UNIVERSIDADE ESTADUAL DO NORTE FLUMINENSE DARCY RIBEIRO

MIGUEL ALEJANDRO SILVA RUA

AVALIAÇÃO DE CARACTERÍSTICAS REPRODUTIVAS DE ÉGUAS DA RAÇA MANGALARGA MARCHADOR

CAMPOS DOS GOYTACAZES - RJ

2018

MIGUEL ALEJANDRO SILVA RUA

AVALIAÇÃO DE CARACTERÍSTICAS REPRODUTIVAS DE ÉGUAS DA RAÇA MANGALARGA MARCHADOR

Tese de doutorado apresentada ao Centro de Ciências e Tecnologias Agropecuárias, da Universidade Estadual do Norte Fluminense Darcy Ribeiro, como requisito final para obtenção de grau de Doutor em Ciência Animal.

ORIENTADORA Profª. Celia Raquel Quirino

Campos dos Goytacazes – RJ

2018

MIGUEL ALEJANDRO SILVA RUA

AVALIAÇÃO DE CARACTERÍSTICAS REPRODUTIVAS DE ÉGUAS DA RAÇA MANGALARGA MARCHADOR

Tese de doutorado apresentada ao Centro de Ciências e Tecnologias Agropecuárias, da Universidade Estadual do Norte Fluminense Darcy Ribeiro, como requisito final para obtenção de grau de Doutor em Ciência Animal.

Aprovada em 19 de fevereiro de 2018

BANCA EXAMINADORA

Prof. José Frederico Straggiotti Silva (Ph.D, Medicina Veterinária) - UENF

Prof. Ricardo Lopes Dias da Costa (Doutor, Ciência Animal) - IZ

Prof. Renato Travasso Beltrame (Doutor, Ciência Animal) - UNESP

Profa. Celia Raquel Quirino (Doutora, Ciência Animal) – UENF
(Orientadora)

AGRADECIMENTOS

Agradeço primeiramente a Deus por guiar meu caminho da melhor forma possível, de uma maneira que nunca pude imaginar que seria tão bom para minha vida.

Aos meus pais que sempre me incentivaram, deram-me todo carinho e suporte para que eu conseguisse alcançar meus objetivos e conquistas.

À minha esposa Paula por estar sempre ao meu lado apoiando-me em todas as horas em que precisei.

À minha orientadora e grande amiga Celia, que me deu todas as oportunidades de que precisei e pela dedicação.

A Ricardo por ter me dado a primeira oportunidade de trabalhar com pesquisa.

A Marcus e Bruno por terem contribuído com os meus conhecimentos sobre reprodução equina e por terem me dado inúmeras oportunidades.

Aos membros dos laboratório de microbiologia e histopatologia da UENF por terem me auxiliados nos experimentos, obrigado Eulógio, Rachel, Mariah, Lourdes e Olney.

Aos meus amigos do laboratório que estiveram comigo durante toda essa jornada, obrigado Ana Carolina (Brita), Ana Cláudia, André, Caroline, Júnior, Júlia, Mariana, Mirela, Wilder e Thiago.

RESUMO

O objetivo do presente estudo foi avaliar os efeitos ambientais na taxa de recuperação embrionária e de prenhez, a dinâmica folicular e repetibilidade do diâmetro do folículo pré-ovulatório de éguas da raça Mangalarga Marchador, assim como avaliar diferentes métodos de diagnósticos que promovem informações sobre o real perfil uterino das éguas. As temperaturas, pluviosidade e o fotoperíodo foram mensurado. As doadoras de embriões apresentaram escore de condição corporal 4 (em escala de 1 a 5) durante todo o estudo. As análises estatísticas foram feitas pelos testes de Qui-quadrado, Kruskal-Wallis e Variância. Efeitos de ano, estação reprodutiva e temperatura foram observados na taxa de recuperação embrionária ($p < 0,05$), porém não houve efeito dos parâmetros ambientais (ano, estação, chuva e fotoperíodo) e tratamento hormonal na taxa de prenhez ($p > 0,05$). A média do diâmetro do folículo pré-ovulatório foi de 39,3 ± 3,8 mm. A estação reprodutiva afetou o diâmetro folicular, com maior média na transição de outono. Em relação às avaliações uterinas, na maioria, não teve associação entre citologia e outros métodos diagnósticos, exceto para cultura bacteriana. A bactéria mais comumente encontrada foi a *Escherichia colli,* no entanto não apresentou associação com presença de neutrófilo na citologia. A presença de *β-hemolitic streptococcus* foi bem caracterizada por citologia positiva e lavado com aspecto turvo. O perfil endometrial foi afetado pela idade tanto em éguas doadoras como receptoras de embriões. O diagnóstico com maior sensibilidade foi o de avaliação do aspecto do fluido intrauterino. O bom escore corporal das éguas foi determinante para evitar influencias negativas do ambiente sobre a recuperação embrionária. Conclui-se que as éguas dessa raça podem ser usadas na reprodução durante todo o ano com sucesso na produção de embriões no clima tropical do Brasil. O uso de tratamento hormonal com progesterona é eficiente na manutenção de resultados similares durante diferentes observações. A Endometrite deve ser diagnosticada por pelo menos dois métodos para aumentar a acurácia.

Palavras-chave: Equino. Transferência de embrião. Reprodução. Útero.

ABSTRACT

The objective of this study was to evaluate the environmental effects on embryo recovery rate and pregnancy rate, follicular dynamics and repeatability of the size of preovulatory follicles of Mangalarga Marchador mares and evaluate different diagnoses to provide more accurate information about endometritis and the real endometrial profile. Temperature, rainfall and photoperiod were measured. The embryo donor mares were considered with body condition score 4 (scale from 1 to 5) during all study long. The statistical analysis were performed using the Chi-square, Kruskal-Wallis and Variance test. Effects were noted of the year, season and temperature on embryo recovery rate ($p < 0.05$), but no effects were observed of the environmental parameters (year, season, hormone treatment, rainfall and photoperiod) on pregnancy rate ($p > 0.05$). The mean of the preovulatory follicle was 39.3 ± 3.8 mm. The reproduction season affected the preovulatory follicle diameter with higher mean in autumnal transition. Regarding the uterine evaluations, most of evaluations showed no association between cytology and other diagnostic methods, except with bacterial culture. The most common bacterium found was *Escherichia colli*, but it was not associated with neutrophil presence in cytology. The presence of *β-hemolitic streptococcus* was well characterized by positive cytology and cloudy lavage fluid aspect. Endometrial profile was affected by age and whether the mare was donor or recipient. The most sensitive diagnostic method was gross aspect of lavage fluid. Good body condition of donor mares appeared to be the main determinant to avoid negative influence of environmental characteristics on ERR. In addition, hormone treatment also supported favorable results of PR in recipient mares during nBS. The conclusion is that mares of this breed can be used in reproduction all year long with successful offspring production in Brazil's tropical environment. The use of hormone treatment is efficient to maintain similar results during different observations. Endometritis should be diagnosed by at least two methods to increase accuracy.

Key-words: Equine, embryo transfer, reproduction and uterus.

SUMÁRIO

1. INTRODUÇÃO

O primeiro relato de transferência de embriões em equinos foi em 1974 por Oguri e Tsutsumi, 1974. Desde então, o número de criadores que se interessam por produzir mais potros por ano vem aumentando. Além do melhoramento genético que se alcança pela obtenção de material genético de garanhões geneticamente superiores, com o uso de sêmen refrigerado ou congelado, a introdução de éguas campeãs que se destacam em competição, como doadoras de embriões, também traz um grande avanço no melhoramento genético em animais de esportes e lazer (Aurich e Aurich, 2006).

Os profissionais que trabalham com reprodução equina, nos últimos anos, têm expressado grande avanço na aplicabilidade da técnica de transferência de embriões (TE), realizando em torno de 27.497 mil transferências por ano em todo mundo, sendo 12.400 mil transferências somente no Brasil, segundo levantamento feito pela Sociedade Internacional de Transferência de Embriões – IETS no ano de 2010 (Stroud e Callesen, 2012). Dentre as fêmeas que são submetidas à biotecnologia, inserem-se jovens, adultas, idosas, assim como éguas com histórico reprodutivo insatisfatório, resultado de isenção da produção de um potro, seja por cobertura natural ou inseminação artificial (IA).

Pouco se sabe sobre os possíveis danos ao endométrio e cérvice dessas éguas que são exaustivamente submetidas à inseminação artificial (IA) e lavagens uterinas para recuperação embrionária em repetidas estações reprodutivas. Segundo Troedsson et al. (1995), éguas em estro submetidas à cópula proporcionam à indução de resposta inflamatória transitória do endométrio. No entanto, não apenas a cópula ou a IA podem induzir a resposta inflamatória intrauterina, mas também a aplicação de fluidos intrauterinos para lavagem e recuperação embrionária (Palm et al., 2008). Além disso, em éguas em fase luteal, a situação é pior, pois, apenas o fato de se realizar artificialmente a abertura da cérvice combinado com a infusão intrauterina de fluido podem carrear agentes patológicos ao lúmen uterino que poderiam, consequentemente, causar inflamação e estímulo da liberação de prostaglandina e eventualmente causar a luteólise prematura (Koblischke et al., 2008). Tal processo torna-se um problema em relação às éguas doadoras de embriões que são

exaustivamente submetidas a várias colheitas de embriões durante uma estação reprodutiva.

Assim como a doadora de embriões, visa-se pelo cuidado com as éguas receptoras de embriões que devem apresentar boa saúde uterina para que levem a gestação à termo. No entanto, sabe-se que são inúmeras as possíveis causas para que haja perda embrionária precoce ou em qualquer outra fase gestacional. Dentre essas causas, leva-se em consideração a idade das éguas, a condição corporal, estresse, os protocolos hormonais, entre outras variáveis que podem tornar uma égua subfértil ou infértil (McKinnon et al., 2011).

Dentre as causas de subfertilidade ou infertilidade em éguas, a endometrite é a mais importante, pois, é a que acarreta mais prejuízos por diminuição da fertilidade. As condições inflamatórias no útero são classificadas como aguda ou crônica, assim como infecciosas causadas por bactérias e fungos. Cada uma dessas classificações é válida para descrever infecções e processos inflamatórios no útero (Hurtgen, 2006).

Diante do exposto, o objetivo desse trabalho é avaliar os fatores ambientais que influenciam na transferência de embriões (TE), fazer avaliações de características reprodutivas como dinâmica folicular, assim como, discutir sobre principais patologias uterinas observadas na rotina reprodutiva e testar três métodos diagnósticos de afecções uterinas de éguas da raça Mangalarga Marchador criadas na região norte do estado do Rio de Janeiro.

2. REVISÃO DE LITERATURA

2.1. INDUTORES HORMONAIS

2.1.1. Sincronização doadora-receptora

Segundo Jacob et al. (2012), para que ocorra prenhez, resultado de uma TE, uma égua receptora precisa ter ovulado um dia antes ou até três dias após a ovulação da doadora. No entanto, para a realização desse procedimento, existem algumas limitações, por causa da sincronização entre doadoras e receptoras, que tornam difícil a obtenção de ao menos 3 receptoras, condição requerida para cada doadora. Um outro problema encontrado é a época do ano a ser realizada a TE, ou seja, no final da estação reprodutiva, é a época em que ocorrem falhas no ciclo estral das éguas. Isso ocorre devido ao fato das éguas apresentarem inadequada secreção de gonadotrofinas no final da estação reprodutiva.

A sincronização da ovulação é um dos fatores mais importantes para tornar uma receptora disponível para ser utilizada em um programa de TE. A administração exógena de P4 em éguas induz o aumento do tônus uterino, que é uma característica do diestro, sendo essa mudança uterina similar ao que ocorre durante a gestação (BACKMAN *et al.*, 2004). No diestro, a parede uterina apresenta-se contraída e sua textura se torna mais homogênea, à medida que decresce o edema, após o estro (AURICH, CHRISTINE, 2011). Pode-se notar também um aumento na densidade uterina, em que os cornos uterinos encontram-se com aspecto mais tubular (MCKINNON *et al.*, 2011).

No momento da inovulação, na TE, realiza-se a escolha da receptora de acordo com a morfoecogeneicidade e tônus uterino. O tônus pode ser avaliado através de palpação retal, e a ecogeneicidade pela visualização ultrassonográfica (FLEURY; ALONSO; BALIEIRO, 2006). Segundo Carnevale et al. (2000), o tônus uterino e cervical no momento da TE é um útil indicador da qualidade da receptora. Um tônus uterino menor é associado a baixas taxas de prenhez e alto risco de perda embrionária.

Um dos protocolos de sincronização de receptoras é o descrito por Botelho et al. (2015) ao realizarem um experimento sobre tratamento hormonal para sincronizar doadoras e receptoras. O tratamento de um grupo de receptora começou 8 dias antes

da TE. Foram feitas injeções intramuscular (IM) de benzoato de estradiol nos dias 8, 7, e 6 antes da TE em doses decrescentes de 5, 3 e 2 mg, respectivamente. Cinco dias prévio à TE, foi administrada uma dose IM de 150 mg de progesterona de longa ação. A mesma dose de progesterona foi repetida no dia da TE, e a cada 7 dias até os 120 dias de gestação. Os mesmos autores observaram taxas de prenhez aceitáveis nos dois grupos, que foi maior que 50%. Além do mais, relataram que houve maior taxa de prenhez no grupo tratado com hormônios em relação ao grupo controle.

2.2. TRANSFERÊNCIA DE EMBRIÃO (TE) E SUA IMPORTÂNCIA NA REPRODUÇÃO EQUINA

Apesar dos equinos serem a primeira espécie doméstica de grandes animais em que se empregou a técnica de IA, provavelmente foi a última espécie ser submetida à transferência de embriões. O primeiro relato de sucesso na técnica de TE em equinos foi por Allen and Rowson (1972) quando transferiram embriões entre jumentos e equinos. Em 1974, Oguri and Tsutsumi (1974) relataram o nascimento de um potro oriundo da técnica de TE não-cirúrgica, em que foi realizada a transferência de um blastocisto para uma égua receptora.

Em relação à quantidade de embriões transferidos, o Brasil e a Argentina lideram o ranking no uso da técnica. No entanto a técnica é bem difundida também e tem grande aceitação na Austrália, Canadá e França (STROUD; CALLESEN, 2012).

O alto custo da técnica de TE restringe a utilização apenas de éguas de genética superior para serem utilizadas como doadoras de embriões. Contribuindo para o alto preço da técnica. São as características biológicas particulares e problemas técnicos que precisam ser superados que limitam o uso da TE em equinos, como a baixa resposta à superovulação ou a falta de um protocolo que proporcione a superovulação de forma eficaz, o que limita o número de embriões recuperados por ciclo estral (que seria apenas um embrião) (SQUIRES; MCCUE; VANDERWALL, 1999). A baixa taxa de recuperação embrionária, na maioria das raças, aumenta o tempo requerido para a obtenção de um produto de uma determinada doadora de embriões, contribuindo para o aumento dos custos da TE em equinos. Estas restrições levam à maioria das éguas doadoras de embriões serem éguas idosas e com histórico de problemas reprodutivos apresentando-se subférteis, sendo incapazes de produzir

um potro por monta natural ou inseminação artificial (SQUIRES; MCCUE; VANDERWALL, 1999).

O maior fator que afeta a recuperação embrionária em éguas é seu status reprodutivo (CARNEVALE *et al.*, 1988; MCKINNON *et al.*, 2011). Éguas idosas com histórico reprodutivo ruim produzem menos embriões. Algumas causas da redução da taxa de recuperação embrionária dessas éguas incluem patologias uterinas e de oviduto, assim como aumento de perda embrionária (BALL *et al.*, 1989).

Diante de vários fatores que podem influenciar negativamente na produção de um potro por ano de forma natural, por uma égua com boa genética, a transferência de embrião torna-se de extrema importância na continuidade da produção de animais com genética superior.

Mesmo que não exista um protocolo efetivo que promova a superovulação em éguas, as taxas de recuperação embrionárias obtidas são satisfatórias devido ao fato da técnica de TE em equino ser bem desenvolvida e empregada por profissionais especializados. Sendo assim, pode-se obter mais de uma cria por doadora por estação reprodutiva. Aurich (2011) relatou em seus estudos uma taxa de recuperação embrionária de 64%. O número de embriões coletados variou de 7 a 26 por égua, com média de 17 ± 2,2 embriões coletados por égua, no período de 2007 a 2009. Os mesmos autores observaram média de intervalo interovulatório de 29,7 ± 1,2 dias, com mínimo de 13 dias.

Em um estudo realizado com éguas da raça Mangalarga Marchador foi verificado recuperação embrionária de 72,8% em 1140 lavados uterinos (LOPES *et al.*, 2013). Raz et al. (2011), obtiveram taxas de recuperação embrionária de 73% em éguas durante uma estação reprodutiva em um programa comercial de TE.

2.2.1. Alguns fatores que interferem na TE

Segundo Squires et al. (1987), a ocorrência de múltiplas ovulações aumenta o número de embriões recuperados. Um dos fatores que mais limita os resultados da TE em éguas é a falta de protocolos hormonais de indução da superovulação que sejam eficientes (RAZ, T.; CARD, 2009; STOUT, 2006). Com isso, diminui a quantidade de produtos de uma mesma égua por estação reprodutiva, aumentando o

custo para que se consiga uma prole. Sendo assim, a ocorrência de múltiplas ovulações espontâneas torna-se um fator requerido em éguas doadoras de embriões. No entanto, a raça, a idade, o status reprodutivo, a estação reprodutiva, o uso de drogas para indução da ovulação, histórico de subfertilidade e dia da recuperação embrionária e condição corporal estariam influenciando na taxa de recuperação embrionária, no número de duplas ovulações e taxa de prenhez em éguas (GINTHER; DOUGLAS; LAWRENCE, 1982; MCKINNON *et al.*, 2011), (PANZANI *et al.*, 2014; STOUT, 2006).

O uso de sêmen fresco, refrigerado ou congelado também influencia na taxa de recuperação embrionária em equinos (SQUIRES; MCCUE; VANDERWALL, 1999; STOUT, 2006).

Outro fator que também poderia influenciar na taxa de recuperação embrionária seria o fato de algumas éguas estarem sendo submetidas à exercício (treinamento e competições), no entanto, esta hipótese ainda não está muito bem elucidada (MORTENSEN *et al.*, 2009).

2.2.2. Condição corporal

Dentre as diversas variáveis que podem influenciar no sucesso em programas de TE, a condição corporal é uma de extrema importância que pode estar ligada diretamente às taxas de recuperação embrionária e prenhez (ZÚCCARI *et al.*, 2013).

Existem evidências de que a inadequada nutrição e a condição corporal têm sido associadas com atrasos no início da ciclicidade em fazes de transição para a estação reprodutiva, diminuição nas taxas de prenhez, aumento de perdas embrionárias e aumento na duração da gestação em éguas. Nas últimas décadas, o papel do tecido adiposo como mediador endócrino tem se tornado evidente. A leptina que é um hormônio produzido principalmente pelo tecido adiposo, tem uma ligação com a função do sistema reprodutivo. Em éguas, assim como em outras espécies, existe uma correlação positiva entre as concentrações de leptina, o plasma sanguíneo e a condição corporal (CARTER *et al.*, 2009; HUFF *et al.*, 2008), sendo que esse hormônio também é envolvido com a modulação da atividade ovariana (FERREIRA-DIAS *et al.*, 2005; GENTRY; THOMPSON; GENTRY; DAVIS; GODKE; *et al.*, 2002).

