness examination was performed before the study, which confirmed that all mares had no free fluid in the uterus, a negative uterine culture and a negative uterine cytology (<5% of PMNs) (Brook, 1993).

2.2. Preparation of PRP

Platelet-rich plasma was prepared by a single centrifugation; in brief, 45 mL blood samples were collected from each animal by puncture of the external jugular vein and conditioned into tubes containing 3.2% sodium citrate anticoagulant (*Vacutainer* 3,2% citrate, Labor Import, SP, Brazil).

Blood samples were homogenized and accommodated in an isothermal box during one hour. After, the samples were centrifuged at 120 x g for 10 minutes. From each centrifuged tube, the upper 50% of the plasma was discarded and the remaining fraction was used as PRP. The tubes with PRP were placed in an isothermal box

(Botuflex, Botupharma, Botucatu/SP, Brazil) to maintain a controlled temperature between 20°C and 25°C, for 1 hour until application.

The platelet concentration was measured using a hemocytometer chamber, the minimal platelet concentration used as a treatment was 250,000 platelets/mL and the PRP was used without activation as proposed by Mccarrel & Fortier.

2.3. Semen collections and preparation of insemination doses

Spermatozoa from a single stallion were used after ejaculate collection using an artificial vagina (Botupharma, Botucatu/SP, Brazil). Gel fraction was removed using a nylon filter and sperm motility was measured by computerized analysis (Ivos 12 - Hamilton Thorne Research, Danvers, USA) and the sperm concentration was measured using a Neubauer chamber. The semen was diluted with a skimmed milk based extender (Botu-semen, Botupharma, Botucatu, SP, Brazil) at a concentration of 50 million sperm per milliliter.

2.4. Treatment protocols

Three cycles of each mare were used and randomly assigned by lottery to control and treated cycles in a crossover study. Mares were examined by transrectal palpation and ultrasonography (Sono Scape A5V, Domed, SP, Brasil) once daily. When a 35-mm follicle or more was detected and endometrial edema at least grade 2 was diagnosed (min. grade 0; max. grade 4) (Mccue et al., 2011), ovulation induction was performed with an intramuscular injection of 1 mg of deslorelin acetate (Sincrorelin, Ouro Fino, Brazil). Inseminations with fresh semen (800 x 10^6 total spermatozoa) were performed 24 hours after induction of ovulation.

In the control cycle (C), the mares had no intrauterine infusion. In the treated cycles, 20 mL of PRP was infused with an insemination pipette (Pipette to mares, Provar Comercial LTDA, SP, Brazil) inserted into the uterine body. The treatments differed at infusion time:

- PreAI - 20 mL of PRP was infused 24 hours before AI (same time of ovulation induction);
- PostAI - 20 mL of PRP was infused four hours after AI.

Mares received only one treatment per estrous cycle. Each mare served as their own control to reduce a sampling effect on data analysis. Before all intrauterine procedures, each mare's perineal region was cleaned and all intrauterine procedures were performed using sterilized material.

2.5. Sampling strategy

2.5.1. Intrauterine fluid evaluation

For the evaluation of uterine fluid accumulation, transrectal ultrasound evaluations were performed 24 hours before and 24 hours after AI. The presence or absence of fluid was recorded, and if present, the height and width of intrauterine fluid column was measured in millimeters in the region of bifurcation of the uterine horn. The amont of uterine fluid was quantify using the height and width multiplication (mm^2).

2.5.2. Endometrial exfoliative cytology

Endometrial exfoliative cytology was performed 24 hours before and 24 hours after AI. The samples were obtained using a disposable cytobrush (Cytological disposable collector to mares, Provar Comercial LTDA, SP, Brazil) as described by Alvarenga and Matos (1990). After collection, the slides were air-dried and stained by Dip Quick (Instant Prov; NewProv, Brazil). The samples were then microscopically examined under oil immersion (1000x), and the percentage of polymorphonuclear neutrophils (PMNs) per 100 epithelial cells was randomly determined. To determine the inflammatory score we used the method described by Brook (1993). The percentage of PMNs present in a cytology was used and classified as a noninflammated (<5%), mild inflammation (5 – <15%); moderate inflammation (≥15 – <30%); and severe inflammation (≥30%).

2.5.3. Conception rates

Ultrasound examination was performed 14 days after the detection of ovulation to diagnose pregnancy. Mares that did not ovulated 24 hours after AI were discarded and the next cycle was used. After the exam, an application of 5 mg (im) of dinoprost tromethamine (Lutalyse®, Zoetis, SP, Brasil) was performed to pregnancy interruption.

2.6. Statistical analysis

To evaluate the Gaussian distribution, data of platelets, PMNs and fluid were evaluated using the Kolmogorov-Smirnov normality test. The ANOVA by Turkey test were used to compare the parametric continuous data. The nonparametric data were tested using Kruskal-Wallis test followed by the Dunns test. Primarily the data were subjected to curve fitting test, checking through the coefficients of determination which the curve that allows better adjustment to the collected scores. A multiple linear regression model and conception rate was evaluated by logistic regression model, considering the pregnancy as the dependent variable and the three treatment groups as explanatory variables. P values less than 0.05 were considered statistically significant.

3. RESULTS

The final mean platelet concentration in PRP increase ($p<0.05$) 2.6 times compared with the platelet blood count. The PMNs mean concentration in PRP were 23.9 times lower than the PMNs blood count (Table 1). All samples obtained more than 250,000 platelets/µL.

Table 1. Platelets and PMNs quantification in whole blood and PRP.

Parameters	Average
Platelets in blood	132,740±24,430/µL[a]
Platelets in PRP	354,236±17,540/µL[b]
PMNs in blood	9,106±804.2/µL[a]
PMNs in PRP	378±74.1/µL[b]

Values expressed as mean ± standard deviation. Lines with different superscript (a,b) are significantly different ($P < 0.05$). PMNs; PRP, platelet-rich plasma.

The presence of intrauterine fluid was not observed before AI in any cycle and no observable difference ($p>0.05$) was seen in the presence of uterine fluid after AI between cycles (mm^2, C - 893±292; PreAI - 587±214; PostAI – 591±213). A significant increase ($p<0.05$) in the number of PMNs was observed in all cycles 24 hours after AI. However, mares in both treated cycles showed a reduction ($p<0.05$) on number of PMNs in the cytology (Fig. 1) and in the inflammatory score (Table 2).

Table 2. Number and percentage of mares classified in each endometrial inflammatory score 24 hours after breed in the cytological exam, without pharmacological interference (Control), treated with PRP 24 hours before AI (PreAI) and treated with PRP four hours after AI (PostAI).

Group	Inflammatory score (%)				
	Non-inflammated	Mild	Moderate	Severe	TOTAL
Control	0	0^{a}	3(23%)	10^{a}(77%)	13
PreAI	0	10^{b}(76.9%)	2(15.3%)	1^{b}(7.7%)	13
PostAI	1(7.7%)	8^{b}(61.5%)	1(7.7%)	2^{b}(15.3%)	12

Non-inflammated - <5%; Mild – 5-<15%; Moderate – ≥15-<30%; Severe - ≥30% PMNs in cytology. Columns with different superscript (a,b,c) are significantly different (P < 0.05). PRP, platelet-rich plasma.

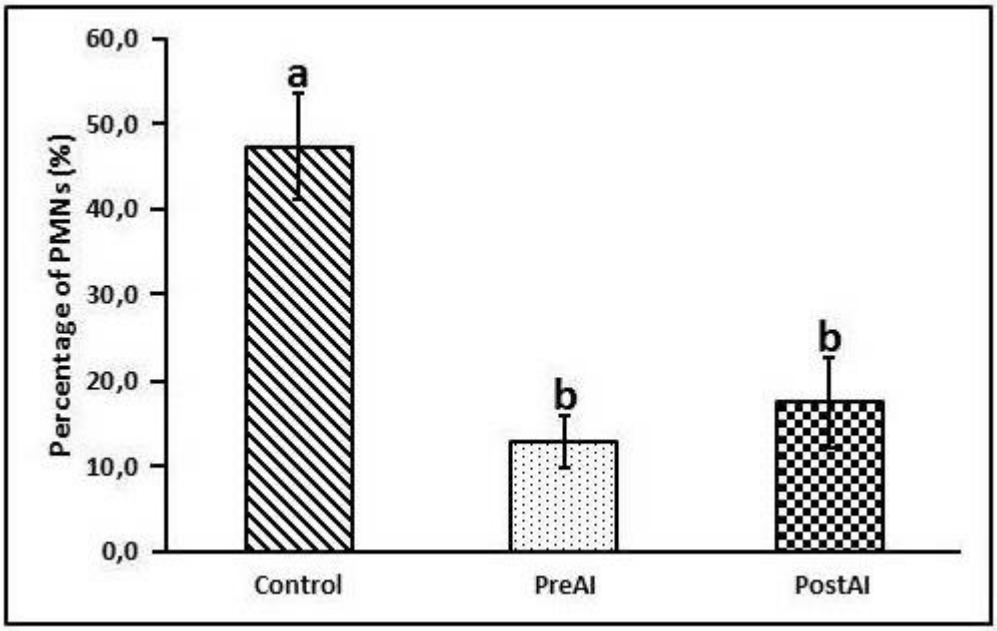

Fig. 1. Percentage of polymorphonuclear cells (PMNs) in uterine exfoliative cytology 24 hours after AI in persistent breeding-induced endometritis mares in nontreated cycle (control); treated cycles with PRP 24 hours before AI (PreAI); treated cycle with PRP four hours after AI (PostAI). Columns with different superscript (a,b) are significantly different (P < 0.05). PRP, platelet-rich plasma.

The conception rate did not differ (p>0.05) between groups (C – 31%; PreAI – 69%; PostAI – 58%). However when the treatment was considered a variable dependently, mares with PBIE exhibited a significantly higher (p<0.05) conception rate after intrauterine PRP infusion treatment compared with the control cycle (Fig. 3).

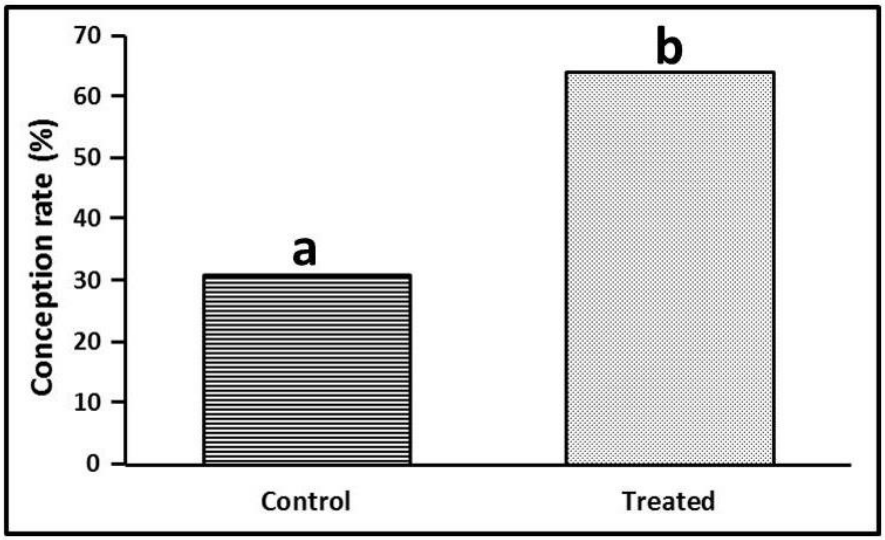

Fig. 2. Conception rate per cycle (%) in persistent breeding-induced endometritis mares in nontreated cycle (control) and in treated (PreAI and PostAI) cycles with PRP (Treated). Columns with different superscript (a,b) are significantly different ($P < 0.05$). PRP, platelet-rich plasma.