Segundo (GENTRY; THOMPSON; GENTRY; DAVIS; GODKE; *et al.*, 2002; GENTRY; THOMPSON; GENTRY; DAVIS; GODKE, 2002), durante o inverno, éguas com baixo escore corporal apresentam menos folículos de tamanhos médios e grandes (≥ 20 mm) em relação à éguas com melhor condição corporal. Aparentemente, esse mecanismo é desencadeado pela glicose, insulina, leptina, hormônios de crescimento e ácidos graxos, que são envolvidos de alguma forma na regulação do eixo reprodutivo (hipotálamo-hipófise-gonadal) (MCKINNON *et al.*, 2011). Gentry et al. (2002) observaram baixas concentrações plasmáticas de leptina e IGF1 (fator de crescimento semelhante à insulina 1) e prolactina em éguas com baixo escore corporal.

2.2.3. Dias de recuperação embrionária

Aurich et al. (2011) realizaram 18 tentativas de coleta de embriões no dia 6 pós-ovulação e obtiveram um resultado significativamente mais baixo (38,9 %) em comparação com os outros dias de coleta de embrião como os dias 7 ao 10.

Panzani et al. (2014) relataram que não houve diferença na taxa de recuperação embrionária entre embriões coletados nos dias 7, 8, 9 e 10 após a ovulação (45,7%, 52,6%, 42,9% e 47,1% respectivamente).

Camargo et al. (2013) relataram que não houve diferença ($p > 0,05$) na taxa de recuperação embrionária entre os dias 7, 8 e 9 pós-ovulação, no entanto, foi verificado diferença ($p < 0,05$) dos dias 6 e 10 em relação aos demais dias pós-ovulação.

2.2.4. Sazonalidade

Segundo a literatura, existem dados de que a transição do outono para o inverno, período de anestro das éguas, indicam que as fêmeas equinas respondem à fatores endógenos que as levam a um declínio reprodutivo sazonal. Esse ritmo de fatores endógenos provavelmente sofre modificações por inúmeros fatores endógenos e exógenos. Alguns desses fatores são o fotoperíodo, temperatura, idade, condição corporal e, possivelmente, outros fatores ambientais. Porém, na indústria equina, os criadores são incentivados, cada vez mais, a produzir maior número de animais por ano. Sendo assim, o entendimento dos processos de transições entre

estações torna-se um instrumento para que se possa manipular o ciclo sazonal das éguas para se produzir potros todo o ano (MCKINNON *et al.*, 2011).

Devido ao fato da terra estar inclinada em seu eixo vertical, durante o ano, ao passar ao redor do sol resulta em diferenças de fotoperíodo e climáticas. Éguas que pariam durante o inverno, quando os recursos alimentares são escassos, estavam em risco de não passarem seus genes para as gerações futuras pois suas proles não apresentavam condições de sobreviver quando nasciam nessa determinada época do ano. Dessa forma, no decorrer do processo evolucionário de seleção natural dos equinos, ocorreu de forma que assegurasse que a "estação reprodutiva", no verão, é o melhor momento para acasalamento, minimizando as chances de nascimento de potros durante o inverno. Dessa forma, o ritmo reprodutivo anual dos equinos foi estabelecido. Assim, o fator ambiental que promove essa precisão é o fotoperíodo e através desse mecanismo, os equinos têm um ritmo reprodutivo circanual inato (SHARP, 2011a).

A melatonina é o hormônio produzido através da transformação de informações de luminosidade em mensagens químicas ao corpo. A mesma é liberada pela glândula pineal durante períodos escuros (noite) (SHARP, 2011b).

Aurich et al. (2011) observaram diferença na atividade ovariana de algumas éguas de acordo com a sazonalidade, sendo que algumas éguas entraram em um estádio anovulatório, enquanto outras continuaram ciclando normalmente durante o inverno. Sendo assim, os mesmos autores puderam observar e relatar que em éguas cíclicas, a taxa de recuperação embrionária não decresceu durante o inverno. Com isso puderam concluir que éguas com ciclos ovulatórios durante o inverno podem ser utilizadas com sucesso como doadoras de embriões. Assim, o uso de éguas como doadoras de embriões durante o inverno pode ser muito interessante porque esse é um período do ano em que há uma redução dos treinamentos e há poucas competições.

Por outro lado, Camargo et al. (2013) ao realizarem uma análise de taxa recuperação embrionária em duas estações diferentes, sendo uma durante a estação reprodutiva (primavera e verão) e outra fora da estação reprodutiva (outono e inverno) relataram que não houve efeito ($p > 0,05$) da estação nos índices de recuperação embrionária. Os mesmos autores ainda ressaltaram que as éguas que ciclaram fora da estação de monta apresentaram índices de recuperação embrionária muito

próximos aos dos índices das colheitas de embriões feitas dentro da estação de monta.

Em se tratando de ciclo estral da égua, sabe-se que as atividades reprodutivas são dependentes da adequada secreção do hormônio GnRH do hipotálamo para estimular a produção e secreção das gonadotrofinas FSH e LH pela hipófise anterior. Esse processo é dependente do fotoperíodo, em que, em dias mais longos (primavera e verão) as atividades hormonais e, consequentemente, ovarianas são maiores, enquanto que no outono e inverno as mesmas diminuem consideravelmente levando as éguas ao anestro estacional (MCKINNON *et al.*, 2011). No caso do estudo desenvolvido por Camargo et al. (2013), os animais encontravam-se em criações correspondentes à altura do paralelo 24, onde a quantidade de horas de luz do dia é maior em relação aos locais situados na altura do paralelo de 30 a 90, indicando que a latitude poderia estar exercendo importante função sobre a secreção hormonal e atividade ovariana, ou seja, a medida que diminui a latitude e, consequentemente, aumenta as horas de luminosidade, favorece à ciclicidade sexual das éguas, associada a uma adequada nutrição a esses animais.

2.3. ENDOMETRITE

A falha no mecanismo de defesa uterina em eliminar antígenos como bactérias, espermatozoides e produtos inflamatórios do útero resultam em endometrite persistente, a qual é a maior causa de redução da fertilidade em éguas em reprodução. O útero, normalmente, é bem protegido da contaminação externa por barreiras físicas consistindo na vulva, no vestíbulo, vagina e cérvice. Qualquer comprometimento de uma dessas barreiras, predispõe a égua à endometrose crônica (PASCOE, 1978).

A endometrite é um das principais causas da redução da infertilidade em éguas, causando a maior parte das perdas econômicas na indústria equina, com prevalência de 25 a 60% das éguas com problemas de fertilidade (TRAUB-DARGATZ; SALMAN; VOSS, 1991). A endometrite induzida pela cópula ou inseminação artificial (IA), é um evento normal que ocorre algumas horas pós-deposição do sêmen no trato reprodutivo da égua. Esse processo é considerado necessário para efetiva remoção de bactérias e excesso de espermatozoide introduzidos no lúmen uterino (TROEDSSON, 2006). Em éguas resistentes, a inflamação uterina termina e a limpeza

uterina ocorre antes do oócito fertilizado entrar no útero após a passagem pelo oviduto, 6 dias após a ovulação aproximadamente; enquanto que no útero de éguas susceptíveis persiste o processo inflamatório (TROEDSSON, 2006).

A inflamação uterina pode ser aguda, crônica, subclínica, pós-parto, de origem bacteriana, fúngica, viral, induzida pelo coito ou IA e persistente. Cada uma dessas classificações tem a validade de descrever a infecção e inflamação que estão afetando o útero. Na avaliação de uma égua com endometrite, é apropriado o uso de mais de um dos termos acima como critério classificatório de positivo ou negativo (HURTGEN, 2006).

A deposição do sêmen por monta natural ou IA é uma fonte de contaminação. As égua são classificadas como susceptíveis e resistentes à infecções uterinas (DIEL DE AMORIM *et al.*, 2015). Alguns mecanismos de defesa uterina são envolvidos na proteção do útero contra endometrite persistente. Os neutrófilos polimorfonucleares (PMNs) são as primeiras células inflamatórias a entrar no lúmen uterino, seguido por estímulo inflamatório (LEHRER *et al.*, 1988).

Acredita-se que uma falha no aspecto mecânico da defesa uterina seria o maior fator na contribuição para o desenvolvimento de uma endometrite persistente. Segundo LeBlanc et al. (1994) existem falhas no processo fisiológico natural de limpeza uterina em éguas susceptíveis em comparação com éguas resistentes. Esse processo está ligado à capacidade do útero em eliminar o agente inflamatório por contrações do miométrio. Alghamdi et al. (2005) observaram que éguas susceptíveis apresentaram aumento de óxido nítrico no lúmen uterino 13 horas após a inseminação. O óxido nítrico faz a mediação do relaxamento do músculo liso, sendo assim, foi sugerido que esse seja o fato de haver falha na expulsão de materiais que possa causar inflamação uterina por contração muscular da parede uterina.

Falhas no relaxamento cervical durante o estro ou insuficiente drenagem linfática também podem contribuir para falhas na limpeza uterina e acúmulo de líquido (LEBLANC, M M *et al.*, 1995).

2.4. DIAGNÓSTICOS DE PATOLOGIAS UTERINAS

2.4.1. Citologia endometrial

Os neutrófilos polimorfonucleares migram para o lúmen uterino em resposta à inflamação, então a endometrite é diagnosticada pelo exame de raspado uterino com escova ginecológica e esfregaço em lâmina de vidro. A amostra pode ser coletada com escova ginecológica, swab ou do líquido recuperado do útero. No esfregaço corado, pode-se observar células epiteliais do endométrio e em alguns casos células de defesa, principalmente neutrófilos PMNs. Sendo assim, a endometrite é estabelecida baseada no número de PMNs encontrados nas lâminas. Mais de um PMN a cada 10 células epiteliais é considerado como endometrite. Riddle et al. (2007) ao avaliarem a citologia uterina de éguas, observaram que éguas com menos de 2 PMNs por campo (x400 objetiva) apresentavam maiores taxas de prenhez (60% por ciclo) comparadas com éguas com 2 a 5 PMNs por campos (x400), que apresentavam taxa de prenhez de 23%. Os mesmos autores relataram que infecções associadas com *E. coli*, eram menos prováveis de serem associadas com citologia positiva comparada com éguas infectadas com *Streptococcus zooepidemicus.*

2.4.2. Biópsia uterina

Segundo Kenney (1978) o útero da égua é formado por três camadas que são: a camada interna ou endométrio que é a mucosa, a camada média que o miométrio, considerada a camada muscular e a camada externa ou perimétrio (camada serosa). O endométrio ou lúmen uterino é revestido por células epiteliais, que podem variar o formato de cúbicas à cilíndricas altas e, abaixo do epitélio está a lâmina própria dividida em estrato compacto e esponjoso.

A biópsia uterina é o único exame em que se pode avaliar a integridade estrutural do endométrio em relação à infiltrados inflamatórios, mudanças fibróticas e dilatação de glândulas endometriais e vasos linfáticos. Assim como também, permite-se avaliar as mudanças cíclicas consistentes no endométrio normal ao decorrer dos efeitos sazonais (MCKINNON *et al.*, 2011).

De acordo com Kenney (1978), a biópsia uterina é indicada para constatação de alterações do trato genital, quando constata-se infertilidade após monta natural ou IA (sêmen de qualidade comprovada) em mais de três ciclos em uma mesma estação reprodutiva, em éguas com histórico de perda embrionária precoce ou aborto, quando

se identifica comportamento de anestro durante a estação de monta, em casos de piometra e mucometra e para avaliação de fertilidade.

O termo endometrose foi introduzido primeiramente por Kenney (1992), o qual observou diferentes alterações no endométrio equino. Kenney (1978), relatou que a endometrose poderia ser definida como fibroses periglandulares e/ou fibrose endometrial estromal ativa ou inativa, incluindo alterações glandulares com focos fibróticos. Os mesmos autores também relataram que glândulas isoladas ou em "ninhos" podem estar afetadas.

O primeiro sinal de endometrose é a diferenciação morfológica e funcional atípica das células estromais do endométrio. O primeiro estágio da fibrose é caracterizado por grandes células estromais poligonal periglandulares que sintetizam fibras de colágeno denso. Em fibroses mais avançadas, não ocorre síntese de colágeno, sendo que há um predomínio de miofibroblastos (WALTER *et al.*, 2001).

De acordo com Walter et al. (2001), a dilatação glandular é outro achado da histopatologia uterina, que pode ocorrer em função da capacidade de contratilidade dos miofibroblastos que apresentam capacidade de afetar a composição e a quantidade de matriz extracelular.

Ferreira et al. (2015) observaram efeito de mudanças degenerativas no útero de éguas no índice de pulsatilidade arterial. O índice de pulsatilidade arterial (PI) é mensurado por ultrassonografia Doppler e tem como objetivo evidenciar sobre a suplementação hemodinâmica de tecidos por artérias (GINTHER, 2007). Um aumento da PI indica aumento na resistência arterial e diminuição do fluxo sanguíneo no tecido (GINTHER; UTT, 2004). Ferreira et al. (2015) observaram altos valores de PI em um grupo de éguas consideradas com alta degeneração uterina pela classificação de Kenney e Doig (1986), indicando que éguas com degeneração endometrial severa e difusa apresentam alta resistência arterial no miométrio e, consequentemente, baixo fluxo sanguíneo após a IA.

O aumento da resistência arterial intrauterina em éguas observada por Ferreira et al. (2015) pode também comprometer a vasodilatação local requerida para a migração de neutrófilos para o lúmen uterino.

2.4.2.1 Indicações para a realização da biópsia

As indicações para que uma égua seja submetida ao exame histopatológico, seriam para éguas com suspeita de anormalidades no endométrio, como éguas idosas ou éguas com histórico de complicações reprodutivas, antes da compra de uma receptora em exame ginecológico, particularmente em éguas mais velhas com histórico de fertilidade desconhecido, histórico de infertilidade mesmo quando acasaladas ou inseminadas com garanhões comprovadamente férteis, éguas não prenhas que estiverem em anestro durante a estação reprodutiva, éguas que irão ser submetidas a algum procedimento cirúrgico como reparação cervical e/ou remoção de cisto endometrial. Nesse último caso, seria interessante a avaliação da biópsia antes da cirurgia para comprovar se a égua será capaz de levar uma gestação a termo depois do procedimento cirúrgico. Dessa forma, se detectado pela biópsia que a égua não será capaz de levar uma gestação a termo mesmo com a realização da cirurgia, não justifica o procedimento cirúrgico (MCKINNON *et al.*, 2011).

2.4.2.2. Contraindicações para realização da biópsia

A principal contraindicação é a remoção de tecido endometrial de éguas prenhes, devido ao risco de ruptura de membranas fetais assim como o risco de ocorrer liberação de prostaglandina e sangue no interior do útero. O período ao qual se vai realizar a coleta de tecido intrauterino também é importante, sendo assim deve-se assegurar que a égua a ser submetida ao procedimento não tenha sido inseminada ou coberta poucos dias antes, pois o procedimento poderia comprometer a recuperação embrionária dessa égua ou uma possível prenhez (MCKINNON *et al.*, 2011).

Apesar da biópsia ser um procedimento pouco invasivo, o mesmo pode causar mudanças no ambiente uterino, pelo fato de ocorrer uma pequena hemorragia no local onde se retira a amostra de tecido, sendo essa a consequência mais comum (SERTICH, 1996).

2.4.2.3. Classificação da biópsia

Kenney (1978), primeiramente categorizou a biópsia endometrial equina em três graus (I, II e III) baseado principalmente na inflamação e fibrose. Uma classificação de grau I é considerada clinicamente insignificante e a de grau III significa que existe alto grau de mudanças patológicas. A categorização de Kenney (1978), apesar de depender de observações do parâmetro tipo, extensão e severidade da inflamação e fibrose, também leva em consideração o estádio do ciclo estral, achados microbiológico e endócrino realizados previamente para interpretação dos achados.

Kenney and Doig, (1986) publicaram um esquema de classificação que se tornou como padrão internacional (Tabela 1).

Tabela 1. Categorias de biópsia endometrial Kenney-Doig e o prognóstico para biópsia uterina em éguas.

Categoria	Achados	Taxa de parição esperada (%)
I	Normal, inflamação ou fibrose leve, escassamente espalhada	80 – 90
IIA	Suave inflamação dispersa, fibrose leve, atrofia do endométrio na estação reprodutiva	50 – 80
IIB	Moderada inflamação dispersa, fibrose moderada	10 – 50
III	Severas alterações irreversíveis, incluindo fibrose e inflamação	<10

É de suma importância na reprodução equina o resultado da biópsia endometrial para predizer a fertilidade de uma égua (SNIDER; SEPOY; HOLYOAK, 2011). Apesar da biópsia endometrial fornecer importantes informações, a mesma não traz todas como, crescimento do folículo e tônus uterino, que são avaliações feitas por palpação retal e ultrassonografia, presença e quantidade de fluido intrauterino, presença de cistos e suas dimensões, que podem ser vistos também por ultrassonografia, identificação exata de qual patógeno os quais são identificados por exame microbiológico, assim como informações de concentrações hormonais. Certamente que a biópsia endometrial fornece uma gama de informações muito úteis,

no entanto deve ser levado em consideração juntamente com outras ferramentas de diagnósticos.

2.4.3. Microbiologia

A endometrite bacteriana é um dos principais problemas em éguas cíclicas com baixa resistência a inflamações persistentes. As bactérias mais comumente isoladas do útero das éguas são: a *Streptococcus β-hemolítico* (*Streptococcus equi* ssp*. zooepidemicus*), *Escherichia coli, Pseudomonas aeruginosa e Kleybsiella pneumoniae*. Outras bactérias aeróbicas isoladas do trato reprodutivo de éguas incluem Streptococcus α-hemolitico, *Corynebacterium* spp., *Actinobacter* spp., *Proteus* spp*. e Citrobacter* spp*.* (MCKINNON *et al.*, 2011).

Buczkowska et al. (2014) ao coletarem amostrar intrauterinas com escova ginecológica e biópsia e em seguida passadas em meios de cultura, observaram que as bactérias encontradas foram *Streptococcus β-hemolítico, Streptococcus α-hemolítico, E. coli, Micrococcus, Staphylococcus epidermidis, Pseudomonas aeroginosa, Corynebacterium* spp*. e Staphylococcus aureus*. Os mesmos autores identificaram também culturas fúngicas como a de *Aspergillus* spp. e *Candida* spp.

Buczkowska et al. (2014) observaram que as bactérias mais frequentemente isoladas foram os *Bacillus*, a qual não é considerada patogênica. Dentre as bactérias patogênicas isoladas, as mais frequentes foram *Streptococcus β-hemolítico*. Os mesmos autores relataram alta frequência de fungos nos materiais coletados, sugerindo que a maioria das éguas que apresentaram crescimento fúngico tinham histórico de frequentes infusões de antibióticos intrauterinos anteriormente à estação reprodutiva. Além disso, observaram que o isolamento de *Streptococcus β-hemolítico* é sempre associado com citologia uterina positiva, assim como ocorreu para *Corynectarium* spp, no entanto não ocorreu para *E. coli.* Segundo Nielsen et al. (2010), quando isolado *Streptococcus β-hemolítico,* ocorrem mais comumente associação com citologia uterina positiva, enquanto o isolamento de *E. coli* e outras bactérias gram-negativas eram menos prováveis de serem associadas com citologia uterina positiva. LeBlanc et al. (2007), sugeriram que a patogenicidade da *E. coli* é diferente da patogenicidade da *Streptococcus β-hemolítico* e, consequentemente, a resposta inflamatória pode variar com diferentes microrganismos.

Buczkowska et al. (2014) observaram um total de 68,3% de éguas com histopatologia positiva para processo inflamatório, pela presença de PMNs no tecido endometrial. A sensibilidade da citologia uterina obtida por escova citológica é superior comparada com a sensibilidade do exame microbiológico obtido por swab. Sendo assim, Buczkowska et al. (2014) relataram resultados em que o exame citológico tem mais acurácia em comparação com o exame microbiológico para diagnóstico de inflamação do endométrio, sugerindo que o exame citológico seja mais indicado para diagnóstico de endometrite, do que o exame bacteriológico e que qualquer citologia positiva pode ser considerada como provável indicativo de endometrite. No entanto, a citologia uterina não promove informações sobre a causa da inflamação. Os mesmos autores relataram que quando associados os dois resultados, aumentam a sensibilidade do exame diagnóstico.

3. BIBLIOGRAFIA

ALGHAMDI, A S *et al.* Nitric Oxide Levels and Nitric Oxide Synthase Expression in Uterine Samples from Mares Susceptible and Resistant to Persistent Breeding-induced Endometritis. **American Journal of Reproductive Immunology** v. 53, n. 5 , 2005.

ALLEN, W R; ROWSON, L E A. Transfer of ova between horses and donkeys. 1972, Munich, Germany: [s.n.], 1972. p.484–487.

AURICH, C.; KÖNIG, N.; BUDIK, S. Effects of repeated embryo collection on embryo recovery rate in fertile mares. **Reproduction in Domestic Animals** v. 46, n. 3, p. 419–422 , 2011.

AURICH, Christine. Reproductive cycles of horses. **Animal Reproduction Science** v. 124, n. 3–4, p. 220–228 , 2011. Disponível em: <http://dx.doi.org/10.1016/j.anireprosci.2011.02.005>.1873-2232 (Electronic)n0378-4320 (Linking).

AURICH, J.; AURICH, C. Developments in European horse breeding and consequences for veterinarians in equine reproduction. **Reproduction in Domestic Animals** v. 41, n. 4, p. 275–279 , 2006.

BACKMAN, T. *et al.* Pregnancy rates of mares inseminated with semen cooled for 18 hours and then frozen. **Journal of Animal Science** v. 82, n. 3, p. 690–694 , 2004.

BALL, B a *et al.* Survival of day-4 embryos from young, normal mares and aged, subfertile mares after transfer to normal recipient mares. **Journal of reproduction and fertility** v. 85, n. 1, p. 187–194 , 1989.

BOTELHO, Jorge H V *et al.* Hormone supplementation protocol using estradiol benzoate and long-acting progesterone is efficient in maintaining pregnancy of anovulatory recipient mares during autumn transitional phase. **Animal Reproduction Science** v. 153, p. 39–43 , 2015. Disponível em: <http://dx.doi.org/10.1016/j.anireprosci.2014.12.014>.

BUCZKOWSKA, Justyna *et al.* Comparison of the biopsy and cytobrush techniques for diagnosis of subclinical endometritis in mares. **Reproductive biology and endocrinology : RB&E** v. 12, p. 27 , 2014. Disponível em: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3986439&tool=pmcentrez&rendertype=abstract>.

CAMARGO, C E *et al.* Aspectos relacionados com a recuperação embrionária em éguas da raça brasileiro de hipismo, utilizadas em programa comercial de transferência de embrião. **Veterinária e Zootecnia** v. 20, n. 4, p. 74–83 , 2013.
CARNEVALE, E. M. *et al.* Factors affecting pregnancy rates and early embryonic death after equine embryo transfer. **Theriogenology** v. 54, n. 6, p. 965–979 , 2000.
CARNEVALE, E M *et al.* Ultrasonographic characteristics of the preovulatory follicle preceding and during ovulation in mares. **Journal of Equine Veterinary Science** v. 8, n. 6, p. 428–431 , 1988. Disponível em: <http://www.sciencedirect.com/science/article/pii/S0737080688800923>.0737-0806.
CARTER, Rebecca A. *et al.* Apparent adiposity assessed by standardised scoring systems and morphometric measurements in horses and ponies. **The Veterinary Journal** v. 179, n. 2, p. 204–210 , 2009.
DIEL DE AMORIM, Mariana *et al.* Comparison of clinical signs, endometrial culture, endometrial cytology, uterine low volume lavage, and uterine biopsy, and combinations in the diagnosis of Equine Endometritis. **Journal of Equine Veterinary Science** v. l , 2015. Disponível em: <http://www.sciencedirect.com/science/article/pii/S0737080615006279>.
FERREIRA-DIAS, G. *et al.* Seasonal reproduction in the mare: Possible role of plasma leptin, body weight and immune status. **Domestic Animal Endocrinology** v. 29, n. 1, p. 203–213 , 2005.0739-7240 (Print)r0739-7240 (Linking).
FERREIRA, J. C. *et al.* Real-time characterization of the uterine blood flow in mares before and after artificial insemination. **Animal Reproduction Science** v. 160, p. 90–96 , 2015. Disponível em: <http://dx.doi.org/10.1016/j.anireprosci.2015.07.008>.
FLEURY, P D C; ALONSO, M A; BALIEIRO, J C C. Avaliação da receptora: efeito de características uterinas e tempo de ovulação. 2006, Araxá: Acta de Ciência Veterinária, 2006. p.502.
GENTRY, L. R.; THOMPSON, D. L.; GENTRY, G. T.; DAVIS, K. A.; GODKE, R. A.; *et al.* The relationship between body condition, leptin, and reproductive and hormonal characteristics of mares during the seasonal anovulatory period. **Journal of Animal Science** v. 80, n. 10, p. 2695–2703 , 2002.
GENTRY, L R; THOMPSON, D L; GENTRY, G T; DAVIS, K A; GODKE, R A. High versus low body condition in mares: Interactions with responses to somatotropin, GnRH analog, and dexamethasone. **Journal of Animal Science** v. 80, n. 12, p.

3277–3285 , 2002.
GINTHER, O. J.; UTT, Matthew D. Doppler ultrasound in equine reproduction: Principles, techniques, and potential. **Journal of Equine Veterinary Science** v. 24, n. 12, p. 516–526 , 2004.0737-0806.
GINTHER, O.J.; DOUGLAS, R.H.; LAWRENCE, Julie R. Twinning in mares: A survey of veterinarians and analyses of theriogenology records. **Theriogenology** v. 18, n. 3, p. 333–347 , set. 1982. Disponível em: <http://linkinghub.elsevier.com/retrieve/pii/0093691X82900115>. Acesso em: 9 ago. 2016.
GINTHER, O J. Ultrasonic imaging and animal reproduction: Color-Doppler ultrasonography. **Cross Plains: Equiservices**. [S.l: s.n.], 2007. p. 258.
HUFF, N.K. *et al.* Hyperleptinemia in Mares: Prevalence in Lactating Mares and Effect on Rebreeding Success. **Journal of Equine Veterinary Science** v. 28, n. 10, p. 579–586 , 2008.
HURTGEN, John P. Pathogenesis and treatment of endometritis in the mare: A review. **Theriogenology** v. 66, n. 3 SPEC. ISS., p. 560–566 , 2006.
JACOB, J. C F *et al.* Effect of embryo age and recipient asynchrony on pregnancy rates in a commercial equine embryo transfer program. **Theriogenology** v. 77, n. 6, p. 1159–1166 , 2012. Disponível em: <http://dx.doi.org/10.1016/j.theriogenology.2011.10.022>.1879-3231 (Electronic)n0093-691X (Linking).
KENNEY, R M. Cyclic and pathologic changes of the mare endometrium as detected by biopsy, with a note on early embryonic death. **Journal of the American Veterinary Medical Association** v. 3, p. 241–262 , 1978. Disponível em: <http://europepmc.org/abstract/med/621166>.
KENNEY, R M. The aetiology, diagnosis and classification of chronic degenerative endometritis. **Equine Veterinary Journal** v. 25, p. 186 , 1992.
KENNEY, R M; DOIG, P A. Equine endometrial biopsy, Current therapy in theriogenology 2. **Current therapy in theriogenology 2**. 3. ed. Philadelphia: Saunders, W B, 1986. p. 723–783.
KOBLISCHKE, P. *et al.* Embryo transfer induces a subclinical endometritis in recipient mares which can be prevented by treatment with non-steroid anti-inflammatory drugs. **Theriogenology** v. 70, n. 7, p. 1147–1158 , 2008.

LEBLANC, M M *et al.* Lymphatic clearance of India ink in reproductively normal mares and mares susceptible to endometritis. **Society for the Study of Reproduction** , 1995.

LEBLANC, M M *et al.* Scintigraphic measurement of uterine clearance in normal mares and mares with recurrent endometritis. **Equine Veterinary Journal** v. 26, n. 2, p. 109–113 , 1994.

LEBLANC, Michelle M.; MAGSIG, Joshua; STROMBERG, Arnold J. Use of a low-volume uterine flush for diagnosing endometritis in chronically infertile mares. **Theriogenology** v. 68, n. 3, p. 403–412 , 2007.0093-691X.

LEHRER, R I *et al.* Neutrophils and host defense. **Annals of Internal Medicine** v. 109, n. 2, p. 127–142 , 1988.

LOPES, E P *et al.* Correlação dos fatores que interferem na eficiência reprodutiva de éguas mangalarga marchador em programas de transferência de embriões. **Rev. Bras. Med. Vet** v. 35, n. 1, p. 69–75 , 2013.

MCKINNON, Angus O *et al.* **Equine Reproduction**. 2. ed. Ames: Blackwell Publishing Ltd, 2011. 3310 p. 1 v. MORTENSEN, C. J. *et al.* Embryo recovery from exercised mares. **Animal Reproduction Science** v. 110, n. 3–4, p. 237–244 , 2009.0378-4320.

NIELSEN, J. M. *et al.* Diagnosis of Endometritis in the Mare Based on Bacteriological and Cytological Examinations of the Endometrium: Comparison of Results Obtained by Swabs and Biopsies. **Journal of Equine Veterinary Science** v. 30, n. 1, p. 27–30 , 2010.

OGURI, N; TSUTSUMI, Y. Non-surgical egg transfer in mares. **Journal of reproduction and fertility** v. 41, n. 2, p. 313–320 , 1974.

PALM, F. *et al.* Influence of different semen extenders and seminal plasma on PMN migration and on expression of IL-1??, IL-6, TNF-?? and COX-2 mRNA in the equine endometrium. **Theriogenology** v. 70, n. 5, p. 843–851 , 2008.0093-691X (Print)r0093-691X (Linking).

PANZANI, Duccio *et al.* Retrospective study of factors affecting multiple ovulations, embryo recovery, quality, and diameter in a commercial equine embryo transfer program. **Theriogenology** v. 82, n. 6, p. 807–814 , 2014. Disponível em: <http://dx.doi.org/10.1016/j.theriogenology.2014.06.020>.

PASCOE, R R. Observations on the length and angle of declination of the vulva and

its relation to fertility in the mare. **Journal of reproduction and fertility** v. 27, p. 299–305 , 1978.
RAZ, T.; CARD, C. Efficiency of superovulation and in vivo embryo production in eFSH-treated donor mares after estrus synchronization with progesterone and estradiol-17β. **Theriogenology** v. 72, n. 2, p. 169–178 , 2009.
RAZ, Tal *et al.* Evaluation of two oestrus synchronization regimens in eFSH-treated donor mares. **The Veterinary Journal** v. 188, n. 1, p. 105–109 , 2011.
RIDDLE, W. T.; LEBLANC, M. M.; STROMBERG, A. J. Relationships between uterine culture, cytology and pregnancy rates in a Thoroughbred practice. **Theriogenology** v. 68, n. 3, p. 395–402 , 2007.
SERTICH, P L. Breeding soundness examination of the mare. 1996, [S.l: s.n.], 1996. p.22.
SHARP, D. C. Photoperiod. **Equine Reproduction**. 2. ed. [S.l.]: McKinnon, A, 2011a. p. 1771–1777.
SHARP, D.C. Melatonin. **Equine Reproduction**. 2. ed. [S.l.]: McKinnon, A, 2011b. p. 1669–1677.
SNIDER, T. A.; SEPOY, C.; HOLYOAK, G. R. Equine endometrial biopsy reviewed: Observation, interpretation, and application of histopathologic data. **Theriogenology** v. 75, n. 9, p. 1567–1581 , 2011. Disponível em: <http://dx.doi.org/10.1016/j.theriogenology.2010.12.013>.4057446265.
SQUIRES, E. L.; MCCUE, P. M.; VANDERWALL, D. The current status of equine embryo transfer. **Theriogenology** v. 51, n. 1, p. 91–104 , 1999.0093-691X (Print)r0093-691X (Linking).
SQUIRES, E.L. *et al.* Spontaneous multiple ovulation in the mare and its effect on the incidence of twin embryo collections. **Theriogenology** v. 28, n. 5, p. 609–613 , nov. 1987. Disponível em: <http://linkinghub.elsevier.com/retrieve/pii/0093691X87902779>. Acesso em: 9 ago. 2016.
STOUT, T A E. Equine embryo transfer: review of developing potential. **Equine veterinary journal** v. 38, n. 5, p. 467–478 , 2006. Disponível em: <http://doi.wiley.com/10.2746/042516406778400529>.14336863 (ISSN).
STROUD, B; CALLESEN, H. IETS statement on worldwide ET statistics for 2010. **Anim. Reprod** p. 210–216 , 2012. Disponível em:

<http://www.cbra.org.br/pages/publicacoes/animalreproduction/issues/download/v9n3/pag210-216(AR510).pdf>.

TRAUB-DARGATZ, J L; SALMAN, M D; VOSS, J L. Medical problems of adult horses, as ranked by equine practitioners. **Journal of the American Veterinary Medical Association** v. 198, n. 10, p. 1745–1747 , 1991.

TROEDSSON, M. H T. Breeding-Induced Endometritis in Mares. **Veterinary Clinics of North America - Equine Practice** v. 22, n. 3, p. 705–712 , 2006.1558-4224 (Electronic)0749-0739 (Linking).

TROEDSSON, M H T; SCOTT, M A; LIU, I K M. Comparative treatment of mares susceptible to chronic uterine infection. **American journal of veterinary research** v. 56, p. 468–472 , 1995.

WALTER, I *et al.* Association of endometrosis in horses with differentiation of periglandular myofibroblasts and changes of extracellular matrix proteins. **Reproduction** v. 121, p. 581–586 , 2001.

ZÚCCARI, Carmem Estefânia Serra Neto *et al.* Eficiência reprodutiva e dinâmica folicular de éguas campolina de acordo com a condição corporal. **Ciencia Animal Brasileira** v. 14, n. 4, p. 406–412 , 2013.

Chapter I - Evaluation of environmental effects on reproductive characteristics of Mangalarga Marchador mares in a commercial embryo transfer program

Abstract

The objective of this study was to evaluate the environmental effects on embryo recovery rate and pregnancy rate. The reproductive characteristics of donor and recipient mares were evaluated during five years in Brazilian tropical environmental conditions. The mares were used in reproduction throughout the year. The body condition of all donor mares were evaluated and all of them were classified as 4 (scale 1 to 5). Seasons were classified as: October to April (breeding season – BS); May (autumnal transition out of the breeding season - ATBS); June to August (non-breeding season – nBS); and September (vernal transition into the breeding season – VTBS). Temperature, rainfall and photoperiod were measured. The embryo recovery rate (ERR) and the pregnancy rate (PR) were observed and frequencies were calculated, along with evaluation of the effect of environmental factors and use of hormonal treatment (benzoate of estradiol and progesterone) on reproductive characteristics of recipient mares by Chi-square test and Kruskal-Wallis test. Effects were noted of the year, season and temperature on ERR ($p < 0.05$), but no effects were observed of the environmental parameters (year, season, hormone treatment, rainfall and photoperiod) on PR ($p > 0.05$). Good body condition of donor mares appeared to be the main determinant to avoid negative influence of environmental characteristics on ERR. In addition, hormone treatment also supported favorable results of PR in recipient mares during nBS. The conclusion is that mares of this breed can be used in reproduction all year long with successful offspring production in Brazil's tropical environment. The use of hormone treatment is efficient to maintain similar results during different observations.

Key-words: Equine, embryo transfer, reproduction.

1. Introduction

Researchers involved in horse reproduction have made great advances in the use of the embryo transfer (ET) technique in recent years. The number of ETs is about 27,500 a year globally, 12,400 in Brazil alone, according to the International Embryo Transfer Society (IETS) (Stroud and Callesen, 2012). The first report of horse embryo transfer was in 1974 by Oguri and Tsutsumi (1974). Since then, the interest of horse breeders in obtaining more than one foal a year per mare has been rising.

Mares of all ages can be submitted to reproductive biotechnologies, whether healthy or suffering from reproductive pathologies, including to overcome subfertility and environmental factors that make embryo production and pregnancy difficult.

Embryo transfer also promotes breed improvement of horses for sports and leisure, through the selective use of genetic material from stallions, as does the introduction of mares with superior genetics as embryo donors, making the offspring more valuable (Aurich and Aurich, 2006). Usually, only one embryo is recovered per mare in each estrous cycle. To obtain a maximum number of embryos from a donor mare with superior genetics, it is necessary perform multiple artificial inseminations and uterine flushes in a breeding season (Squires et al., 1999; Woodward et al., 2015). In Brazil, the Mangalarga Marchador breed is used in reproduction throughout the year, even in the winter, normally a period of seasonal anestrus or non-breeding season. In some regions, the donor mares do not go into seasonal anestrus, or artificial illumination is used to avoid this phase. However, there are different environmental factors that can influence reproductive management and production of offspring (McKinnon et al., 2011).

The aim of this study was to evaluate the effect of year, season, photoperiod, temperature and rainfall on reproductive characteristics (embryo recovery rate and pregnancy

rate), as well as to evaluate the effect of the use of hormonal treatment on pregnancy rate in a commercial embryo transfer program in northern Rio de Janeiro state, Brazil.

2. Material and methods

2.1. Animals and study design

This experiment was performed with the approval of the animal use ethics committee of North Fluminense State University (UENF), in accordance with the rules of the Brazilian Society of Laboratory Animal Science/Brazilian College of Animal Experimentation (SBCAL/COBEA), under protocol number 307.

Data of the commercial embryo transfer center were collected and evaluated during 2012 to 2016, from January to December, in northern Rio de Janeiro (latitude: − 21º45'15", longitude: −41º19'28", altitude: 13 m). The predominant climate in this region is tropical, with hot and humid summers and cooler and dryer winters.

The reproductive seasons (RS) were defined according to the practice in the region where the study was conducted. October to April is considered the breading season (BS), May is considered the autumnal transition out of the breeding season (ATBS), June to August is the anestrus or non-breeding season (nBS), and September is the vernal transition into the breeding season (VTBS).

In this study 2,595 records were evaluated from adult donor mares (n = 195) and recipient mares (n = 581). The donor mares were kept in an open shelter and outdoor paddock with access to pasture, fed with hay and 2.0 kg of pellet feed (Equitech, Brazil) containing 12% crude protein and 2,700 kcal of digestible energy, twice/day, with amino acid supplementation of 25 ml/day (Glicopan Energy®, Vetnil, São Paulo, Brazil). Trace-mineralized salt with water were available *ad libitum*. The recipient mares were kept in an outdoor paddock with access to pasture and trace-mineralized salt with water *ad libitum.* The body condition of all donor mares

were evaluated monthly and all of them were classified as 4 (scale 1 to 5) in all observations, then they were in good body condition during all years of evaluation.

Data of temperature, rainfall and photoperiod were collected during five years and were used to calculate the means (monthly for temperature and rainfall and daily for photoperiod).

The rainfall data were separated in classes, whereby 6 to 100 mm^3 was considered "R1" and 101 mm^3 to 200 mm^3 was considered "R2". The same was done with temperature, where 21 to 24^o C was considered "T1" and 24.1 to 28^o C was considered "T2". Finally, the photoperiod was separated in two classes, one with 10:39 to 11:39 hours of daylight, considered "P1" and the other with 11:40 to 13:18 hours of daylight, considered "P2".

The reproductive tract (uterus, and ovaries) was examined by transrectal ultrasonography (Mindray® DP-2200 system, Shenzhen, China) with a 5 MHz linear transducer to determine the estrous cycle stage, presence and measurement of follicles, uterine edema and intrauterine fluid. The evaluations were performed every day from the moment when a follicle 30 mm in diameter was detected. When a follicle equal or greater than 35 mm diameter was detected, 1.0 mg of deslorelin acetate (BioRelease®, BET Laboratories, Rio de Janeiro, Brazil) was administered to induce ovulation.