4. DISCUSSION

The average concentration of platelets in PRP processed by the proposed protocol was greater than the minimum value (300,000 platelets/µL) recommended by Anitua et al., (2004) and had 2.6 times more platelets than in the blood (Marx, 2001). Furthermore, the platelets concentration in our study was also greater than the platelets values used by Reghini et al. (2016) to treat the uterine inflammation of mares with chronic degenerative endometritis (CDE). This protocol also needs less volume of blood to prepare a PRP dose to intrauterine infusion than the protocol used by Reghini et al. (2016).

In our study, both PRP treatments were able to reduce the number of PMNs into the uterus 24 hours after AI, regardless of whether the inflammatory reaction was already triggered (PostAI) or not (PreAI). The decrease in PMNs number may be explained by the immunomodulation effect of PRP via inhibition of NF-κB (Bendinelli et al., 2010; Van Buul et al., 2011), consequently leading a inhibition on the inflammatory cells migration to the inflamed tissue by downregulation of vascular cell adhesion molecules (Mazzoca et al., 2013), and proinflammatory cytokines expression (Kim et al., 2014; Sundmn et al., 2013; Woodall et al., 2008; Wu et al., 2011) in the mares endometrium (Metcalf et al., 2012). The reduction in intrauterine PMNs was also showed by Reghini et al. (2016), using an activated PRP after AI, in mares with CDE,

confirming the beneficial effect of PRP to control the intrauterine inflammation.

Metcalf (2014) and Reghini et al. (2016) observed reduction in the intrauterine fluid accumulation in barren mares and CDE mares, respectively, using intrauterine PRP treatment. However, in the currently study no statistical difference was found in this parameter after PRP therapy, because there is great variability between mares with a high standard deviation among results. Some studies (Fumuso et al., 2003; Rogan et al., 2007) using immunomodulatory products showed a similar results, modulating the inflammatory markers but without changes in intrauterine fluid, probably caused by failure in myometrial contractility (Leblanc et al., 1998; Reitzenstein et al., 2002; Troedssonn et al., 1993).

The exacerbated inflammation that happens into the susceptible mares uterus, beyond 24 hours after breeding, is harmful to their fertility (Troedsson et al., 1998). The decrease in the inflammatory response into these mares endometrium after PRP treatment could help to increase the fertility in this group of animals. In this study, the conception rates of 31% and 64% were found for PBIE mares in the control and treated cycles, respectively. This results show a significant increment on the conception rates to susceptibles mares treated with intrauterine PRP infusion when the treated groups were combine in spite of large numerical difference on pregnancy among control and both treated groups. Metcalf (2014) also observed a similar increase on the barren mares fertility rates after PRP treatment, using these therapy before AI. Others studies, using immunomodulatory therapies, such as glucocorticoids (Bucca et al., 2008; Dell'Aqua et al., 2006; Papa et al., 2008), mycobacterium cell wall extract (Fumuso et al., 2003) and *Propionibacterium acnes* (Rohrbach et al., 2007) also showed an increased the fertility rates on susceptible mares, associated with the downregulation of inflammatory response, supporting our results. The PRP treatment has the advantaged to be safer, low cost and with no side effects when compared with other immunomodulatory therapies.

We could observe that both PRP protocols showed similar results, independently if the inflammatory reaction was trigged or not. Some reports in orthopedic cases using PRP in injured conditions (Arguelle et al., 2008; Carmona et al., 2007; Lopes et al., 2010; Maia et al., 2009; Prades et al., 2006) or during surgery to reduce the postoperative inflammation and accelerates tissue regeneration (Gobbi et al., 2012; Rafols et al., 2015; Smith et al., 2007) strengthening our result, showing that either one

these protocols may be used to help on the susceptible mares treatment. The uterine inflammatory peak happens 6 hours after sperm contact with the endometrium (Katila, 2001), since either one of these protocols used in the current study acts before the highest inflammation and showed efficient results to regulate this process. Reghini et al. (2016) using activated PRP after AI (4 hours after AI) to treat mares with CDE, observed a similar results in the PMNs influx to the uterine lumen, however showed a significant reduction in intrauterine fluid. Maybe, the fast release of growth factors after pharmacologically activation (Kevy and Jacobson, 2004; Pietrzak and Eppley, 2005) of PRP could increase the anti-inflammatory action when the inflammatory process is already initiated.

In this study, we used intrauterine PRP treatment to regulate the uterine inflammation in susceptible mares to PBIE. This injure is a real problem in the horse market, since it cause an economic loss and poor fertility rates in affected mares. Also, this condition is related to increasing age, becoming a real problem to reproductive assisted programs, since old mares represent around 30% of mares in these managements (Alvarenga and Carmo, 2009) and worth much with considerable interest in their offspring. Thus, there is encouragement to use advanced techniques that increase these animals fertility, could this therapy to be used as a further management.

In conclusion, both proposed treatments with PRP were able to reduce the uterine inflammatory process after breed in PBIE mares, with a similarly response between them, increasing the conception rate in these animals.

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ARTIGO II

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Effect of platelet rich-plasma on leucocyte migration and amount of COX-2 protein in endometrial tissue of susceptible mares after insemination.