The donor mares were matched or inseminated 24 h after Deslorelin administration and with 2-day intervals until ovulation detection. The stallions' semen was diluted in commercial semen extender (BotuSemen®, Botupharma, Botucatu, Brazil) to allow insemination of more than one mare per day.

The embryo collections were performed 6, 7, 8, 9 and 10 days after detection of mares' ovulation (D6, D7, D8, D9 and D10). The uterus was flushed with Ringer's lactate solution (1 to 3 liters) at environmental temperature. After uterine flush, 7.5 mg of prostaglandin-F2α analogue was administered intramuscularly (Lutalyse®, Pfizer, São Paulo, Brazil) to induce faster return to estrus. The recovered fluid was carefully evaluated using a stereomicroscope

(Olympus, Tokyo, Japan) to find embryos, and embryo recovery was classified as negative (0), positive with one embryo (1) or positive with two embryos (2). The embryos were manipulated in holding medium (BotuEmbryo®, Botupharma, São Paulo, Brazil), and all embryos graded as 1 and 2 (excellent and good quality) were transferred to recipient mares. Twelve days after embryo transfer, pregnancy was diagnosed as positive or negative.

We evaluated two reproductive traits (RT) that have a strong influence on success of commercial embryo transfer programs: embryo recovery rate (ERR) and pregnancy rate (PR), along with three environmental factors that can affect these reproductive traits: temperature, rainfall and photoperiod.

2.2. Experiment 1

Environmental effects on reproductive traits of both donor and recipient mares.

The effects of year of observation, reproductive season, temperature and photoperiod on embryo recovery rate (ERR) and pregnancy rate (PR) were evaluated.

2.3. Experiment 2

The effect of the day of uterine flush after ovulation (D6, D7, D8, D9 and D10) on ERR and PR was evaluated, as well as the effect of using recipient mares when they were naturally cycling, during the vernal transition into the breeding season, during the breeding season (progesterone secreted by corpus luteum), during autumnal transition out of the breeding season or during the non-breeding season, using a hormonal protocol.

The hormonal protocol used on recipient mares was: administration of 3 mg of estradiol benzoate (Estrogin®, Farmavet, São Paulo, Brazil), and 3 days later, if the mares presented uterine edema grade 3 by ultrasound evaluation, 5 mL (300 mg/mL) of progesterone (P4,

Farmavet, São Paulo, Brazil), in both cases injected intramuscularly (-2 days to +7 days of donor mare ovulation).

We also evaluated the effect of the day of embryo transfer on PR of recipient mares after ovulation or hormonal treatment, from day 2 to day 9.

2.4. Statistical analysis

Descriptive statistics were calculated from all data collected. The environmental effect (temperature, rainfall and photoperiod) and the reproductive traits (ERR and PR) were arranged in contingency tables and analyzed by the Chi-square and Kruskal-Wallis tests, at 5% probability (SAS, 2009).

3. Results

From 1,446 uterine flushes performed during evaluation of the embryo donor mares for five years, a 68% embryo recovery rate (ERR) was observed. The percentage of double embryo recovery was 3.3%. There was a significant effect of year on this characteristic ($p < 0.05$). The years with highest embryo recovery rates were 2012 and 2015 and the lowest was 2014. The pregnancy rate of the recipient mares was of 66.7% overall and was not affected by evaluation year ($p > 0.05$) (Table 1).

Table 1. Kruskal-Wallis test of embryo recovery rate (ERR) and pregnancy rate (PR) according to year of observation.

ERR (%)	year					
	2012	2013	2014	2015	2016	p
N	(323)	(341)	(389)	(129)	(264)	< 0.05
0	28.2	36.1	42.9	30.2	31.44	
1	69.0	60.1	54.5	66.7	63.6	
2	2.8	3.8	2.6	3.1	4.9	
PR (%)	year					
	2012	2013	2014	2015	2016	p
N	(199)	(152)	(248)	(68)	(158)	> 0.05
negative	31.7	41.5	32.3	41.2	25.9	
positive	68.3	58.6	67.7	58.2	74.1	

Number of observation (N), no embryos recovered (0), one embryo recovered (1), two embryos recovered (2). p = 5% probability.

Regarding the climate factors, the environmental temperature was different between seasons ($p < 0.05$), with higher temperature during the breeding season and lower temperature during the non-breeding season. Rainfall did not differ between the seasons ($p > 0.05$) (Table 2).

Table 2. Means and standard deviations of environmental effect (EE) with reproduction season (RS).

EE	RS				
	BS	ATBS	nBS	VTBS	p
Mean Temp	26.3 ± 1.4^{a}	23.2 ± 0.6^{b}	22.3 ± 0.6^{b}	23.5 ± 0.5^{b}	< 0.001
Min Temp	22.7 ± 1.2^{a}	19.4 ± 0.5^{b}	18.3 ± 0.8^{b}	19.5 ± 0.8^{b}	< 0.001
Max Temp	31.8 ± 1.9^{a}	28.3 ± 0.8^{b}	27.9 ± 0.9^{b}	28.8 ± 0.6^{b}	< 0.001
Rainfall (mm^3)	85.7 ± 66.1^{a}	78.9 ± 64.0^{a}	52.1 ± 43.9^{a}	40.2 ± 30.3^{a}	> 0.05
Humidity (%)	73.8 ± 3.5^{a}	77.2 ± 2.7^{a}	76.6 ± 2.9^{a}	72.1 ± 3.9^{a}	> 0.05

Mean temperature (Mean Temp), minimum temperature (Min Temp), maximum temperature (Max Temp), rainfall and humidity. Breeding season (BS), autumnal transition out of breeding season (ATBS), non-breeding season (nBS) and vernal transition into the breeding season (VTBS). p = 5% probability.

Table 3 shows the frequencies of the embryo recovery rate and pregnancy rate in different seasons. There was a significant effect of season on ERR ($p < 0.05$). The highest ERR and double embryo recovery rates were observed in the non-breeding season and the lowest during the breeding season. However, the PR was not affected by the reproductive season.

Table 3. Chi-square test of embryo recovery rate (ERR) and pregnancy rate (PR) according to reproduction season (RS).

ERR	RS				
	BS	ATBS	nBS	VTBS	p
N	(694)	(137)	(482)	(166)	< 0.05
0	39.8	38.7	27.8	35.5	
1	57.5	58.4	67.8	61.4	
2	2.7	2.9	4.4	3.0	
PR	RS				
	BS	ATBS	nBS	VTBS	p
N	(329)	(81)	(332)	(93)	> 0.05
Negative	31.9	35.8	33.1	36.6	
Positive	68.1	64.2	66.9	63.4	

Number of observation (N), no embryo recovery (0), one embryo recovery (1), two embryos recovery (2), breeding season (BS), autumnal transition out of the breeding season (ATBS), non-breeding season (nBS), vernal transition into the breeding season (VTBS). p = 5% probability.

Table 4 shows the embryo recovery rate and pregnancy rate in relation to the number of days from uterine flush to embryo recovery (DFER). There was a significant effect of DFER on ERR ($p < 0.05$). The highest embryo recovery rate was registered when the uterus was flushed on days 6, 7 and 8 after mare ovulation and the lowest ERR was observed on day 9 and mainly day 10 after ovulation. The rate of double embryo recovery was highest on day 7 and lowest on days 6 and 10. On the other hand, PR was not affected by DFER ($p > 0.05$).

Table 4. Kruskal-Wallis test of embryo recovery rate (ERR) and pregnancy rate (PR) according to days between ovulation and embryo recovery (DFER).

ERR (%)	DFER					
	D6	D7	D8	D9	D10	p
N	(9)	(187)	(641)	(494)	(62)	< 0.05
0	11.1	31.5	32.1	37.7	41.9	
1	88.9	63.1	64.7	59.3	56.5	
2	0	5.4	3.1	3.0	1.6	
PR (%)	DFER					
	D6	D7	D8	D9	D10	p
N	(33)	(174)	(359)	(170)	(15)	> 0.05
Negative	42.4	38.5	33.2	34.1	33.3	
Positive	57.6	61.5	66.9	65.9	66.7	

Number of observations (N), no embryos recovered (0), one embryo recovered (1), two embryos recovered (2), days after ovulation (D6, D7, D8, D9 and D10). p = 5% probability.

The use of the hormonal protocol with progesterone (P4) on the recipient mares and the use of recipient mares that were naturally cycling (ovulation) did not affect the pregnancy rate ($p > 0.05$) (Table 5).

Table 5. Chi-square test of pregnancy rate (PR) according to natural recipient cycle (Ovulation) or hormonal treatment (Progesterone).

PR (%)	Hormonal Treatment		
	Ovulation	Progesterone	p
N	(240)	(455)	> 0.05
Negative	34.6	33.0	
Positive	65.4	67.0	

p = 5% probability.

Table 6. shows the frequency of negative and positive pregnancy of recipient mares when they were used on different days after ovulation (DA) or after hormonal treatment with progesterone. There was no effect of different days on pregnancy rate ($p > 0.05$).

Table 6. Kruskal-Wallis test of pregnancy rate (PR) according to days after hormonal treatment or ovulation (DA).

PR (%)	DA								
	D2	D3	D4	D5	D6	D7	D8	D9	p
N	(36)	(91)	(125)	(110)	(90)	(118)	(55)	(13)	> 0.05
Negative	36.1	35.2	32.0	36.4	34.4	33.9	40.0	30.8	
Positive	63.9	64.8	68.0	63.6	65.6	66.1	60.0	69.2	

p = 5% probability.

Table 7 and 8 report the frequency of ERR and PR in relation to rainfall class and temperature class. Rainfall class did not affect the embryo recovery rate or pregnancy rate ($p > 0.05$). The temperature class also did not affect the pregnancy rate ($p > 0.05$), but did affect the ERR ($p < 0.05$): the highest ERR occurred when the temperature was lower.

Table 7. Chi-square test of embryo recovery rate (ERR) according to mean rainfall and mean temperature.

Rainfall	ERR			
	0	1	2	p
N	(437)	(814)	(42)	> 0.05
R1	33.4	63.4	3.2	
R2	35.3	61.2	3.5	
Mean temperature	ERR			
	0	1	2	p
N	(491)	(870)	(47)	< 0.05
T1	32.8	63.5	3.8	
T2	39.0	58.5	2.5	

Number of observations (N), rainfall ranging from 0 to 100 mm^3 (R1) and from 101 to 235 mm^3 (R2); mean temperature ranging from 21^o C to 24 ^{o}C (T1) and from 25 to 28 ^{o}C (T2). p = 5% probability.

Table 8. Chi-square test of pregnancy rate (PR) according to mean rainfall and mean temperature.

Rainfall	PR (%)		
	Negative	Positive	p
N	(241)	(490)	> 0.05
R1	32.8	67.2	
R2	33.5	66.5	
Temperature	PR (%)		
	Negative	Positive	p
N	(268)	(531)	> 0.05
T1	33.4	66.6	
T2	33.9	66.1	

Number of observations (N), rainfall ranging from 0 to 100 mm^3 (R1) and from 101 to 235 mm^3 (R2); mean temperature ranging from 21 ºC to 24 ºC (T1) and from 25 ºC to 28 ºC (T2). p = 5% probability.

The embryo recovery rate was affected by photoperiod class ($p < 0.05$), with slightly higher embryo recovery rate in P1 than in P2. However, the photoperiod class did not affect the pregnancy rate ($p > 0.05$) (Table 9).

Table 9. Chi-square test of embryo recovery rate (ERR) and pregnancy rate (PR) according to photoperiod.

	Photoperiod			
ERR	N	P1	P2	p
0	(515)	31.9	38.1	< 0.05
1	(901)	64.5	58.9	
2	(48)	3.6	2.9	
	Photoperiod			
PR	N	P1	P2	p
Negative	(273)	34.5	31.6	> 0.05
Positive	(549)	65.5	68.3	

Number of observations (N), no embryos recovered (0), one embryo recovered (1), two embryos recovered (2); day length from 10h to 11h:40m (F1) and from 11h:41m to 13h:18m (F2). P = 5% probability.

4. Discussion

The embryo recovery rate observed (68%) was similar to that reported by other researchers (Aurich et al., 2011; Lopes et al., 2013). However, in this study we evaluated some environmental effects during five consecutive years, finding a significant effect of year on embryo recovery (Table 1). This probably occurred because the effect of year of evaluation is due to climate factors, mainly rainfall, temperature and air humidity, on the animals and pastures. However, during the years these variables did not vary significantly ($p > 0.05$), but variations were observed when these environmental effects were evaluated by reproductive

season. Other factors could also cause the ERR difference. Genetic differences of the donor mares is one of the them, and in this study the donor mares were not the same as the previous year most of time. Therefore, the improvement of the mares' reproductive characteristics should a subject for further investigation in order to improve these traits, as done for other species (Ortega et al., 2016; Terakado et al., 2015). Modifications of the installations and the management in general can also be important factors that influence reproductive characteristics. All these facts make the interaction of year and reproductive traits more complex (Pereira, 2012).

Regarding the environmental conditions, the temperature was relatively high during all years, with no significant variations ($p > 0.05$) (Table 2), with minimum average yearly temperature 21.1 ^{o}C and maximum of 30.3 ^{o}C. However, when temperature was evaluated in different reproduction seasons, there was variation, with higher mean in BS and lower in nBS. Therefore, the climate where the experiment was conducted is warm throughout the year and the day length does not change substantially, there was significance variation in the photoperiod by season, where the highest mean day length was in BS compared to the others.

Considering the effect of the season on the mares' reproductive characteristics (Table 3), the best ERR results occurred during the non-breeding season, even without use of artificial light treatment to suppress melatonin (Walsh et al., 2013), as happens during the summer. This result is different those found in countries or regions farther from the Equator (Williams et al., 2012). This fact can be explained because the difference of day length during the year in all seasons (breeding season, autumnal transition out of the breeding season, non-breeding season and vernal transition into the breeding season) were not sufficient to cause influence the endogenous circannual rhythm (Williams et al., 2012), and suppress the gonadotropins that cause mares to enter anestrus (McKinnon et al., 2011). The minimum day length (in nBS) was 10h:43m, which is sufficient for mare cycling (Mariz et al., 2008). Another important aspect is that the mares

were supplemented throughout year with hay and concentrated feed. In the last three weeks of the BS (summer) and ATBS, the mares were supplemented with concentrate with high percentage of energy and protein. Therefore, the mares did not enter into the transition phase without nutritional support and they did not suffer a possible decrease in the quantity and quality of forage. This is indicated because the nBS presented lower mean (52.1 ± 43.9 mm^3) rainfall compared with BS (85.7 ± 66.1 mm^3). So, the donor mares had good body condition during the entire experiment. According to Zúccari et al. (2013), among the different variables that can influence commercial ET programs, the body condition is the most relevant, because it is directly related to embryo recovery rate and pregnancy rate. Inadequate nutrition and poor body condition can be associated with delay in cyclicity during the transition phase to BS, causing high embryo loss and low pregnancy rate (Carter et al., 2009; Huff et al., 2008). In the present study, a small number of the mares submitted to insufficient nutrition stopped cycling and entered seasonal anestrus, and it was not possible to reverse this situation and cause them to return to the cycle the next season (nBS).

On the other hand, all mares that received adequate nutrition supplementation continued to cycle all year long.

The day of uterine flush for embryo recovery is a very important aspect of ET in horses because it can vary from D6 to D10 after ovulation (Table 4). In this study, the highest embryo recovery rate was day 6, different from other studies (Camargo et al., 2013; Panzani et al., 2014), even with the small size of the embryo in the development stage on this day (Squires and McCue, 2016). This might have occurred because the 6-day-old embryos had just passed through the oviduct and arrived in the uterus, where they could be collected before suffering from probable uterine disease and early death (Overbeck et al., 2011). In this respect, 51.3% of the mares had some uterine disease, according to previous history. The embryo recovery on days 7 and 8 after ovulation had similar percentage and embryo recovery on days 9 and 10 had

lower recovery rates. This suggests that embryo collection should be done mainly on day 7 or 8, because even though the embryos on day 6 had the highest recovery rate, they were very small, which can complicate the search and manipulation. The advantage of embryo collection on D6 is that in this development stage, the embryo enters the uterus from the oviduct with morphology of the morula stage or early blastocyst stage, which enables it to survive the freezing and thawing of cryopreservation (Squires and McCue, 2016). Furthermore, the embryos on day 10, besides having the lowest recovery rate, were larger, making it harder to manipulate them, leading to damage to embryo capsule and death (McKinnon et al., 2011). In some cases, the embryos recovered on day 10 were damaged, with severe capsule lesions, which possibly occurred during uterine flushes. Therefore, while embryo collection on D10 is possible, the chances of damage and loss are considerably higher.

The pregnancy rate (PR) of the recipient mares was not affected by the year of observation, season or day of embryo collection, unlike observed for the ERR trait (Table 1). Despite the environmental changes, which affected ERR, PR remained constant during the experiment, possibly because of the hormone treatment of the recipient mares to prepare the uterus for embryo implantation and maintenance of gestation until progesterone was produced and secreted in sufficient concentration by the placenta (Ousey et al., 2005). In this study, recipient mares were used during all seasons, but these mares were not supplemented with hay and concentrate like the donor mares were. However, during the anestrus and transition phases (nBS, ATBS and VTBS), the recipient mares were synchronized with donor mares artificially with the hormonal protocol. There were no differences on the pregnancy rate when the recipient mares were cycling (BS) and when they were in anestrus but treated with hormones (Table 5). This allowed good results of the TE program. This result was similar to that reported by Kaercher et al. (2013). They reported that the administration of exogenous progesterone can

provide the recipient mares sufficient levels of serum P4 to receive an embryo and maintain gestation.

The embryos were transferred to the recipient mares from day 2 until day 9 after ovulation or after hormonal treatment (progesterone) (Table 6). Even with considerable time differences, they presented similar pregnancy rates. This indicates that the use of recipient mares could be optimized by making them available for use in embryo transfer for a longer period (from 2 to 9 days after hormone treatment) for each cycle of a donor. This would require fewer recipient mares per donor and reduce the cost. Therefore, the use of hormonal treatment with progesterone during seasonal anestrus of mares or during the transitional phase is an alternative to diminish the number of recipient mares per donor due to the longer window of availability (from D -1 until D +6 regarding day of donor's mare ovulation), reducing the costs of artificial light and supplementation with concentrate and hay.

The rainfall in the region where the experiment was performed is low throughout the year long and did not change during the years of observation. Thus, there were no differences in the quality and quantity of forage in the pasture. This can explain the fact there were no effects of rainfall on ERR and PR (Tables 7 and 8).

Regarding the temperature measured during experiment, the climate is considered warm year-round, with mean maximum temperature of 30.3^{o} C and mean minimum temperature of 21.1^{o} C. This climate favors ERR and PR during all seasons, as suggested by Mariz et al. (2008). However, high temperatures cause thermal stress, when the rectal temperature rises more than 2 ^{o}C, and consequently decreases ERR (Mortensen et al., 2009). Thermal stress caused by high temperatures (Rua et al., 2013) can promote secretion of cortisol (Juliane et al., 2016), a hormone that acts directly to suppress GnRH and consequently suppresses gonadotropin release (Campbell, 2014). Higher temperatures influenced the ERR slightly. In this respect, we observed lower ERR when temperatures ranged from 25 to 28 ^{o}C. However, temperature did

not affect the pregnancy rate, suggesting that the temperature oscillations were not sufficient to affect this characteristic, However, temperature did not affect the pregnancy rate, suggesting that the temperature oscillations were not sufficient to affect this characteristic. The fact that this rate is less affected by climate means embryo implantation and maintenance of gestation can be successfully controlled by the hormonal protocol. This fact was expected because recipient mares that were cycling (during BS) and treated with hormone (in anestrus, during nBS) presented similar pregnancy rates. This suggests that hormonal treatment of the recipient mares during anestrus (nBS) was sufficient to achieve a pregnancy rate similar to that of the cycling mares during BS.