Abstract

The Post-Mating Induced Endometritis (PMIE) is characterized by the exacerbated influx of polymorphonuclear cells (PMNs) into the uterus and inability to eliminate intrauterine fluid after breeding, resulting in an unfavourable environment to embryo growth. The platelet rich-plasma (PRP) is an inexpensive and accessible product that has been used in the treatment of localized inflammations. Thus, the present study aimed to determine if the intrauterine infusion of PRP could modulate the uterine inflammatory response in susceptible mares. A total of 13 mares classified as susceptible to PMIE based on presence of intrauterine fluid 48 hours after artificial insemination (AI), more than 20% of polymorphonuclear cells (PMNs) 48 hours after AI and historic of less than 30% of embryo recovery rates were used for this study. The mares were inseminated with fresh semen in three consecutive cycles in a cross-over study design. PRP was prepared by single centrifugation protocol and just samples with a minimum concentration of 250,000 platelets/μL were used for treatments. The cycles were classified as control cycle (C): no pharmacological interference; PreAI: 20 mL of PRP was infused 24 hours before AI; PostAI: 20 mL of PRP was infused four hours after AI. Follicular dynamic was monitored daily by transrectal ultrasound and when a >35 mm follicle and endometrial edema at least grade 2 (min. 0 and max. 4) were detected, the ovulation induction was performed with deslorelin acetate (1mg, IM). AI was performed 24 hours after ovulation induction with the semen deposition (800 x 10^6 total sperm) in the uterine body. The percentage of PMNs was observed in the biopsy

(HIP) samples collected 24 hours after AI, five fields were count and the average number of PMNs obtained, samples with an average of ≥18 PMNs cells were positive for endometritis; the number of COX-2 positive cells was evaluated by immunohistochemistry using a score system (0 – negative to COX-2; 1 – mild presence of positive cells; 2 – moderate presence of positive cells; 3 – severe presence of positive cells). To evaluate the normality distribution the Shapiro-Wilks test was used. Data were considered non-parametric and Kruskal-Wallis test followed by Tukey-Krammer test were used, considering a 5% significance level. The number of positive mares to endometritis in the HIP, decrease ($p<0.05$) in both treated cycles (Positive: C – 69%; PreAI – 15%; PostAI – 23%). Intensely ($p<0.05$) positive COX-2 labelling was observed in the control cycle compared to the PreAI and PostAI cycles. In conclusion, PRP is benefit to susceptible mares, downregulating the PMNs migration to the uterus and the amount of COX-2 protein in the endometrium after breed, becoming an option as a complementary treatment in these mares.

Key-worlds: Endometritis, equine, embryo transfer, PRP.

1. Introduction

The platelet-rich plasma (PRP) is a whole blood derived that has a high platelet concentration, consequently contains diverse growth factors that act in injured tissue by the mitogenic, neovascular and antiinflamatoory effects [1-4]. After platelet activation, the growth factors are released in the injured area, altering the chemotactic gradient, reducing the leucocyte (PMNs) attraction to the inflamed tissue [5, 6]. Some studies [7, 8] showed the downregulation on intrauterine inflammatory markers using PRP in mares with uterine inflammation.

The uterine inflammation is a physiological process that happens after artificial insemination (AI) or natural breeding, aiming clears the semen excess and microorganisms to the uterine lumen [9]. Mares considered resistant to persistent mating induced endometritis (PMIE) are able to reduce this inflammatory process within another 12-24 hours [10], however mares that fail this mechanism are classified as susceptible to PMIE. These animals are characterized by an exacerbate number of PMNs and fluid accumulation inside the uterus [11-13]. Additionally, the endometrium of susceptible mares express more pro-inflammatory cytokines and has lower

expression of anti-inflammatory cytokines [14, 15], that are regulatory molecules of the acute inflammation [15–17], than in resistant mares.

The acute inflammatory process starts after bacterial or semen recognition by the Toll-like receptors (TLRs) in the endometrial cells [14, 15, 19-23]. After the TLRs activation, the nuclear factor-kappa B (NF-κB) is expressed, whose acts activating pro-inflammatory citokines, chemoquines and cyclooxigenase-2 (COX-2) [24, 25]. These molecules regulate the inflammatory signals to the immune cells [15, 26].

The therapies usually used to treat endometritis, could be considered support treatments, since they aim to reduce the predisposing factors and do not act in the inflammatory process directly. Some immunomodulatory therapies have been used to modulate the uterine inflammatory process [13, 14, 27, 28]. The PRP acts inhibiting of the NF-κB [29, 30] and was shown to be capable of downregulating pro-inflammatory cytokines expression in the mares endometrium. Thus, in this study, we investigated the effect of platelet-rich plasma on endometrial PMNs migration as well as the amount of COX-2 protein in susceptible mares.

2. Material and methods

This study was approved by the Animal Care and Use Committee, São Paulo State University – UNESP Botucatu, SP, Brazil.

Thirteen crossbred mares from Department of Animal Reproduction and Veterinary Radiology, São Paulo State University, UNESP Botucatu, SP/Brazil, aging eight to 20 years old and chose by reproductive history and had all these characteristics: presence of fluid accumulation 24 hours after AI (>10 mm of fluid column); exacerbated neutrophil cells (>20%) 48 hours after AI in the uterine cytology; poor embryo recovery rates (<30%). The mares were remained in similar handling and pasture, receiving 10 kg of silage, water and mineral salt *ad libitum*.

Before start the study, a breeding soundness was performed to confirm that all experimental mares had a negative uterine culture, negative cytology (<5% of PMNs) [31] and no free fluid into the uterine lumen.