Another factor that influenced mares' hormonal parameters and cyclicity is the photoperiod (Table 9). Mares are classified as seasonal polyestric, with suspension of reproductive activity usually in autumn (McKinnon et al., 2011). Contrasting with this fact, we observed that the mares continued cycling in winter (nBS) and presented higher ERR in this season compared to summer (BS). We can suggest that the season when the temperature was most favorable (in addition to the influence of temperature on ERR), was nBS, to these two environmental characteristics affected the same reproductive characteristic. Mariz et al. (2008) reported that day length of more than 7.7 hours can cause mares to have high estrus rate. In this study, the minimum day length observed was 10h:43m, which was sufficient luminosity to keep donor mares cycling and producing embryos all year long. The good body condition might have been one of the most important factors contributing to this. As expected, the length of the day did not affect the pregnancy rate of the recipient mares. The recipient mares were not fed with any supplement in any phase of the experiment. When they were in the autumnal transition out of the breeding season, most of them entered the seasonal anestrus phase, but all of them used in ET received hormonal treatment. The use of the hormonal protocol presented favorable results, allowing the recipient mares to have similar pregnancy rates in BS and nBS.

Conclusion

Good feed supplementation and body condition are sufficient to keep mares cycling and producing embryos in the specific temperature and photoperiod conditions of the Brazilian tropical environment. In addition, hormonal treatment to synchronize cycles and maintain gestation provides the same results as naturally cycling recipient mares in commercial embryo transfer programs.

Bibliography

Aurich, C., König, N., Budik, S., 2011. Effects of repeated embryo collection on embryo recovery rate in fertile mares. Reprod. Domest. Anim. 46, 419–422. doi:10.1111/j.1439-0531.2010.01682.x

Aurich, J., Aurich, C., 2006. Developments in European horse breeding and consequences for veterinarians in equine reproduction. Reprod. Domest. Anim. 41, 275–279. doi:10.1111/j.1439-0531.2006.00719.x

Camargo, C.E., Weiss, R.R., Kozicki, L.E., Duarte, M.P., Duarte, M.C.G., Bertol, M.A.F., Gaievski, F.R., Bastos, G. de M., 2013. Some relevant factors regarding embryonic recovery rates in a program of embryo transfer in Brazilian jumping horse mares/Aspectos relacionados com a recuperacao embrionaria em eguas da raca Brasileiro de hipismo, utilizadas em programa comercial de trans. Vet. e Zootec. 20, 74–84.

Campbell, M.L.H., 2014. Embryo transfer in competition horses: Managing mares and expectations. Equine Vet. Educ. 26, 322–327. doi:10.1111/eve.12182

Carter, R.A., Geor, R.J., Burton Staniar, W., Cubitt, T.A., Harris, P.A., 2009. Apparent adiposity assessed by standardised scoring systems and morphometric measurements in horses and ponies. Vet. J. 179, 204–210. doi:10.1016/j.tvjl.2008.02.029

Huff, N.K., Thompson, D.L., Gentry, L.R., Depew, C.G., 2008. Hyperleptinemia in Mares: Prevalence in Lactating Mares and Effect on Rebreeding Success. J. Equine Vet. Sci. 28, 579–586. doi:10.1016/j.jevs.2008.08.008

Juliane, K., Christina, N., Natascha, I., Jörg, E.A., Christine, A., 2016. The PGF2α agonists luprostiol and d-cloprostenol reliably induce luteolysis in luteal phase mares without evoking clinical side effects or a stress response. Anim. Reprod. Sci. 168, 92–99. doi:10.1016/J.ANIREPROSCI.2016.02.031

Kaercher, F., Kozicki, L.E., Camargo, C.E., Weiss, R.R., dos Santos, I.W., Muradas, P.R., Bertol, M.A.F., de Abreu, R.A., 2013. Embryo Transfer in Anovulatory Recipient Mares Treated with Estradiol Benzoate and Long-Acting Progesterone. J. Equine Vet. Sci. 33, 205–209. doi:10.1016/j.jevs.2012.06.009

Lopes, E.P., Pinho, R.., Siqueira, J.., Rocha, A.., Pereira, J.V.T., Martins, L.., de Freitas, B.., Guimarães, J.D., 2013. Correlação dos fatores que interferem na eficiência reprodutiva de éguas mangalarga marchador em programas de transferência de embriões. Rev. Bras. Med. Vet 35, 69–75.

Mariz, T.M. de A., Anjos, A.G. dos, Flor, J.M., Flor, L.M. de A.M., Lima, C.B., Givisiez, P.E.N., Azevedo, P.S. de, 2008. Influências Do Clima Sobre a Atividade Reprodutiva De Éguas Da Raça Mangalarga Marchador No Estado De Sergipe. Acta Vet. Bras. 2, 39–43.

McKinnon, A.O., Squires, E.L., Vaala, W.E., Varner, D.D., 2011. Equine Reproduction, 2nd ed. Blackwell Publishing Ltd, Ames.

Mortensen, C.J., Choi, Y.H., Hinrichs, K., Ing, N.H., Kraemer, D.C., Vogelsang, S.G., Vogelsang, M.M., 2009. Embryo recovery from exercised mares. Anim. Reprod. Sci.

110, 237–244. doi:10.1016/j.anireprosci.2008.01.015

Oguri, N., Tsutsumi, Y., 1974. Non-surgical egg transfer in mares. J. Reprod. Fertil. 41, 313–320. doi:10.1530/jrf.0.0410313

Ortega, M.S., Denicol, A.C., Cole, J.B., Null, D.J., Hansen, P.J., 2016. Use of single nucleotide polymorphisms in candidate genes associated with daughter pregnancy rate for prediction of genetic merit for reproduction in Holstein cows. Anim. Genet. 47, 288–297. doi:10.1111/age.12420

Ousey, J.C., Houghton, E., Grainger, L., Rossdale, P.D., Fowden, A.L., 2005. Progestagen profiles during the last trimester of gestation in Thoroughbred mares with normal or compromised pregnancies. Theriogenology 63, 1844–1856. doi:10.1016/j.theriogenology.2004.08.010

Overbeck, W., Witte, T.S., Heuwieser, W., 2011. Comparison of three diagnostic methods to identify subclinical endometritis in mares. Theriogenology 75, 1311–1318. doi:10.1016/j.theriogenology.2010.12.002

Panzani, D., Rota, A., Marmorini, P., Vannozzi, I., Camillo, F., 2014. Retrospective study of factors affecting multiple ovulations, embryo recovery, quality, and diameter in a commercial equine embryo transfer program. Theriogenology 82, 807–814. doi:10.1016/j.theriogenology.2014.06.020

Pereira, J.C.C., 2012. Melhoramento Genético aplicado à produção animal, 6th ed. Belo Horizonte.

Rua, M.A.S., Quirino, C.R., Pacheco, A., Vega, W.H.O., Ribeiro, M.S., Matos, L.F., 2013. Caracterização fisiológica e seminal de pôneis do Norte do Estado do Rio de Janeiro-Brasil. Actas Iberoam. Conserv. Anim. 3, 51–58.

SAS, 2009. Statistical analyses system.

Squires, E.L., McCue, P.M., 2016. Cryopreservation of Equine Embryos. J. Equine Vet. Sci.

41, 7–12. doi:10.1016/j.jevs.2016.03.009

Squires, E.L., McCue, P.M., Vanderwall, D., 1999. The current status of equine embryo transfer. Theriogenology 51, 91–104. doi:10.1016/S0093-691X(98)00234-9

Stroud, B., Callesen, H., 2012. IETS statement on worldwide ET statistics for 2010. Anim. Reprod 210–216.

Terakado, A.P.N., Boligon, A.A., Baldi, F., Silva, J.A.I. V., Albuquerque, L.G., 2015. Genetic associations between scrotal circumference and female reproductive traits in Nelore cattle. J. Anim. Sci. 93, 2706. doi:10.2527/jas.2014-8817

Walsh, C.M., Prendergast, R.L., Sheridan, J.T., Murphy, B.A., 2013. Blue light from light-emitting diodes directed at a single eye elicits a dose-dependent suppression of melatonin in horses. Vet. J. 196, 231–235. doi:10.1016/j.tvjl.2012.09.003

Williams, G.L., Thorson, J.F., Prezotto, L.D., Velez, I.C., Cardoso, R.C., Amstalden, M., 2012. Reproductive seasonality in the mare: Neuroendocrine basis and pharmacologic control. Domest. Anim. Endocrinol. 43, 103–115. doi:10.1016/j.domaniend.2012.04.001

Woodward, E.M., Christoffersen, M., Horohov, D., Squires, E.L., Troedsson, M.H.T., 2015. The effect of treatment with immune modulators on endometrial cytokine expression in mares susceptible to persistent breeding-induced endometritis. Equine Vet. J. 47, 235–239. doi:10.1111/evj.12266

Zúccari, C.E.S.N., Bender, E.S.C., Silva, E.V.C.E., Saturnino, H.M., 2013. Eficiência reprodutiva e dinâmica folicular de éguas campolina de acordo com a condição corporal. Cienc. Anim. Bras. 14, 406–412. doi:10.5216/cab.v14i4.17693

Chapter II. **Environmental effects and repeatability of the follicular diameter in mares**

Abstract

The aim of this study was to evaluate the effect of environment on follicular diameter and the repeatability of the size of preovulatory follicles of mares. The follicular dynamics of Mangalarga Marchador mares were evaluated year-round for five years in Brazil. The mares were used in reproduction throughout the year. Reproduction seasons (RS) were classified as breeding season (BS); autumnal transition out of the breeding season (ATBS); non-breeding season (nBS); and vernal transition into the breeding season (VTBS). The temperature, rainfall and photoperiod were measured and their effects were evaluated on follicular dynamics. The data were studied by analysis of variance of follicular size and environment traits. During the five years, 7% double ovulations and 159 anovulatory follicles were recorded. The mean preovulatory follicle diameters of left and right ovaries were 39.3 ± 3.8 and 39.2 ± 3.5 mm respectively. There was no effect of evaluation year on follicular diameter ($p > 0.05$). The RS affected the right preovulatory follicle diameter with higher mean in ATBS. There was no effect of temperature and rainfall on follicular diameter ($p > 0.05$). Repeatability values of the preovulatory follicular diameter were low ($t = 0.07$). In the subtropical climate studied, the temperature and rainfall caused no effect on follicular dynamics and photoperiod had only a small effect on dominant preovulatory follicular diameter. The repeatability of the follicular

diameter was low, so this characteristic should be evaluated daily to predict ovulation and the optimal time to inseminate the mare.

Key words: Horse, female, reproduction.

1. Introduction

The widespread use of reproductive biotechnologies for horse breeding is a reality and is increasing worldwide with the need to produce animals with superior genetics to compete in the market [1]. With this need for breeding improvement, the use of semen of stallions with superior genetic traits is of great importance, and knowledge of the mare's reproductive characteristics is necessary to obtain good embryo recovery during a given breeding season.

The follicular dynamics and preovulatory diameter are essential characteristics to accurately predict the ovulation moment. This can be observed, but it takes lengthy evaluation time during the reproduction management in horse embryo transfer centers, in most cases making it unfeasible [2].

Therefore, the use of a standard to classify a follicle as preovulatory is an alternative to predict the most favorable moment to inseminate embryo donor mares. However, there are individual differences and effects of environment on reproductive characteristics, such as age of mare, year of observation, season and climate. So, evaluation of repeatability would be a useful tool to estimate if this characteristic tends to repeat over observations. Repeatability is genetic parameter that can estimate the genotypic value of a trait that can be spread to other generations [3]. Therefore, this type of evaluation could help reproduction management of mares and improve breeding regarding the selection of donor mares [4].

The aim of this study was to evaluate the preovulatory follicular diameter, the daily growth of follicular diameter, the environmental factors that can influence the preovulatory follicular diameter and to estimate the repeatability of preovulatory follicle diameter of embryo donor mares in an embryo reproduction center.

2. Materials and methods

2.1. Animals and study design

This experiment was performed with the approval of the Committee on Ethical Use of Animals (CEUA-UENF) in accordance with the Brazilian Society of Laboratory Animal Science/Brazilian College of Animal Experimentation (SBCAL/COBEA) under protocol number 307.

A total of 195 embryo donor mares of the Mangalarga Marchador breed were evaluated during five years from 2012 to 2016. The mares were in an embryo transfer center located in northern Rio de Janero, Brazil (latitude: 21º45'15", longitude: 41º19'28", altitude: 13 m). All mares were evaluated throughout the year, so they did not enter into seasonal anestrus in winter. They were evaluated in different reproductive seasons (RS). The period from October to April is considered the breading season (BS), May is considered the autumnal transition out of breeding season (ATBS), the period from June to August is considered the seasonal anestrus, or non-breeding season (nBS); and September is considered the vernal transition into breeding season (VTBS) in Brazil's tropical climate.

The animals were kept in an open shelter and outdoor paddock with access to pasture and supplemented twice a day with hay and 2.0 kg of pellet feed (Equitech, Brazil) containing

12% crude protein and 2,700 kcal of digestible energy, with amino acid supplementation of 25 ml/day (Glicopan Energy®, Vetnil, São Paulo, Brazil). Trace-mineralized salt and water were available *ad libitum*.

The reproductive tract (uterus, and ovaries) was examined by transrectal ultrasonography (Mindray® DP-2200 system, Shenzhen, China) with a 5-MHz linear transducer to determine the estrous cycle stage, presence and measurement of follicles, uterine edema and intrauterine fluid. The evaluations were performed every day from the moment when a follicle having 30 mm diameter was detected. When a follicle with diameter 35 mm or greater was detected, 1.0 mg of deslorelin acetate was administered intramuscularly (BioRelease, BET Laboratories, Rio de Janeiro, Brazil) to induce ovulation. Each mare was evaluated repeatedly during the year.

During five years the data on average monthly temperature and rainfall were collected, along with daily photoperiod, to calculate the respective reproductive seasonal averages.

The rainfall data were separated into classes, whereby 6 mm^3 to 100 mm^3 was considered "R1" and 101 mm^3 to 200 mm^3 was considered "R2". The same was done with average temperature, where 21 to 24 ºC was considered "T1" and 24.1 to 28 ºC was considered "T2". Finally, the photoperiod was separated into two classes, one with 10:39 to 11:39 hours of daylight, considered "P1", and the other with 11:40 to 13:18 hours of daylight, considered "P2".

We evaluated the mean preovulatory follicle diameter one day before ovulation (D-1) and the daily growth from five days before ovulation (D-5) until the day of ovulation (D0). We also evaluated the effect of the year of evaluation, reproductive season, temperature, rainfall and photoperiod on preovulatory follicular diameter (PFD). Finally, we calculated the repeatability of preovulatory follicle diameter.

2.2. Statistical analysis

Descriptive statistics were calculated from all data collected. The environmental effect (temperature, rainfall and photoperiod) and the follicular dynamics (dominant follicular diameter, daily dominant follicular growth and preovulatory follicular diameter) were arranged in contingency tables and analyzed by the PROC MIXED routine, at 5% probability [5].

The repeatability (t) of the preovulatory follicle diameter was calculated from the estimated variance (VAR) with the REML method of the VARCOM procedure [5]. Repeatability (t) of preovulatory follicular diameter was calculated using PROC VARCOMP-REML [5] with:

$$t = \frac{\sigma^2 m}{\sigma^2 m + \sigma^2 w}$$

where: $\sigma^2 m$ = variance among individuals

$\sigma^2 w$ = variance within individuals

3. Results

The total number of ovulations was 2,124 during all years of evaluation. There were 1975 single ovulations (83%) and 149 double ovulations (7%), of them 38 in the right ovary (RO) and 45 in the left ovary (LO), plus 66 double ovulations, in which one dominant follicle grows in each ovary simultaneously and both follicles ovulate (from left and right ovaries).

Finally, there were three triple ovulations, and during the five years, 159 anovulatory follicles were recorded.

The mean diameter of the preovulatory follicles (D-1) was similar in the left and right ovaries (39.3 ± 3.8; 39.2 ± 3.5 mm, respectively).

Table 1 shows the mean follicular diameters of the right and left ovaries from day -5 to day -1 (prior to ovulation). The table also reports the daily follicular growth until one day before ovulation (-1). There was a progressive increase of the follicle diameter until one day before ovulation. This increase was higher from day -4 to day -3, with mean of 2.8 mm for dominant follicular growth, and growth decelerated from day -1 onward. Growth was similar in the left and right ovaries.

Table 1. Mean and standard deviation of follicular diameter on days (-5, -4, -3, -2 and -1) before ovulation.

Ovary	N	-5	-4	-3	-2	-1
RO	(3050)	30.8 ± 4.7[a]	32.8 ± 4.1[a]	35.6 ± 4.0[a]	37.9 ± 3.6[a]	39.3 ± 3.8[a]
LO	(2744)	30.5 ± 5.0[a]	32.5 ± 4.3[a]	35.3 ± 3.9[a]	37.7 ± 3.7[a]	39.2 ± 3.5[a]
DFG			(5-4)	(4-3)	(3-2)	(2-1)
RO			2 ± 0.6	2.8 ± 0.1	2.3 ± 0.4	1.5 ± 0.2
LO			2 ± 0.7	2.8 ± 0.4	2.4 ± 0.2	1.5 ± 0.2

Number of observations (N); right ovary (RO); left ovary (LO); dominant follicular growth diameter (mm) (DFG). Means with a different superscript (a and b) are different ($p < 0.05$).

Table 2 reports the means and standard deviations of the preovulatory follicle diameter throughout the years of evaluation. There was no difference in preovulatory follicular diameter among the years ($p > 0.05$). The same result was observed in both ovaries.

Table 2. Effect of year of evaluation on preovulatory follicular diameter.

Ovary	Years of evaluation				
	2012	2013	2014	2015	2016

N		(387)	(419)	(499)	(131)	(283)
	RO	39.4 ± 3.2^a	39.4 ± 4.4^a	39.4 ± 3.4^a	39.1 ± 3.7^a	39.3 ± 4.0^a
	LO	39.5 ± 3.7^a	39.5 ± 3.7^a	39.4 ± 3.6^a	39.2 ± 2.8^a	38.6 ± 3.2^a

Number of observations (N); right ovary (RO); left ovary (LO). Means with a different superscript (a and b) are different ($p < 0.05$).

Table 3 shows the effect of the reproduction season on the diameter of the preovulatory follicles. There was an effect only on the diameter of the preovulatory follicle in the right ovary ($p < 0.05$), with highest mean recorded in the autumnal transition out of breeding season.

Table 3. Effect of reproduction season (RS) on preovulatory follicular diameter.

Ovary	RS			
	BS	ATBS	nBS	VTBS
N	(899)	(142)	(531)	(192)
RO	39.0 ± 3.7^b	40.7 ± 4.3^a	39.5 ± 3.6^b	39.4 ± 3.8^b
LO	39.2 ± 3.9^a	39.2 ± 3.2^a	39.1 ± 2.9^a	39.3 ± 3.4^a

Number of observations (N); right ovary (RO); left ovary (LO); breeding season (BS), autumnal transition out of breeding season (ATBS), non-breeding season (nBS), vernal transition into breeding season (VTBS). Means with a different superscript (a and b) are different ($p < 0.05$).

Table 4 reports the mean preovulatory follicular diameters according to different temperature and rainfall values. There was no difference in preovulatory follicular diameter with different temperature (T1 and T2) and rainfall intervals (R1 and R2) ($p > 0.05$).

Table 4. Effect of rainfall and temperature on preovulatory follicular diameter.

EE		Temperature (^oC)		Rainfall (mm^3)	
Ovary	N	T1	T2	R1	R2
RO	(792)	39.4 ± 3.9^a	39.3 ± 3.5^a	39.4 ± 4.0^a	38.9 ± 3.3^a
LO	(704)	39.1 ± 3.2^a	39.5 ± 3.7^a	39.3 ± 3.4^a	39.3 ± 3.6^a

Environmental effect (EE); Number of observations (N); right ovary (RO); left ovary (LO); mean temperature ranging from 21º C to 24 ºC (T1) and from 25 to 28 ºC (T2); rainfall ranging from 0 to 100 mm^3 (R1) and from 101 to 235 mm^3 (R2). Means with a different superscript (a and b) are different ($p < 0.05$).

The photoperiod had an effect on preovulatory follicular diameter of the right ovary ($p < 0.05$), with greater mean follicular diameter observed when daylight was shorter (P1). However, there was no effect of photoperiod on preovulatory follicular diameter of the left ovary ($p > 0.05$) (Table 5).

Table 5. Effect of photoperiod on preovulatory follicular diameter.

Photoperiod		N	P1 (hours)	P2 (hours)
Ovary	RO	(824)	39.6 ± 3.7^a	39.1 ± 3.8^b
	LO	(743)	39.2 ± 3.0^a	39.3 ± 3.9^a

Number of observations (N); right ovary (RO); left ovary (LO); day length from 10h to 11h:40min (P1) and from 11h:41min to 13h:18min (P2). Means with a different superscript (a and b) are different ($p < 0.05$).

The estimated variance values for preovulatory follicle diameter were VAR (mare) = 0.042 and VAR (residual + mare) = 0.587, with the result t = 0.07.

Regarding the frequencies of the preovulatory follicle diameters, the highest frequency was 40 mm and the lowest 46 mm (Table 6).

Table 6. Frequencies of preovulatory follicular diameter categories.

FD (mm)	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	48	50
Frequency (%)	0.9	0.6	2.0	5.0	9.5	5.5	20	2.5	34.6	0.9	6.9	1.1	0.9	6.8	0.4	0.5	2.2

Follicular diameter (FD).

4. Discussion

The rate of double ovulation of Mangalarga Marchador mares in this study was low and not significant for an embryo transfer program, because the rate (7%) of double ovulation using deslorelin did not increase the embryo recovery rate considering all the ovulations during the evaluation period. Azevedo et al. [6] reported a good response to deslorelin treatment to induce double ovulation in Quarter Horse mares. Squires [7] also reported that mare breeds like Thoroughbreds, Warmbloods and Draft horses have higher rates of spontaneous double ovulation compared to others, so they have a greater embryo recovery rate per estrous cycle and are more productive in embryo transfer programs. Since double ovulation increases embryo production, the selection of Mangalarga Marchador donor mares with this natural trait can promote the genetic improvement of this breed for reproductive characteristics. It can thus be a criterion for selection of reproductive characteristics, as established in other countries such as Germany [4], associated with the morphofunctional traits that are already used for genetic improvement of horses [8].

Knowledge of the pattern of follicular diameter variation can help make choices in embryo transfer programs, by predicting the moment nearest to ovulation. It is unfeasible to evaluate ovulation by ultrasound of all mares during their cycles due the excessive time this would take. The mares are usually evaluated once a day or every other day, so we observed a pattern of daily follicular growth from day -5 until ovulation (Table 1). Another observation that can be a tool to predict ovulation is that as the moment of ovulation nears, the daily follicular growth decreases, indicating that ovulation is about to happen. Ginther et al. [10], observed that follicles continue to grow at a steady rate until two days before ovulation, when follicular size reaches a plateau of approximately 40 mm. This decreasing growth can be due to the peak production of inhibin near to ovulation [11].

Regarding the effect of year of evaluation, there was no influence ($p > 0.05$) on mares' follicular dynamics (Table 2). There were differences of temperature, rainfall and quality/quantity of pasture, but none of those environmental effects caused changes in ovary traits in this study.

The reproductive season defined in this study into four categories (BS, ATBS, nBS and VTBS) can be used as a model in tropical regions of the Southern Hemisphere if climate and photoperiod do not have abrupt changes. The RS did not affect the follicular diameter in most evaluations (Table 3), except in autumnal transition into non-breeding season, where the average preovulatory follicular diameter was slightly larger than other RS. This suggests that during this transitional phase, inadequate gonadotropin stimulation, mainly by LH with low circulating concentration, can act to delay ovulation, leading to a greater follicular growth until ovulation. Other authors have observed low concentration of LH in this phase, as well as no follicular diameter growth above 16 mm and no dominant follicle development [12,13]. King et al. [14] reported that the vernal transitional phase into breeding season is characterized by the resumption of follicular deviation, with one to three anovulatory follicular waves before ovulation, and that during the winter (considering as the non-breeding season), mares enter seasonal anestrous. In northern Rio de Janeiro state, mares continue cycling during the year, probably because the concentration of LH during transitional phases and non-breeding season are sufficient to promote signals for final follicular development and ovulation (Table 3).

Environmental effects (EE) like temperature and rainfall did not influence the follicular dynamics (Table 4). This is probably because these two parameters do not have a sufficient variation to influence follicular dynamics, suggesting there are other factors that can affect the dominant follicular diameter. Warriach et al. [15] reported that mares continue cycling throughout the year in subtropical regions even with lower temperatures than in the present study in winter or nBS. Higher temperatures and humidity, during summer cause lower

reproductive rates of mares, suggesting that the higher temperature can cause thermal stress, with negative effects on reproductive performance by stimulating release of corticotrophins and affecting the hypothalamus-hypophysis-gonad axis, decreasing the synthesis and release of gonadotropin FSH and LH [15,16]. Even with the high summer temperatures, when the mares were evaluated in this study they responded very well to hormonal treatment to induce ovulation, with small number of anovulatory follicles and usually one ovulatory wave. These observations suggest that the mares studied are adapted to tropical climate, because Mangalarga Marchador is a Brazilian breed, causing them to have a favorable response to deslorelin administration (Table 4).

Regarding the effect of photoperiod on follicular diameter (Table 5), we observed that the period when daylight hours was lowest (P1 - 10:39 to 11:39 hours) corresponded to the non-breeding season and vernal transition into breeding season (winter and spring), and the mean follicular diameter (day -1) was bigger than in P2 (11:40 to 13:18 hours of daylight), which corresponds to breeding season and autumnal transition out of breeding season (summer and autumn). During periods with shorter daylight, the pineal gland is stimulated to synthesize and release more melatonin, and this hormone can suppress the hypothalamus, causing release of GnRH, so there is less stimulus on the hypophysis to release gonadotropin, mainly LH. Therefore, in winter and transitional phases, the concentrations of LH might not be sufficient to promote follicular development and ovulation of a dominant follicle. In the mares of this study, the photoperiod was not sufficient to interrupt ovulation, suggesting there were other factors favorable to ovary dynamics [13]. The nutritional supplementation associated with good body condition of the mares [17], climate [18] and genetic adaptations of the Mangalarga Marchador breed to this environment [4] are likely factors causing mares to continue cycling year-round. However, the observation the follicle diameter was bigger in shorter photoperiod can be explained by the fact that even though the hours of darkness were not sufficient to

suppress follicular development, the dominant follicle spent more time to ovulate and consequently increased in size compared to what happened in the longer photoperiod, probably due to lower concentration of LH [13].

Regarding the repeatability of the preovulatory follicle diameter during the five years of evaluation, we observed a low value of t = 0.07, reflecting considerable variation and suggesting that follicle diameter cannot be expected to be the same one day before ovulation in every evaluation. Lefrançois and Bruyas [19] also observed low repeatability of the preovulatory follicular diameter in mares, and that knowledge of mare history regarding the diameter preceding ovulation would not be useful to estimate the best follicle diameter at which to breed the mare at the optimal time near ovulation. Furthermore, in this study, we evaluated the frequency of the diameter of the preovulatory follicle and observed that 40 mm was the most frequent diameter one day before ovulation (34.6% frequency).

It is not possible to be certain that ovulation will occur on the day after observing a follicle with diameter of 40 mm or more, but this does indicate that ovulation is near. So, we can suggest that the low repeatability of follicular diameter indicates that the size of the preovulatory follicle is affected more by environmental factors than by genetics of the mare [3].

5. Conclusion

In subtropical climates, the environment does not affect follicular dynamics. The low repeatability of the follicular diameter suggests that this characteristic must be evaluated daily when a follicle with diameter of 30 mm or larger is detected to predict the moment of ovulation and the optimal time to inseminate the mare.

Acknowledgements

We are thankful for the support of CAPES and CNPQ, which are development agencies for Brazilian research.

6. References

[1] Stroud B, Callesen H. IETS statement on worldwide ET statistics for 2010. Anim Reprod 2012:210–6.

[2] Melo CM, Papa FO, Filho JNPP, Araújo GH, Dell'Áquua Jr JA, Alvarenga MA. Eficiência do acetato de deslorelina e do extrato de pituitária equina na indução da ovulação em éguas. Veterinária E Zootec 2012;3:392–8.

[3] Pereira JCC. Melhoramento Genético aplicado à produção animal. 6th ed. Belo Horizonte: 2012.

[4] Sitzenstock F, Rathke I, Ytournel F, Simianer H. The potential of embryo transfer in a German horse-breeding programme. J Anim Breed Genet 2013;130:199–208. doi:10.1111/jbg.12003.

[5] SAS. Statistical analyses system 2009.

[6] Azevedo MV, Souza NM, Ferreira-Silva JC, Batista IO, Sales FABM, Alvarenga MA, et al. Indução de ovulações duplas utilizando baixa dose de deslorelina em éguas. Ciência Veterinária Nos Trópicos 2014;17:42–7.

[7] Squires EL, McCue PM. Superovulation in mares. Anim Reprod Sci 2007;99:1–8. doi:10.1016/j.anireprosci.2006.04.054.

[8] Andersson LS, Larhammar M, Memic F, Wootz H, Schwochow D, Rubin C-J, et al.

Mutations in DMRT3 affect locomotion in horses and spinal circuit function in mice. Nature 2012;488:642–6. doi:10.1038/nature11399.

[9] Ginther OJ, Gastal MO, Gastal EL, Jacob JC, Siddiqui MAR, Beg MA. Effects of age on follicle and hormone dynamics during the oestrous cycle in mares. Reprod Fertil Dev 2008;20:955–63. doi:10.1071/RD08121.

[10] Ginther OJ, Gastal EL, Gastal MO, Beg MA. Dynamics of the Equine Preovulatory Follicle and Periovulatory Hormones: What's New? J Equine Vet Sci 2008;28:454–60. doi:10.1016/J.JEVS.2008.07.008.

[11] Satué K, Jc G. A Review of the Estrous Cycle and the Neuroendocrine Mechanisms in the Mare. J Steroids Horm Sci 2013;4. doi:10.4172/2157-7536.1000115.

[12] Donadeu FX, Watson ED. Seasonal changes in ovarian activity: Lessons learnt from the horse. Anim Reprod Sci 2007;100:225–42. doi:10.1016/j.anireprosci.2006.12.001.

[13] McKinnon AO, Squires EL, Vaala WE, Varner DD. Equine Reproduction. vol. 1. 2nd ed. Ames: Blackwell Publishing Ltd; 2011.

[14] King SS, Douglas BL, Roser JF, Silvia WJ, Jones KL. Differential luteolytic function between the physiological breeding season, autumn transition and persistent winter cyclicity in the mare. Anim Reprod Sci 2010;117:232–40. doi:10.1016/j.anireprosci.2009.04.012.

[15] Warriach HM, Memon MA, Ahmad N, Norman ST, Ghafar A, Arif M. Reproductive Performance of Arabian and Thoroughbred Mares under Subtropical Conditions of Pakistan 2014;27:932–6.

[16] Kelley DE, Gibbons JR, Smith R, Vernon KL, Pratt-Phillip SE, Mortensen CJ. Exercise affects both ovarian follicular dynamics and hormone concentrations in mares. Theriogenology 2011;76:615–22. doi:10.1016/j.theriogenology.2011.03.014.

[17] Aurich C. Reproductive cycles of horses. Anim Reprod Sci 2011;124:220–8.

doi:10.1016/j.anireprosci.2011.02.005.

[18] Oliveira JP, Jacob JCF, de Jesus VLT, Silva PC de A. Influência da temperatura e umidade ambiente em um programa de transferência de embriões equinos, na Baixada Fluminense, Rio de Janeiro. Rev Bras Med Veterinária 2015;1:158–62.

[19] Lefrançois C, Bruyas J-F. Is there a repeatability in the size of follicles at the time of ovulation within individuals in mares? J Equine Vet Sci 2016;41:83. doi:10.1016/j.jevs.2016.04.086.

Chapter III. Diagnostic methods to detect uterus illnesses in mares

Abstract

Uterine illnesses are the major problem in horse reproductive programs. The diagnosis of these pathologies is often neglected or does not provide enough information about the cause. The aim of this study was evaluate different diagnoses to provide more accurate information about endometritis and the real endometrial profile at the beginning of a breeding season. Fifty-one mares with reproductive failure were evaluated. Two different uterine cytology diagnoses, culture of uterine flush material and biopsy assay, were performed. The data were analyzed by the Chi-squared test to compare different diagnostic methods for the same sample from each mare, and sensitivity and specificity tests were performed. Most of evaluations showed no association between cytology and other diagnostic methods, except with bacterial culture. The most common bacterium found was *Escherichia colli*, but it was not associated with neutrophil presence in cytology. The presence of *β-hemolitic streptococcus* was well characterized by positive cytology and cloudy lavage fluid aspect. Endometrial profile was affected by age and whether the mare was donor or recipient. The most sensitive diagnostic method was gross

aspect of lavage fluid. Endometritis should be diagnosed by at least two methods to increase accuracy. Endometritis is not the only uterine disease that causes embryo loss, since endometrial fibrosis can also be a cause.

Keywords: Biopsy, Female, Horse and Reproduction.

1. Introduction

Endometritis is an inflammatory process that is one of the major problems in equine reproductive practice (24). This pathology of the endometrium is a common cause of subfertility and infertility in mares, leading to failure to conceive, embryonic losses and shortened luteal phase (10). It has significant economic impact on the equine industry (25), because of the intensive breeding management and the fact that mares require more cycles to become pregnant (9).

Among the problems diagnosed in the endometrium of mares, acute infectious, chronic infectious and chronic degenerative endometritis are the most common (14).

The correct moment to diagnose endometritis is very important to determine the cause and choose the most adequate treatment. There are several diagnostic methods available to identify the extension of uterine illness. They include clinical examination, transrectal palpation with ultrasound examination of the reproductive tract, vagina and cervix examination with speculum, uterine cytology, culture and endometrial biopsy (5).

Uterine cytology and culture are techniques for diagnosis of endometritis by detection of inflammatory cells and intrauterine pathogens respectively (21). However, these techniques often present unsatisfactory sensitivity and specificity (11). Therefore, uterine biopsy has been used as the “gold standard” for endometritis diagnosis (5, 23). In 1978, Kenney reported that

endometrial biopsy was the principal procedure for the assessment of equine uterine health (6), and this is still the case.

Usually, one embryo per mare is recovered per estrous cycle, so for one mare produce more than one foal during one breeding season (BS), the technic of embryo transfer must be performed in the same mare multiple times (18). However, there is little knowledge about the damage to the endometrium that can be caused by the use of this reproductive biotechnology.

Mating-induced endometritis is considered a transient inflammation of the uterus after exposure of the endometrium to spermatozoa (26). However, the uterine inflammation response can also be caused by intrauterine fluid infusion for flushes and embryo recovery (17).

Endometritis can also be influenced by the pathogenicity of the bacterium and the immune response to it. This can result in different clinical signs observed by ultrasonography and laboratory findings (15).

A healthy uterus is the main determinant for the success of reproductive programs using reproductive biotechnologies like artificial insemination and embryo transfer. The aim of this study was to evaluate different diagnostic methods to predict uterus health of embryo donor and recipient mares.

2. Materials and methods

2.1. Animals and experimental groups

This experiment was performed with the approval of the Committee on Ethical Use of Animals of Norte Fluminense State University (UENF), according to the guidelines of the Brazilian Society of Animal Laboratory Science/Brazilian College of Animal Experimentation (SBCAL/COBEA) under protocol number 307, 2015.

Fifty-one mares from reproduction centers in northern Rio de Janeiro state, Brazil (latitude 21º 45'15', longitude 41º19'28', and 13 meters above sea level) were used. The mares were 3 to 22 years of age.

The mares were kept in an open shelter and outdoor paddock, fed with hay and pellet feed and trace-mineralized salt with water available *ad libitum.*

The experiment was performed in February, during the reproductive season, which South America occurs from September to May.

The embryo donor mares were considered subfertile because all of them had a history of uterine fluid, endometritis, embryo loss and/or very low embryo recovery rate. The recipient mares had unknown reproductive status, but they had a history of at least three embryo losses in the previous embryo transfer attempts (three consecutive months).

The reproductive tracts of mares were evaluated by transrectal palpation and ultrasound (Mindray® DP – 2200, Shenzhen, China) with a 5-MHz linear transducer to determine the estrous cycle stage, presence and measurement of follicles, uterine edema and intrauterine fluid. Uterine edema was graded as 0 (without edema), 1 (slight), 2 (moderate) and 3 (significant). When a corpus luteum (CL) was visualized and the uterus had no edema, the mare was considered to be in diestrus, while when a mare had uterine edema score of 2.5 to 3 and average follicle length ≥ 35 mm, she was considered to be in estrus.

After the transrectal palpation and ultrasonography, the perineum and vulva were washed with soap and water and dried with paper towels. The diagnostic samples were collected in the following order: first a guarded endometrial brushing for cytology evaluation, then a 500 ml lavage for culture and cytology, and finally an endometrial biopsy for histopathology evaluation.

2.2. Cytology

Samples for cytological evaluation were taken from each mare by using two methods: a commercial uterine cytological brush (Cytobrush) (Minitube®, Germany) and by centrifuge of the uterine lavage.

The instruments for uterine cytology were passed through the vagina and cervix into the uterus guided by hand. With the cytological brush in contact with the endometrium of the uterine body, it was rotated three times and then the instrument was carefully retracted out of the vagina. The material on the Cytobrush was then immediately smeared on a glass slide by using a gentle rolling motion. The slides were stained using the Diff Quick® system (Hemal Stain, Danbury, North Carolina, USA).

The material from each uterine lavage was centrifuged in a cytocentrifuge (Presvac®, Balneário Camburiú, SC – Brazil), stained by the panoptic method and analyzed with an optical microscope (Olympus – U-MDOB3, Japan).

All slides for endometrial cytology were evaluated by light microscopy at x400 magnification for the presence of polymorphonuclear neutrophils (PMNs). The evaluation involved calculating the neutrophil-to-epithelial cell ratio after evaluating a total of 200 cells.

2.3. Lavage fluid culture (LvC) for microbiology evaluation

To obtain the 500 ml lavage sample, a uterine catheter (Bivona, Partnar Animal Health, Ilderton, Ontario, Canada) was passed transcervically into the uterine body, the balloon was inflated and 500 ml of Ringer's lactate solution (JP, Riberão Preto, SP, Brazil) was infused into the uterus. The fluid was left in the uterus for 30 seconds, during which the uterus was manipulated and massaged by transrectal palpation, and then the fluid was recovered by gravity flow reflux to the same bottle in a closed system. The gross aspect of the lavage fluid (GALv) was recorded and classified as clear or cloudy. The pH of the LvC was measured with pH indicator strips (Merck, Darmstadt, Germany).

All samples were sent to the laboratory for microbiology analysis. The recovered fluid bottle was sealed with Scotch tape and transported in boxes with ice at a temperature of 5 ºC to the laboratory within 2 hours and suspended for sedimentation by gravity for 30 minutes. One simple of 15 mL was aspired with a sterile needle from the most ventral part of the bottle into a 15 mL Falcon tube (Fisher Scientific, Hampton, NH, USA) and centrifuged at 400 g for 10 minutes. Then the supernatant was removed and the pellet was retrieved for sterile culturing on sheep blood agar.