2.1. Detection of oestrus and treatment protocol

Mares were examined by transrectal palpation and ultrasonography (Sono Scape A5V, Domed, SP, Brasil) once daily. When a follicle measuring ≥ 35 mm and

endometrial edema at least grade 2 was diagnosed (min. grade 0; max. grade 4) [63], intramuscular injection of 1 mg of deslorelin acetate (Sincrorelin, Ouro Fino, Brazil) was performed to ovulation induction. Artificial inseminations were performed 24 hours after induction of ovulation with fresh semen (800 10^6 total spermatozoa).

Three cycles of each mare were used and randomly assigned by lottery to control and treated cycles in a crossover study. The biopsy samples were taken 24 hours after AI in each mare. The cycles were sorted: Control - no intrauterine infusion or pharmacological interference; PreAI - 20 mL of PRP was infused 24 hours before AI (during the ovulation induction); PostAI - 20 mL of PRP was infused four hours after AI. To PRP infusions an insemination pipette (Pipette to mares, Provar Comercial LTDA, SP, Brazil) inserted into the uterine body was used.

The perineal region was cleaned using mild detergent, rinsed with clean water and dried with paper towels. To the intrauterine procedures, sterilized materials were used to minimize the contamination potential. Mares received only one treatment per estrous cycle.

2.2. Semen collection

Semen from one stallion was used for inseminations. The ejaculate collections were performed using an artificial vagina (Botupharma, Botucatu/SP, Brazil). Gel fraction was removed using a nylon filter. The sperm concentration was measured using a Neubauer chamber and the sperm motility was measured by computerized analysis (Hamilton Thorne Research, Danvers, USA). The semen was diluted at a concentration of 50 million sperm per milliliter using a skimmed milk based extender (Botu-semen, Botupharma, Botucatu, SP, Brazil).

2.3. Platelet-rich plasma preparation

Platelet-rich plasma was prepared by modifications to the method described by Carmona [32] using only one centrifugation step. For this, 45 mL blood samples were collected using tubes containing 3.2% sodium citrate anticoagulant, from each animal by puncture of the external jugular vein. Blood samples were homogenized and accommodated in a isotherma box during a hour.

To prepare the PRP, samples were centrifuged at 120 x g for 10 minutes. The upper 50% of the plasma was discarded from each centrifuged tube and the remaining

fraction was used as PRP. The tubes with PRP were placed in an isothermal box (Botuflex, Botupharma, Botucatu/SP, Brazil) for 1 hour until application to maintain a controlled temperature (20-25°C). The minimal platelet concentration considered to treat mares in this study was 250,000 platelets/mL and the PRP was used without activation [33].

2.4. Endometrial biopsy

Biopsy samples were taken from the anterior part of the uterine body, using a sterilized alligator jaw biopsy forceps (Botupharma, Botucatu, Brazil) covered with a sterile glove, introduced through the vagina and placed in the caudal part of the cervical canal. After collection, the biopsies samples were removed and stored in 10% neutral buffered formalin and embedded in paraffin for histology [35].

Tissue sections were cut at a thickness of 5 µm and stained with haematoxylin/eosin (H/E) [36]. Five fields per sample without artefacts, intact blood vessels or damaged epithelium were examined by light microscopy (Primo Star, Zeiss, Brazil) (magnification x 400). The number, types and location of the PMNs and lymphocytes were recorded, and the means for the five fields calculated. Samples with an average of 0 to 17 PMN cell in the stratum compactum were classified as negative and sample with average of ≥18 PMN cells were positive for endometritis [37].

2.5. COX-2 immunohistochemistry

The technique described by Vilés [38] was used to this study. Amount of COX-2 protein was measured using a standard streptavidin–avidin–biotin immunoperoxidase technique. Paraffin sections were deparaffinized and rehydrated. Endogenous peroxidases were inhibited by exposure to 3% H_2O_2 in distilled water for 5 min. Antigen retrieval was performed by heating tissue at 90–100 ◦C in citrate buffer (pH 6.0) for 10 min and after that cooling at room temperature during 30 min and the non-specific protein binding was blocked using 2% bovine serum albumin (Sigma–Aldrich, St. Louis, Missouri, USA). Sections were incubated overnight at 4°C with a polyclonal rabbit anti-murine COX-2 antibody (Cayman Chemical, Ann Arbor, Michigan, USA) diluted 1:500, and then washed with PBS three times. The slides were then incubated with biotiny lated polyclonal goat anti-rabbit secondary antibody (Dako, Glostrup, Denmark) for 1 h, followed by incubation for 1 h with an avidin–biotin-peroxidase

complex (ImmunoPure, Thermo Fisher Scientific, Rockford, Illinois, USA). The COX-2 labelling was visualized using 3,3-diaminobenzidine tetrahydrochloride (Sigma–Aldrich, Madrid, Spain), counterstaining with Mayer's haematoxylin. Normal rabbit serum was used for negative control and canine squamous cell carcinoma were prepared [39] as a positive control.

The number of COX-2 positive cells was evaluated by light microscopy (Primo Star, Zeiss, Brazil) (magnification ×400) in five randomly selected fields. A modified scoring system proposed by Jiwakanon [40] was used to jointly record the number of labelled cells, the intensity of labelling, and the location of labelled cells (0 – negative to COX-2; 1 – mild presence of positive cells; 2 – moderate presence of positive cells; 3 – severe presence of positive cells).

2.6. Statistical analyses

The data were evaluated by Shapiro-Wilks test, to test its normality distribution. Data were not normally distributed and the non-parametric Kruskall–Wallis test was used to examine the differences between groups. When significant differences were observed, the corresponding data were rank-transformed and means compared using the Tukey–Kramer test. Significance was set at $P \leq 0.05$ for all tests and P values between 0.05 - 0.1 were considered statistical trend.

3. Results

3.1.1. Biopsy

A diffuse PMN infiltration into the luminal epithelium and stratum compactum was observed in all samples. The mean number of PMNs did not differ in the stratum compactum between the control cycle and PostAI cycle, but in the PreAI cycle a statistical trend ($p=0.063$) to reduce the number of these cells was observed (Table 1). However in both treated cycles the number of mares classified as positive to endometritis were less ($p<0.05$) than in the control cycle (Fig. 1). The number of PMNs in the stratum spongiosum was also significantly ($p<0.05$) high in the control cycle than in treated cycles. Lymphocytes concentration did not differ ($p>0.05$) in endometrial biopsies at any cycle (Table 1). Representative images of biopsies are showed in Fig. 2.

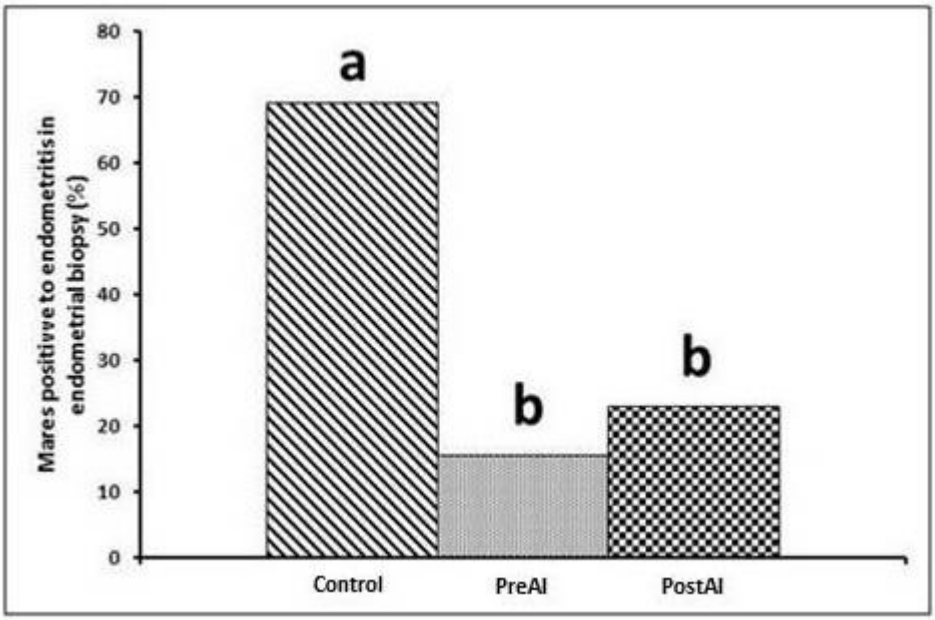

Fig. 1. Percentage of mares positive to endometritis in endometrial biopsies (an average ≥18 PMNs per fiel at 400x magnification) evaluated 24 hours after AI in the control cycle – no intrauterine treatment; PreAI – Treated with PRP 24 hours before AI; PostAI – treated with PRP 4 hours after AI. Columns with different superscript (a,b) are significantly different ($P < 0.05$).

Table 1. Leucocyte concentration in the biopsies without pharmacological interference (Control), treated with PRP 24 hours before AI (PreAI) and treated with PRP four hours after AI (PostAI).

Cycle	**PMNs**		**Lymphocytes**	
	Stratum Compactum	**Stratum spongiosum**	**Stratum compactum**	**Stratum spongiosum**
Control	22.3 ± 6.1^{c}	4.20 ± 1.45^{a}	30.61±6.61	14.96±3.5
PreAI	8.9 ± 2.4^{d}	0.97 ± 0.84^{b}	30.36±5.90	15.13±4.21
PostAI	9.7 ± 2.6^{cd}	1.06 ± 0.67^{b}	31.15±5.41	14.89±4.50

Columns with different superscript (a,b) are significantly different ($P < 0.05$) and (c, d) are statistical trend ($P = 0.063$).