The bacterial and fungal identification was performed using CLSI standard methods. Briefly, isolates were first evaluated based in plate morphology after overnight growth, typically 24 hours after the sample was received. Colonies were confirmed with the Pastorex Staph-Plus latex agglutination test (Bio-Rad, Marnes-la-Coquette, France) and coagulase testing.

Another simple of 15 mL was aspired from the most ventral part of the bottle into a 15 mL Falcon tube and was centrifuged (Cito-centrifuga – Preservac CT – 12, Balneário Camboriú, SC, Brazil). The slides were stained by the panotic method and evaluated under an optical microscope (Olympus U-MDOB3, Japan) for the presence of PMNs.

Culture media were incubated at 37 ºC in air atmospheric and growth of microorganisms was evaluated after 24 hours. Culture plates with no growth were incubated and re-examined at 48 hours for the presence of bacteria or fungi. If mixed cultures of more than three pathogens were observed, this was considered as confirming contamination, as suggested by Overbeck et al. (2011).

2.4. Endometrial biopsy

An endometrial sample was obtained transcervically from each mare using a sterilized guarded biopsy punch (Equi-Vet®, Kruuse, Marselv, Denmark). Following separation of the

vulva labia, the biopsy punch was carefully introduced into the vagina while being held with a hand protecting the edge where the jaw is located. After passing through the cervix, the jaw was opened and closed to obtain a sample of the anterior part of the uterine body. Then the instrument was retracted from the vagina, and the sample was placed in a 15 mL Falcon tube, fixed in 10% tamponed formalin and sent to the laboratory for histological preparation and examination. After fixing for 24 hours in formalin, the samples were processed routinely by paraffin embedding, sectioned at 3 to 4 µm, and stained with hematoxylin and eosin. The histopathological examination of the biopsies was based on the grading scheme of Kenney and Doig (1986).

The effect of the age on uterine characteristics was evaluated. The endometriums of 51 mares were evaluated to predict the degenerative changes. All mares were separated in two age categories (AC): young mares (≤ 10 years old) and old mares (≥ 11 years old). The effects of age on the endometrial characteristics were evaluated according to biopsy classification: I (mild focal inflammation or fibrosis), IIA (moderate inflammation, multifocal fibrosis, <2 fibrotic nests per 5 mm linear field), IIB (moderate inflammation, multifocal or diffuse fibrosis, 2 to 4 fibrotic nests per 5 mm linear field) and III (severe inflammation and 5 or more nests per 5 mm linear field) (7), GALv, pH, Cytobrush, CCtr, LvC, endometrial leucocyte infiltrate (BiopLeuc), fluid (presence of liquid intrauterine) and fibrosis (0 – absence of fibrosis; 1 – mild fibrosis; 2 - <2 fibrotic nests per 5 mm linear field and 3 - 2 – 4 fibrotic nests per 5 mm linear field). The effect of AC on neutrophil count from the Cytobrush was also evaluated.

The cytology diagnostic methods were also compared according to other variables: CCtr, reproductive cycle, mare category (embryo donor or recipient), GALv and uterine edema grade (EDa – 0 to 2; EDb – 2.5 to 3).

Biopsy was compared with the other diagnostic methods. The biopsy results were considered the “gold standard” to determine the relative importance of the culture and cytology

for presence or absence of uterine illness, for comparison of the results. The presence of neutrophil infiltration in the luminal epithelium (stratum compactum) in uterine biopsy was used to determine endometritis, and then compared with the other diagnostic methods: Cytobrush, centrifuge cytology, gross character of lavage fluid, neutrophil-to-epithelial cell ratio, centrifuge cytology, microbiology culture of lavage fluid, presence of bacteria and presence of fungi.

2.5. Statistical analysis

All comparisons were made by the Chi-squared test and significance was set at $p < 0.05$ (22). Cytobrush, centrifuge cytology, gross character of lavage fluid, neutrophil-to-epithelial cell ratio, microbiology culture of lavage fluid, endometrial edema by ultrasonography and biopsy were compared.

Sensitivity, specificity and positive and negative predictive value (PPV and NPV respectively) were compared among the different diagnostic methods. Data were calculated from frequency tables as suggested by Buczkowska et al. (2014). Sensitivity is the percentage of mares correctly diagnosed with endometritis. Specificity is the percentage of healthy mares correctly diagnosed. The positive predictive value (PPV) is the probability that positive results are true, and negative predictive value (NPV) is the probability that negative results are true.

3. Results

3.1. Cytology and pH

From the 51 mares evaluated, 13 were positive for cytology (Cytobrush). The average of neutrophils per 200 cells counted was 10.1%. Age did not affect ($p > 0.05$) the percentage of neutrophils.

Table 1 shows the association between cytology by Cytobrush and cytology by centrifuge ($p < 0.05$). Table 2 shows the comparison between Cytobrush and the other diagnostic methods: lavage fluid culture, reproductive cycle, mare category, growth aspect of lavage fluid and edema grade.

Table 1. Number (N) and percentage (%) of observations of Cytobrush and CCTr. Comparison between two methods of diagnoses for endometritis.

		CCtr		
Cytobrush		Negative	Positive	P value
Negative	N	14	2	0.0098
	%	70.0	10.0	
Positive	N	1	3	
	%	5.0	15.0	

Cytology diagnoses performed with the use of endometrial brush specific for cytology smears (Cytobrush), centrifuge cytology (CCtr). No presence of neutrophils (N), positive, presence of neutrophils in the smear (P).

The presence of PMNs on slides obtained from the Cytobrush (positive cytology) was not associated ($p > 0.05$) with presence of uterine pathogens (positive culture). A total of 12.2% of the slides were positive for cytology and positive for LvC, and 10.2% of the slices were positive for cytology and negative for LvC. Association between CCtr and LvC was observed ($p < 0.05$). From 21 observations, 87.5% were positive for both variables.

Table 2. Number (N) and percentage (%) of observations of cytology and other diagnoses methods.

				P value
Cytology (Cytobrush)		LvC		
		Negative	Positive	
Negative	N	28	10	0.0787
	%	57.1	20.4	
Positive	N	5	6	
	%	10.2	12.2	
		Reproductive cycle		
		Diestrus	Estrus	
Negative	N	10	24	0.6561
	%	23.8	57.1	
Positive	N	3	5	
	%	7.1	11.9	
		Mare category		
		Donor	Recipient	
Negative	N	8	30	0.007
	%	16.3	61.2	
Positive	N	7	4	
	%	14.2	8.1	
		GALv		
		Clean	Cloundy	
Negative	N	25	13	0.4957
	%	51.0	26.5	
Positive	N	6	5	
	%	12.24	10.2	
		EdemaGrade		
		RDa	EDb	
Negative	N	17	21	0.1020
	%	34.7	42.9	
Positive	N	8	3	
	%	16.3	6.12	

Cytology smears (Cytobrush; Negative and Positive for presence or absence of neutrophil on smear), lavage culture (LvC; Negative and Positive for presence or absence of microorganisms on lavage), period of reproductive cycle of the mare (Reproductive Cycle), category of donor or recipient mare (Mare category), gross aspect of lavage (GALv) and uterine edema grade RDa 0 – 2; RDb 2.5 – 3 Edema Grade).

Comparing the cytology with mare reproductive cycle, they were not associated ($p > 0.05$). From a total of 42 mares, 57.1% were in estrus and had negative cytology.

The presence of neutrophils and mare category (donor or recipient) was associated ($p < 0.05$). Of 15 donor mares, 53.3% had negative cytology and 46.7% had positive cytology. From 34 recipient mares, 88.2% had negative cytology and only 11.8% had positive cytology. So, 63.6% of the positive cytology results were from donor mares and 36.4% from recipient mares.

No association was detected between the cytology results and gross aspect of the lavage fluid ($p > 0.05$). From 31 clear LvC, 80.7% were cytology negative and 19.3% were cytology positive, and from 18 cloudy LvC, 77.2% were cytology negative and 27.8% were cytology positive. Only 10.2% of 49 evaluations were cytology positive and cloudy.

There was no association ($p > 0.05$) between cytology and biopsy edema. Endometrial biopsy with EDa had 68% negative cytology and 32% positive cytology. On the other hand, endometrial biopsy with EDb had 87.5% negative cytology and 12.5% positive cytology. Between the two edema categories, 51% was EDa and 49% was EDb.

There were no associations between Cytobrush with pH, and between LvC with pH ($p > 0.05$) (Table 3). Of 35 observations, only 14.3% had pH 7, with 8.6% negative and 5.7% positive for Cytobrush. Most samples had pH 6 (57.1%). From 36 observations of LvC with pH analysis, only 16.7% were pH 7 and most had pH 6 (55.6%).

Table 3. Number (N) and percentage (%) of observations of Cytology and pH. Comparison among pH of uterine lavage with Cytobrush method and microbiological culture.

Cytology (Cytobrush)		pH 6	6.5	7	P value
Negative	N	16	9	2	0.3916
	%	45.7	25.7	8.6	
Positive	N	4	1	2	
	%	11.4	2.9	5.7	
LvC					
Negative	N	13	7	5	0.6931
	%	36.1	19.4	13.9	
Positive	N	7	3	1	
	%	19.4	8.3	2.8	

Cytology diagnoses performed with the use of endometrial brush specific for cytology smears (Cytobrush). No presence of neutrophils (Negative) and presence of neutrophils in the smear (Positive), lavage culture (LvC; Negative and Positive for presence or absence of microorganisms on lavage).

3.2. Microbiology of LvC

Microorganism growth was detected in only 17 of 51 cultures. Samples from two mares had Enterobacteriaceae, but the species could not be determined during laboratorial analysis. Four mares were detected with monoculture of Enterobacteriaceae, one with two types of Enterobacteriaceae, three with monoculture of *Escherichia coli*, one with monoculture of *Morganella morgani*, one with *Edwarsiella tarda*, one with *Cedecea sp*, two with the β-hemolitic streptococcus, one with Acinetobacter sp.

Two mares presented positive LvC culture with mixed growth, one with *Escherichia coli*, Enterobacteriaceae and *Staphylococcus*, and the other with *Escherichia coli*, *Pseudomonas aeruoginosa* and *Acinetobacter* sp. Four mares had growth of fungal elements, three with *Candida* spp and one with *Aspergillus* spp.

Table 4 shows the bacterial and fungal culture results and their concordance between cytology and microbiology. *Edwarsiella tarda*, *Cedecea sp* and the mixed culture of

Escherichia coli, *Pseudomonas aeruoginosa* and *Acinetobacter sp* showed no concordance with cytology findings, but Morganella morgani, β-hemolitic streptococcus, the mixed culture of *Escherichia coli*, Enterobacteriaceae, Staphylococcus and *Aspergillus spp* showed high concordance, and monoculture of *Escherichia coli* and *Candida spp* showed low concordance with cytology findings.

Table 4. Bacteria and fungi detected in culture of the uterine lavage and its comparison with cytology finds.

Microorganisms	Cytobrush Cytology		
	Positive cytology	Negative cytology	Concordance between cytology and microbiology (100%)
Escherichia coli	1	2	1/3 (33.3)
Morganella morgani	1	0	1/1 (100)
Edwarsiella tarda	0	1	0/1 (0)
Cedecea sp	0	1	0/1 (0)
β-hemolitic streptococcus	2	0	2/2 (100)
Acinetobacter sp	0	1	0/1 (0)
Escherichia coli, *Enterobacteriaceae* and *Staphylococcus*	1	0	1/1 (100)
Escherichia coli, *Pseudomonas aeruoginosa* and *Acinetobacter sp*.	1	0	1/1 (100)
Candida spp	1	2	1/3 (33.3)
Aspergillus spp	1	0	1/1 (100)

3.3. Biopsy findings

When the biopsies were evaluated, at least one endometrial alteration was found in 92% of the 51 mares, with the IIA category having the highest frequency (61.5%). Eight mares were diagnosed with acute endometritis according to the inflammation detected in endometrial biopsy analysis (presence of PMNs), 14 with chronic endometritis, i.e., detection of mononuclear cells, and 4 mares with both (PMNs and mononuclear cells).

Figure 1 shows the endometrial cytology (Cytobrush) results with presence of neutrophils; endometrial biopsy grade I, IIA and IIB. In all endometrial biopsies graded as IIA and IIB, periglandular fibrosis was observed, considered as moderate or severe, and only 21%

of mares with endometrial biopsy graded I did not present fibrosis. Lymphocyte and neutrophil infiltrates were also observed in almost all samples.

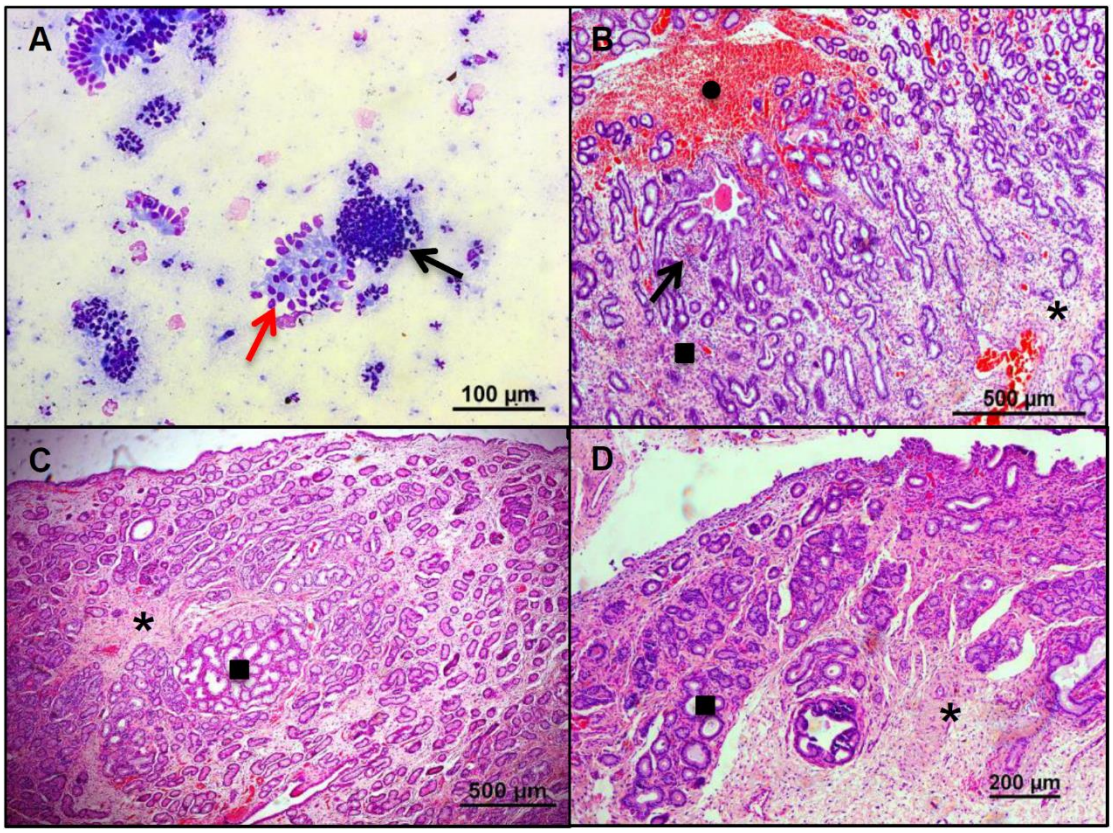

Figure 1: A. Number of neutrophils (black arrow) associated to endometrial cells (red arrow). H.E. **B.** Endometrium with discreet inflammatory infiltrate represented by neutrophils (arrow), edema (*), slight fibrosis (■) and bleeding (•). H.E. **C.** Discreet proliferation of fibrous conjunctive tissue (*) forming nets of endometrial glands (■). H.E. **D.** Severe fibrosis (*) forming nets of endometrial glands (■). H.E.

Table 5 shows the comparison between endometrial biopsy and mare category (donor or recipient). Associations between the two variables were observed ($p < 0.05$). The donor mares had more frequency of IIB biopsy (12.2%) than recipient mares (8.2%). From all biopsies classified as IIB, 60% were donor mares. On the other hand of all biopsies classified as I, 86.4% were recipient mares and only 13.6% were donor mares.

Table 5. Number (N) and percentage (%) of observations of each biopsy category and mare category. Comparison between biopsy classification and mare category.

		Biopsy Classification			
Mare Category		I	IIA	IIB	P value
Donor	N	3	6	6	0.027
	%	6.1	12.2	12.2	
Recipient	N	19	11	4	
	%	38.8	22.4	8.2	

Donor mares (Donor) and recipient mares (Recipient). Frequency, percent, Row pct and Col pct.

3.4. Age

Table 6. Number (N) and percentage (%) of observations and effect of age category on the mares endometrial traits.

		Age Category		
Biopsy		Adult	Old	P value
I	N	15	5	0.0202
	%	33.3	11.1	
IIA	N	11	5	
	%	24.4	11.1	
IIB	N	2	7	
	%	4.4	15.6	
Fibrosis				
0	N	8	1	0.0871
	%	17.8	2.2	
1	N	12	8	
	%	26.7	17.8	
2	N	7	4	
	%	15.6	8.9	
3	N	1	4	
	%	2.2	8.9	
BiopLeuc				
Negative	N	17	10	0.9001
	%	37.8	22.2	
Positive	N	11	7	
	%	24.4	15.6	

Adult mares (≤ 10 years old) and Old mares (≥ 11 years old); grade of fibroses on endometrial biopsy (0 – 3; absence to severe fibrosis) presence or absence (Positive and Negative) of neutrophil infiltrate on biopsy (BiopLeuc).

The age category of the mares only affected the biopsy category and fibroses level ($p < 0.05$). The presence of leucocytes observed in endometrial biopsy (PMNs and mononuclear cells) was not affected by age ($p > 0.05$) (Table 6). Of the samples from old mares, 41.2% had biopsy classified as IIB and only 7.4% from young mares. Of 20 mares with endometrial biopsy categorized as I, 15 were young and 5 were old. Of the old mares, 23.5% had fibrosis grade 3, and only 5.9% had no fibrosis. Of the young mares, only 3.6% had fibrosis grade 3 and 28.6% had no fibrosis. Of all 45 observations, 44.4% had fibrosis grade 1.

Table 7. Number (N) and percentage (%) of observations and evaluation of the effect of age category on uterine flush and cytology.

	Age Category		
GALv	Adult	Old	P value
Clear	19	10	0.5394
	42.2	22.2	
Cloud	9	7	
	20.0	15.6	
LvC			
Negative	21	9	0.1280
	46.7	20.0	
Positive	7	8	
	15.6	17.8	
Cytobrush			
Negative	23	13	0.6447
	51.1	28.9	
Positive	5	4	
	11.1	8.9	

Gross aspect of the lavage (GALv), culture of the uterine lavage (LvC) and Cytology diagnoses performed with the use of endometrial brush specific for cytology smears (Cytobrush).

The characteristics GALv, LvC and Cytobrush were not affected by age category ($p > 0.05$) (Table 7). The means of neutrophils in young and old mares were similar ($p > 0.05$). The pH and fluid also were not affected by age category ($p > 0.05$) (Table 8).

Table 8. Number (N) and percentage (%) of observations and evaluation of the effect of age category on the pH and uterine trait.

	Age Category		
pH	Adult	Old	P value
6	13	7	0.7532
	39.4	21.2	
6.5	6	2	
	18.2	6.1	
7	4	1	
	12.1	3.0	
Fluid			
Negative	20	7	0.9699
	66.7	21.2	
Positive	3	1	
	9.1	3.0	

Presence or absence of liquid intrauterine (Fluid).