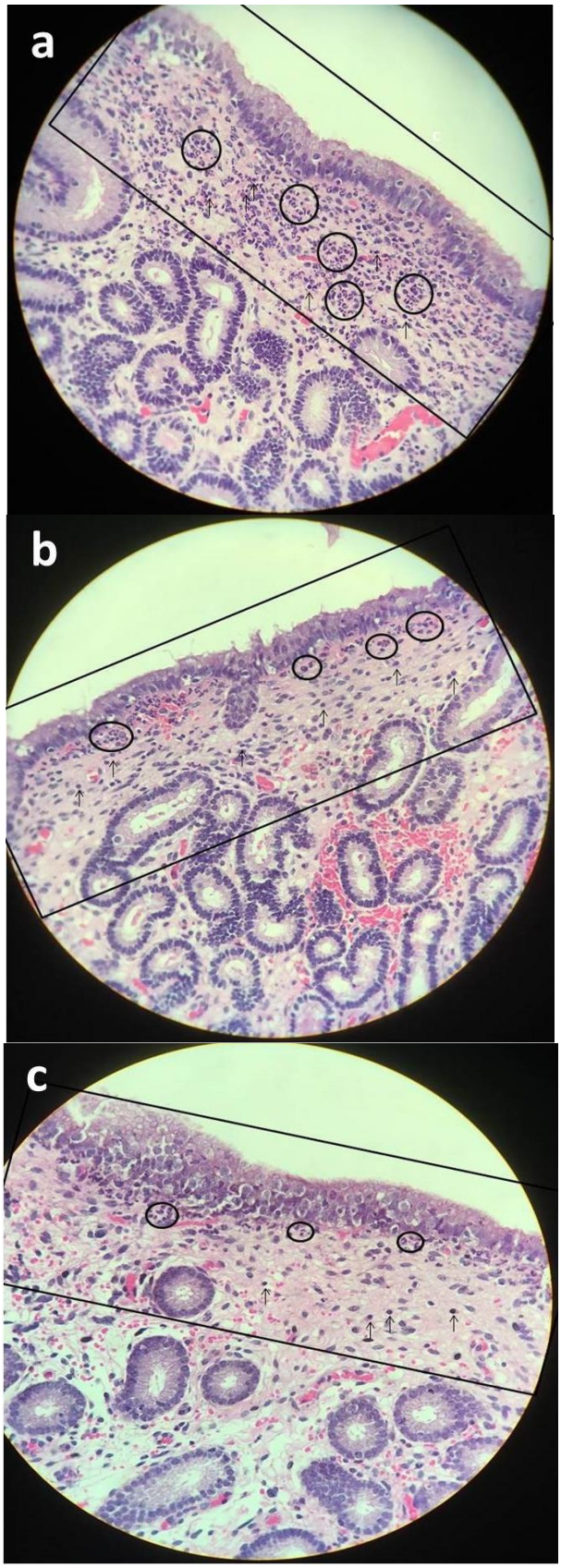

Fig. 2. Representative images of H/E-stained biopsies showing the inflammatory cells infiltration 24 hours after AI in the control cycle (a); PreAI cycle (b); PostAI cycle (c). Arrows show lymphocytes and circles neutrophils.

3.1.2. Cox-2 immunhistochemistry

Negative to COX-2 samples were not observed in any cycle before AI. However, greater score of positive COX-2 cells were observed ($p<0.05$) in the control cycle samples compared to both treated cycles (Table 2). Fig. 3 shows representative images of the COX-2immunohistochemistry results for each treatment group.

Table 2. Median scores for COX-2 immunohistochemical labelling in the biopsies from mares 24 hours after insemination and number of mares in each score, without pharmacological interference (Control), treated with PRP 24 hours before AI (PreAI) and treated with PRP four hours after AI (PostAI).

Treatment	**Score**	**Number of mares (%)**			
		Mild	**Moderate**	**Severe**	**TOTAL**
Control	$3\pm0,48^{a}$	0	4(30.8%)	9(69.2%)	13(100%)
PreAI	$2\pm0,37^{b}$	4(15.4%)	9(84.6%)	0	13(100%)
PósAI	$2\pm0,68^{b}$	4(30.8%)	5(53.8%)	4(15.4%)	13(100%)

Colunas com diferente sobescrito (^{a,b}) representam diferença estatística ($p<0,05$).

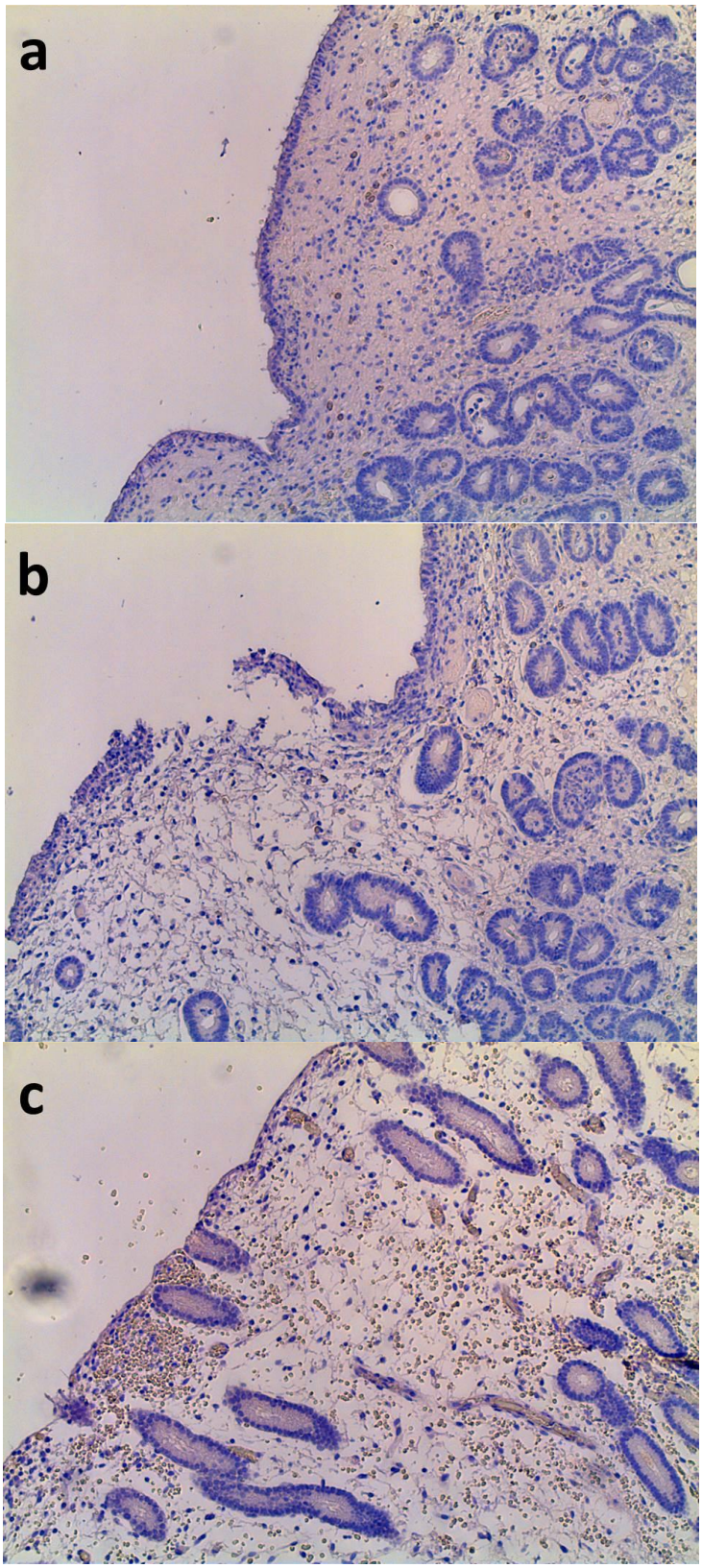

Fig. 3. Representative images of COX-2 immunohistochemical labelling in mares 24 hours after AI in the PreAI cycle (a); PostAI cycle (b); control cycle (c).