From the 51 mares evaluated, 31 had some abnormal clinical finding, such as positive cytology, microorganism growth on culture and lavage fluid with cloudy appearance. Of the 20 mares with no clinical signs that could be considered as endometritis, only one mare had presence of PMNs in the biopsy. From the eight mares diagnosed with acute endometritis by biopsy analysis (presence of PMNs), three had negative cytology. However, two of them had other signs that could be related to endometritis: one had cloudy lavage fluid and positive LvC with growth of bacteria and the other had cloudy lavage fluid. The third mare had no other clinical signs.

3.1. Sensitivity and specificity.

Table 9 shows the results of sensitivity and specificity when comparing the Cytobrush, CCt, LvC, GALv and uterine fluid against the endometrial biopsy as gold standard for detecting endometritis.

Gross aspect of the lavage fluid and Cytobrush cytology were the most sensitive diagnostic methods (80% and 70% respectively). In comparison, intrauterine fluid aspect had low sensitivity and but high specificity. However, the GALv was the most sensitive and lease specific diagnostic method. Intrauterine fluid presented the highest positive predictive value (0.91) and GALv the lowest PPV (0.67). The CCtr diagnostic method showed the lowest value of NPV (0.16).

Table 9. Sensitivity and specificity of the diagnoses methods in relation to the gold standard (Biopsy).

DM	Tp (%)	Fn (%)	PPV	NPV	Sensitivity %	Specificity %
Cytobrush	16.6	42.8	0.84	0.57	70	86
CCtr	16.7	83.3	0.83	0.16	50	86
LvC	29	61.1	0.71	0.40	62	78
GALv	33.3	44.4	0.67	0.56	80	75
Fluid	9.5	56.2	0.91	0.44	55	92

Diagnoses methods (DM), cytology diagnoses performed with the use of endometrial brush specific for cytology smears (Cytobrush), centrifuge cytology (CCtr), centrifuge cytology (CCtr), Lavage culture (LvC) and intrauterine fluid (Fluid). True positive (Tp), false negative (Fn), positive predictive value (PPV) and negative predictive value (NPV).

4. Discussion

Ultrasonography, cytology performed with Cytobrush and uterine culture are the most common and practical diagnostic methods to evaluate some uterine diseases, such as endometritis. However, these techniques do not provide enough information about the disease. Most of the time, clinical signs of inflammation are absent, and these diagnostic methods can also produce false negative results (10). So, accurate diagnosis of subclinical endometritis is important to predict if a mare should be an embryo donor or a recipient in an embryo transfer program during a given breeding season.

The presence of 2% neutrophils was considered positive for cytology (Cytobrush and CCtr). Diel de Amorim et al. (2016) reported that there is no consensus in the literature of what defines equine endometritis based on endometrial cytology. The same authors considered more than two neutrophils per hpf in cytology.

The adequate diagnostic method is important to evaluate the prognosis and choose the best treatment for mares. This study was designed to compare the diagnostic performance of ultrasonography, pH, gross aspect of lavage fluid, cytology by Cytobrush and centrifuge, intrauterine lavage culture and biopsy to diagnose endometritis. For the culture, the specimens were harvested under field conditions and samples were examined bacteriologically and cytologically. Furthermore, histological evaluations of endometrium samples were regarded as "best standard" and compared with other diagnostic methods.

The positive association ($p < 0.05$) between Cytobrush and CCtr meets one of the goals of this study. Usually during a given breeding season, a considerable number of donors mares present low embryo recovery rate so they are submitted to several uterine flushes. The resulting fluid can easily be used as samples for microbiological analysis with low cost and optimized time. Since the embryo collection from horses using the transcervical method must be sterile (4), it provides adequate samples for culture analysis.

Although the Cytobrush test is one of the must performed diagnostic methods to detect endometritis, we observed that this technique was not associated with detection of pathogens in cultures. However, the uterus can present inflammation not necessarily caused by pathogens (9). Also, some bacteria tightly adhere to the epithelium and are harder to remove when collecting samples for culture, and others can create a biofilm (2, 11), thus impairing the identification of microbes during culture analysis. Also, inflammation detected by cytology can be caused by a pathogen that does not grow in a culture. In this study, the percentage of positive findings for both Cytobrush and LvC was similar to that for positive Cytobrush and negative

LvC (12.2 and 10.2% respectively). Therefore, there is a low positive percentage for both methods simultaneously. For a more precise and accurate evaluation, the association of multiple diagnostic methods is required (5).

The stage of estrous cycle and edema grade were not considered causes of presence or not of neutrophils because mares in both cycles (estrus and diestrus) showed no association with PMNs. Overbeck et al. (2011) considered endometritis to be present when PMN was greater than 2%, because most healthy mares showed constant amounts of PMNs in the uterus and could be considered normal physiologically. According to Woodward et al. (2013), mares present higher numbers of PMNs after breeding or insemination as a response to dead spermatozoa and some semen proteins. However, susceptible mares have an increase in the number of PMNs in endometrial tissue during the estrous cycle after breeding, and even in diestrus, as shown by prolonged inflammatory signs, in addition to delayed inflammatory response to spermatozoa compared with resistant mares. They are also less capable to clear uterine inflammation than resistant mares. However, in this study the presence of PMNs during estrus compared to diestrus did not bring enough information about the presence of inflammation.

The presence of neutrophils in Cytobrush was associated with mare category. Between the two categories, we observed that the donors presented more positive Cytobrush in relation to recipients, suggesting that donor mares have more probability of having uterine inflammation. Those mares were mated several times by artificial insemination, followed by uterine flushes to recover embryos, in more than three breeding seasons consecutively. This could trigger some uterine clearance failure, leading to recurrent endometritis (17).

The centrifuged lavage sample fluid evaluated for the presence of PMNs was associated with microbiological culture ($p < 0.of$), but the Cytobrush method was not associated with that culture.

This can be explained by the fact the brush used to collect samples must not access focal infections, while the 500 mL of fluid infused and recovered from the uterus probably contacted the entire intrauterine surface (12). So, if a microorganism infected the uterus, it was detected by the lavage fluid culture. Although the neutrophils detected in CCtr were degenerated, this could be interpreted. Diel de Amorim et al. (2016) also reported that neutrophils were more degenerated from lavage samples comparing with swab samples, where cell integrity was very good. Overbeck et al. (2011) reported that only 18.2% of the cytologically positive mares also showed positive microbiological culture. However, they collected samples using a swab, and this technique does not always detect focal infection.

The bacteria isolated from the 500 mL lavage fluid cultures were *Escherichia coli*, *Morganella morgana*, *Edwarsiella tarda*, *Cedecea sp*, *β-hemolitic streptococcus*, *Acinetobacter sp* and *Staphylococcus* and *Enterobacteriaceae*. Two positive fungal cultures were observed, identified as *Candida* spp and *Aspergillus* spp. Of all microorganisms detected in this study, the most commonly reported in literature are *Escherichia coli* and *β-hemolitic streptococcus* (2, 4, 5, 9). The two fungi found in this study have also been reported in literature as common in mare uterine infections (3, 20). In this study, the bacterium *β-hemolitic streptococcus* was associated with positive cytology (Cytobrush and CCtr) and with the presence of intrauterine fluid detected by ultrasonography. The gross aspect of the lavage fluid of mares infected by this bacterium was cloudy with a creamy or mucoid aspect, different from other bacterial infections. *E. coli* showed low concordance with cytology. Overbeck et al. (2011) observed growth of *E. coli* in monoculture, and this bacterium was not associated with positive cytological findings ($r = -0.23$; $P = 0.87$). M. LeBlanc (2010) reported that not all uterine pathogens produce a neutrophilic response and intra-uterine fluid, so they suggested using diagnostic methods other than culture to identify uterine illness. Another suggestion is that the pathogenicity of *Escherichia coli* appears to be different from that of *β-hemolitic*

streptococcus, so the uterine inflammatory response can vary with different microorganisms (12).

M. M. LeBlanc (2010) observed that pathogens associated with uterine fluid were more likely to have neutrophils in cytology while pathogens not associated with uterine fluid tended to show negative neutrophil results in cytology. This suggests that not all uterine microorganisms induce acute neutrophilic response, and intrauterine fluid can indicate acute inflammation and not necessarily pathogen infection. The same authors related that there are other causes of uterine inflammation that promote acute neutrophil response, such as pneumovagina and urine and semen in the uterus.

We considered the mares to have acute endometritis when at least one PMN per power field was detected in endometrial biopsy ("gold standard") when neutrophils were observed in the stratum compactum.

The mares' reproductive status can be another factor leading to uterine disease (5), but in this study the reproductive category was not known. That is one of the problems veterinarians have observed for the selection of recipient mares. The history of all recipient mares evaluated in this study showed that they had at least three embryos lost in the three months of the last breeding season, but there was no information about the number of barren mares, maiden mares and parturition. So, random evaluations of mares with poor reproductive history are difficult to define if they have endometritis or other uterine diseases. In this study the endometrial biopsy was considered the gold standard for diagnosis of endometritis and other endometrial diseases, according to Christoffersen et al. (2015) and Diel de Amorim et al. (2016). Of the 34 recipient mares, 20 had endometrial biopsy grade I, so these mares should have presented a foaling rate of 80 to 90%. (7). However, this was not what was in the history, suggesting there were other causes of embryo loss. Most of these mares with classified biopsy as I presented slight and sparsely scattered fibrosis and/or leukocyte infiltration. So, we suggest that even with minimal

lesion or inflammation, the uterus can be unfavorable for embryo implantation and development. LeBlanc et al. (2007) also evaluated mares that had been bred three of more times unsuccessfully in the same breeding season and had a history of more than two years of reproductive failure, with more than two unsuccessful embryo recovery attempts in two consecutives cycles. However, they observed only 26% of mares with neutrophils in cytological smears. Another advantage of biopsy is the identification of fibrosis, which has a severe effect on a mare carrying a fetus to term. This method facilitates the selection of the best recipient mares in a given breeding season. It also significantly reduces the costs and improves the success of a breeding program.

In this study we evaluated the effect of age on the detection of PMNs in cytology using Cytobrush. The results were not significant, but similar averages of PMNs were observed in young and old mares in this study. However, Pycock (2000) reported that old mares had higher probability of having uterine disease like endometritis, compromising their fertility. Mares older than 12 years typically have an elongated, closed and fibrous cervix during estrus. The uterus of these mares can accumulate fluid, which is likely favorable to pathogenic microorganism growth. Adams et al. (2008) reported that advanced age in horses is associated with alterations in the immune system, including cellular senescence and inflamm-aging. In the other hand, since we observed no difference in cytology in the two age categories ($p > 0.05$), other characteristics should be considered as having more influence on endometritis in mares. Furthermore the evaluation of more characteristics with different diagnostic methods can provide more precise results, as reported by (5), who presented a checklist of diagnostic methods as a gold standard to detect endometritis.

Uterine illness can affect mares of all ages, due to various causes. In this study, donor embryo mares had been submitted to artificial insemination and uterine flushes to for embryo recovery many times during more than two breeding seasons, and independently of age category

the use of this technique can stimulate an intrauterine inflammatory response. When persistent, uterine inflammation can cause deleterious effects by releasing molecules that can damage the endometrium tissue around the inflamed area and contribute to degeneration (13). Kozdrowski et al. (2013) reported that in samples for cytological evaluation, the presence of more than 5% PMNs or more than 2 PMNs per power field indicates an inflammatory process, so the mare should be subjected to other diagnostic methods to decide on treatment

According to Diel de Amorim et al. (2016), endometrial biopsy associated with other clinical findings is more likely to correctly diagnose affected mares. Furthermore, endometrial biopsy remains the only method to reliably diagnose mares with chronic endometritis. Therefore, we considered biopsy as the gold standard to calculate sensitivity and specificity of all diagnostic methods evaluated.

In this study, 40% of the mares were diagnosed with endometritis according to the presence of leucocyte infiltration ($\geq$ 2hpf) in the stratum compactum by biopsy evaluation. This result was compared with other diagnostic methods and the sensitivity, specificity, positive predictive value and negative predictive value were calculated. Cyitobrush had sentitivity of 70%, similar to finding of Buczkowska et al. (2014) (73% sensitivity). The sensitivity of Cytobrush was superior to the sensitivity of lavage fluid culture. These results show that cytological examination by Cytobrush is more accurate than LvC for diagnosis of endometritis. However, the sensitivity of cytology by centrifugation of lavage fluid was lower than the sensitivity of LvC. This can be explained by the low detection of neutrophils in CCtr, and it might be associated with the dilution effect or the amount of debris, and centrifugation of the efflux (12). Diel de Amorim et al. (2016) also reported that cytology samples prepared from uterine lavage sometimes exhibited poor preservation of cells and more debris, making interpretation difficult. In this study, some smears prepared from CCtr had poor cell preservation, suggesting that the Cytobrsh method is more accurate than the CCtr method.

The growth aspect of the lavage fluid cultures had the greatest sensitivity, meaning that if the recovered uterine fluid is cloudy, it is likely to be positive for endometritis in biopsy evaluation. The presence of intrauterine fluid had low sensitivity, detecting only 55% of the mares with endometritis.

Conclusion

Mares with uterine disease can be found in all age categories. Endometritis should be diagnosed by at least two methods to increase accuracy. Endometritis is not the only uterine disease that can cause embryo loss. Endometrial fibrosis also is an indication of poor uterine health. Therefore, selection of recipient mares should be more judicious in order to improve the results of positive pregnancy diagnosis.

Acknowledgements

We are thankful for the support of CAPES and CNPQ, which are development agencies for Brazilian research.

Bibliography

1. **Adams AA**, **Breathnach CC**, **Katepalli MP**, **Kohler K**, **Horohov DW**. Advanced age in horses affects divisional history of T cells and inflammatory cytokine production. *Mech Ageing Dev* 129: 656–664, 2008.

2. **Beehan DP**, **Wolfsdorf K**, **Elam J**, **Krekeler N**, **Paccamonti D**, **Lyle SK**. The Evaluation of Biofilm-Forming Potential of Escherichia coli Collected From the Equine Female Reproductive Tract. *J Equine Vet Sci* 35: 935–939, 2015.

3. **Buczkowska J**, **Kozdrowski R**, **Nowak M**, **Raś A**, **Staroniewicz Z**, **Siemieniuch MJ**. Comparison of the biopsy and cytobrush techniques for diagnosis of subclinical endometritis in mares. *Reprod Biol Endocrinol* 12: 27, 2014.

4. **Christoffersen M**, **Brandis L**, **Samuelsson J**, **Bojesen AM**, **Troedsson MHT**, **Petersen MR**. Diagnostic double-guarded low-volume uterine lavage in mares. *Theriogenology* 83: 222–227, 2015.

5. **Diel de Amorim M**, **Gartley CJ**, **Hill A**, **Scholtz EL**, **Chenier TS**, **Foster RA**, **Hayes A**, **Diel de Amorim M**. Comparison of Clinical Signs, Endometrial Culture, Endometrial Cytology, Uterine Low-Volume Lavage, and Uterine Biopsy and Combinations in the Diagnosis of Equine Endometritis. *J Equine Vet Sci* 44: 54–61, 2016.

6. **Kenney RM**. Cyclic and pathologic changes of the mare endometrium as detected by biopsy, with a note on early embryonic death. [Online]. *J Am Vet Med Assoc* 3: 241–262, 1978. http://europepmc.org/abstract/med/621166.

7. **Kenney RM**, **Doig PA**. Equine endometrial biopsy, Current therapy in theriogenology 2. In: *Current therapy in theriogenology 2*. Philadelphia: Saunders, W B, 1986, p. 723–783.

8. **Kozdrowski R**, **Gumienna J**, **Sikora M**, **Andrzejewski K**, **Nowak M**. Comparison of the Cytology Brush and Cotton Swab in the Cytological Evaluation of the Endometrium in Mares with Regard to Fertility. *J Equine Vet Sci* 33: 1008–1011, 2013.

9. **LeBlanc M**. Advances in the Diagnosis and Treatment of Chronic Infectious and Post-Mating-Induced Endometritis in the Mare. *Reprod Domest Anim* 45: 21–27, 2010.

10. **LeBlanc M**, **Causey R**. Clinical and Subclinical Endometritis in the Mare: Both

Threats to Fertility. *Reprod Domest Anim* 44: 10–22, 2009.

11. **LeBlanc MM**. Advances in the diagnosis and treatment of chronic infectious and post-mating-induced endometritis in the mare. *Reprod Domest Anim* 45: 21–27, 2010.

12. **LeBlanc MM**, **Magsig J**, **Stromberg AJ**. Use of a low-volume uterine flush for diagnosing endometritis in chronically infertile mares. *Theriogenology* 68: 403–412, 2007.

13. **Lögters T**, **Margraf S**, **Altrichter J**, **Cinatl J**, **Mitzner S**, **Windolf J**, **Scholz M**. The clinical value of neutrophil extracellular traps. *Med Microbiol Immunol* 198: 211–219, 2009.

14. **McKinnon AO**, **Squires EL**, **Vaala WE**, **Varner DD**. *Equine Reproduction*. 2nd ed. Ames: Blackwell Publishing Ltd, 2011.

15. **Otto M**. Bacterial Evasion of Antimicrobial Peptides by Biofilm Formation. In: *Antimicrobial Peptides and Human Disease*. Springer Berlin Heidelberg, p. 251–258.

16. **Overbeck W**, **Witte TS**, **Heuwieser W**. Comparison of three diagnostic methods to identify subclinical endometritis in mares. *Theriogenology* 75: 1311–1318, 2011.

17. **Palm F**, **Walter I**, **Budik S**, **Kolodziejek J**, **Nowotny N**, **Aurich C**. Influence of different semen extenders and seminal plasma on PMN migration and on expression of IL-1??, IL-6, TNF-?? and COX-2 mRNA in the equine endometrium. *Theriogenology* 70: 843–851, 2008.

18. **Panzani D**, **Rota A**, **Marmorini P**, **Vannozzi I**, **Camillo F**. Retrospective study of factors affecting multiple ovulations, embryo recovery, quality, and diameter in a commercial equine embryo transfer program. *Theriogenology* 82: 807–814, 2014.

19. **Pycock JF**. Breeding management of the problem mare (the old maiden mare

syndrome). In: *Equine Breeding Management and Artificial Insemination*. Philadelphia: Samper, J C, 2000, p. 222.

20. **Ribas JA**, **Carvalho EQ**, **Stussi JP**. Endometrite fúngica em éguas : diagnóstico e implicações clínico-patológicas * Fungal endometritis in mares : diagnosis and clinicopathological aspects. 21: 204–212, 2014.

21. **Riddle WT**, **LeBlanc MM**, **Stromberg AJ**. Relationships between uterine culture, cytology and pregnancy rates in a Thoroughbred practice. *Theriogenology* 68: 395–402, 2007.

22. **SAS**. Statistical analyses system. Institute Inc.: 2009.

23. **Snider TA**, **Sepoy C**, **Holyoak GR**. Equine endometrial biopsy reviewed: Observation, interpretation, and application of histopathologic data. *Theriogenology* 75: 1567–1581, 2011.

24. **Traub-Dargatz JL**, **Salman MD**, **Voss JL**. Medical problems of adult horses, as ranked by equine practitioners. [Online]. *J Am Vet Med Assoc* 198: 1745–7, 1991. http://www.ncbi.nlm.nih.gov/pubmed/2071472 [1 Feb. 2017].

25. **Troedsson MHT**. Therapeutic considerations for mating-induced endometritis. *Pferdeheikunde* 13: 516–520, 1997.

26. **Troedsson MHT**, **Scott MA**, **Liu IKM**. Comparative treatment of mares susceptible to chronic uterine infection. *Am J Vet Res* 56: 468–472, 1995.

27. **Woodward EM**, **Christoffersen M**, **Campos J**, **Betancourt A**, **Horohov D**, **Scoggin KE**, **Squires EL**, **Troedsson MHT**. Endometrial inflammatory markers of the early immune response in mares susceptible or resistant to persistent breeding-induced endometritis. *Reproduction* 145: 289–296, 2013.