4. Discussion

Endometritis is the third most common infirmity of horses and considered the most common cause of infertility in mares [41]. The spermatozoa is the main predisposing factor for the onset of the inflammatory reaction into the uterus [10], starting the acute inflammatory response by TLRs activation and consequently releasing pro-inflammatory markers [19, 23, 42, 43]. This inflammation is transient and should be solved until 48 hours [10], mares that could not solve this process by themselves are classified as susceptible to PMIE [44]. Clinical findings in these mares are an exacerbated influx of PMNs to the uterus, fluid accumulation into the uterine lumen and endometrial inflammation, characterized by excessive edema after breed [13, 45, 46]. This exacerbated inflammation result in an unfavourable environment to embryo growth [42], causing significant economic loss in the horse market [11, 47].

An exacerbated inflammatory process was observed in the mares during the control cycle, with presence of large number of PMNs in the tissue samples. However, when the treatment with intrauterine infusion of PRP was used the number of mares classified as susceptible to endometritis decrease significantly. The PRP acts in the inflammations through the release growth factors that are restored into the α-granules of the platelets [48, 49]. Some growth factors released by platelets after activation in the inflamed tissue: transforming factor ß, insulin-like growth factor-I, platelet derived growth factor, fibroblast growth factor, epidermal growth factor, vascular endothelial growth factor; are important in tissue repair owing to their mitogenic, anti-inflammatory, chemotactic and neovascular effects [1-4]. The capacity of PRP to decrease the number of PMNs in the endometrial exfoliative cytology was showed by Reghini [8], using an activated PRP into the uterus of mares with chronic degenerative endometritis (CDE) after breed. However, we could observe that the pre-treatment with PRP was most effective to reduce the PMNs infiltration in the stratum compactum of the susceptible mares after artificial insemination than the post-AI treatment. The dexamethasone also reduce the inflammatory markers when used before breeding in susceptible mares [13], but no benefits on fertility rate [50] or inflammatory markers [51] were observed when this therapy was used after breed. Probably this occurs because using the PRP therapy before the sperm contact with the endometrium, this product acts modulating the beginning of the inflammatory cascade that was not started yet. Differently, when we used the PRP treatment four hours after AI, the inflammatory

reaction was already active [52] and the pro-inflammatory cytokines were previously expressed immediately after the antigen recognition [14, 15, 19-25].

The PRP acts in the inflammation by inhibition of NF- κB [29, 30], which is the key valve of the inflammatory process [30, 53, 54]. In addition, the PRP increase lipoxin A4 and some chemokines that cause a reduction in the PMNs attraction to the inflamed tissue [5, 6]. These effects cause an in vitro downregulation on cytokines (TNF-α, IL-1) and COX-2 expression [4, 55-57] and also reduce in vivo endometrial expression of IL-1b, IL-6, IL-8 [7] in susceptible mares. In the current study an intense expression of COX-2 protein in the endometrium of susceptible mares was observed in the control cycle, however after PRP treatment a lower expression of COX-2 protein was observed endometrium of these mares. The COX-2 expression as well as the other pro-inflammatory cytokines are regulated by the NF- κB [53, 54, 58] and it is important to the prostaglandins (PGF2α) synthesis from arachidonic acid, released by damaged tissues [59]. COX-2 also modulate the PGF2α expression in mares endometrium, being correlated with luteolysis during the late dioestrus [59]. In exacerbated inflammatory situations the increase on PGF2α synthesis may cause premature luteolysis and embryonic loss [47]. Also, some studies using exogenous PGF2α applications in the periovulatory and early postovulatory period showed a negative effect on luteal function and resulted in decrease of progesterone levels [60] and pregnancy rate [61] in mares. Maybe the exacerbate expression of COX-2 that happens in the susceptible mares endometrium after breed causes an intense synthesis of PGF2α and can also harm the luteal function and pregnancy rate.

The PRP was able to reduce the COX-2 expression in the endometrium of susceptible mares and showed a statistical trend in the pre-treated cycle group to reduce the number of PMNs in tissue in this study, however in the post-treated cycle group did not show reduce the influx of PMNs to the endometrium after breed. Vilés [38] also showed similar results in jennies inseminated with frozen semen plus seminal plasma that reduce the COX-2 expression but did not reduce the PMNs migration on the endometrium. Additionally, it was reported that post-thawed semen associated with seminal plasma result in an increase in jenny fertility rates [62]. Thus, these results strengthen the benefit of PRP therapy on fertility rate of susceptible mares to PMIE showed in previous studies [63, 64], since the exacerbated uterine inflammation is correlated with poor fertility rate [9]. Furthermore, PRP is an inexpensive, safety and

accessible biological therapy that may be use associated with usual endometritis treatments, being economic gains to the horse market.

With these results, we can conclude that PRP treatment benefits mares susceptible to PMIE, reducing the inflammatory response after breed. However, the key mechanism to start the uterine inflammatory process in mares is not well elucidate, as well as the failure on uterine clearance mechanisms in susceptible mares.

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