VICTORIA KANADANI CAMPOS POLTRONIERI

EFEITOS DO ESTRÓGENO EXÓGENO E DA INSUFLAÇÃO UTERINA COM OZÔNIO SOBRE PARÂMETROS HEMODINÂMICOS ENDOMETRIAIS E DE ESTRESSE OXIDATIVO EM ÉGUAS

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Medicina Veterinária, para obtenção do título de *MagisterScientiae*.

Orientadora: Bruna Waddington de Freitas

Coorientadores: José Domingos Guimarães
Jair Camargo Ferreira

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Orientadora

A meu avô, que dedicou sua vida à pesquisa e formação de novos cientistas.

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“O homem nasceu para aprender tanto quanto a vida lhe permita”. (Guimarães Rosa)

RESUMO

POLTRONIERI, Victoria, M.Sc., Universidade Federal de Viçosa, fevereiro de 2023. **Efeitos do estrógeno exógeno e da insuflação uterina com ozônio sobre parâmetros hemodinâmicos endometriais e de estresse oxidativo em éguas.** Orientadora: Bruna Waddington de Freitas. Coorientadores: José Domingos Guimarães e Jair Camargo Ferreira.

É postulado que o ozônio (O_3) age no organismo de forma a provocar um quadro de estresse oxidativo (EO), o que leva à indução de resposta imune, antioxidante e circulatória. Da mesma forma, a aplicação exógena de estrógeno, amplamente utilizada nos protocolos aplicados na rotina da reprodução equina, possui efeito direto sobre o controle da atividade oxidante-antioxidante. Em vista disso, o presente trabalho objetivou caracterizar os efeitos da insuflação uterina com O_3 ou O_2, sobre o fluxo sanguíneo endometrial e parâmetros de EO teciduais/séricos. Ademais, buscou-se verificar no estudo o impacto da aplicação de Benzoato de Estradiol (BE) sobre parâmetros hemodinâmicos uterinos e de EO sistêmicos. Para tanto, dois experimentos foram realizados, sendo que no primeiro (**E I**) foram utilizadas 19 éguas em em atividade cíclica fisiológica e no segundo (**E II**), 16 éguas em anestro sazonal que receberam 10 mg de BE IM. No **E I**, o útero das éguas foi insuflado com misturas gasosas contendo 44 μg O_3 mL^{-1} e 0 μg O_3 mL^{-1} (100 % O_2), constituindo respectivamente os grupos tratado e controle. A insuflação uterina foi realizada a cada 48hrs, totalizando 3 aplicações. Amostras de tecido endometrial foram obtidas dos ciclos imediatamente anterior e imediatamente posterior aos protocolos experimentais por meio de biópsia uterina. Lavados uterinos de baixo volume (LBV) foram efetuados imediatamente antes dos tratamentos e amostras de sangue coletadas em dias alternados, até 7 dias após a finalização dos protocolos. No **E II,** amostras de sangue foram obtidas imediatamente antes da aplicação de BE, 48h e 96 horas após. Ao longo de todo o período experimental, em ambos os experimentos, as éguas foram avaliadas por meio de ultrassonografia colorida Doppler, a fim de avaliar a vascularização uterina e os padrões de fluxo das artérias uterinas médias. As amostras obtidas em **E I** e **E II** foram submetidas a quantificações das atividades de substâncias oxidantes e antioxidantes (superóxido dismutase-SOD, catalase- CAT, malondaldeído-MDA, capacidade antioxidante total-FRAP, proteína total-PT óxido nítrico-NO). No **E I**, maiores valores de NO foram observados em todas as amostras de LBV obtidas após a insuflação uterina com O_3 (P<0,05). Entretanto, alterações séricas e/ou hemodinâmicas uterinas não foram observadas entre grupos. No **E II,** a aplicação de BE resultou na alteração dos marcadores séricos de EO avaliados. Maiores valores de FRAP,

CAT e MDA foram observados passadas 96h após aplicação do fármaco (P<0,01). Menores índices de pulsatilidade (PI) da artéria uterina esquerda e direita foram observados 96h horas após a aplicação de BE ($p<0.05$). Ainda, maior fluxo sanguíneo endometrial foi obtido passadas 48 e 96h da aplicação de BE ($p<0.01$). Nas condições do presente estudo, a administração exógena de estrógeno foi capaz de alterar o status oxidativo sistêmico e a hemodinâmica uterina de fêmeas equinas em anestro. Entretanto, comportamento semelhante não foi observado nas éguas submetidas a ozonioterapia intrauterina.

Palavras-Chave: Atividade antioxidante. Doppler. Equino. Endometrite. Ozonioterapia.

ABSTRACT

POLTRONIERI, Victoria, M.Sc., Universidade Federal de Viçosa, February 2023. **Effect of exogenous estrogen and uterine insufflation with ozone on endometrial vascular parameters and oxidative stress biomarkers in mares.** Adviser: Bruna Waddington de Freitas. Co-advisers: José Domingos Guimarães and Jair Camargo Ferreira.

The Ozone (O_3) acts in the body causing oxidative stress (OS), which leads to an immune, antioxidant and circulatory response. Likewise, the exogenous application of estrogen, widely used in protocols applied in the routine of equine reproduction, has a direct effect on the control of oxidant-antioxidant activity. In view of this, the present work aimed to characterize the effects of uterine insufflation with O_3 or O_2 on endometrial blood flow and tissue/serum OS biomarkers. Furthermore, we sought to verify in the study the impact of applying Estradiol Benzoate (EB) on uterine hemodynamic parameters and systemic OS. For that, two experiments were carried out. In the first one (**E I**), mares (*n*=19) in a period of physiological cyclical activity were used. In Experiment 2 (**E II**), mares in seasonal anestrus (*n*=16) induced with 10 mg of EB were used. In **E I**, the mares' uterus was exposed to gaseous mixtures containing 44 µg O3 mL-1 0 µg O3 mL-1 (100 % O2), respectively constituting the treated and control groups. Uterine insufflation was performed every 48 hours, totaling 3 applications. Endometrial tissue samples were obtained from the cycles immediately before and immediately after the experimental protocols through uterine biopsy. Low volume uterine flushes were performed immediately before the treatments and blood samples collected previously, between and 7 days after the protocols. In **E II**, blood samples were collected immediately before EB application, 48 hours and 96 hours after. Throughout the entire experimental period, in both experiments, the mares were evaluated using color Doppler ultrasonography, in order to assess uterine vascularity and flow patterns of the middle uterine arteries. The samples collected in **E I** and **E II,** including LVL and endometrial tissue, were submitted to quantification of the activities of oxidizing and antioxidant substances (superoxide dismutase-SOD, catalase-CAT, malonaldehyde-MDA, total antioxidant capacity-FRAP, total protein-PT, nitric oxide-NO). In **E I**, higher NO values were observed in all LBV samples collected after uterine insufflation with O3 ($P<0.05$). However, uterine serum and/or hemodynamic changes were not observed between groups. In **E II**, the application of BE was shown, by itself, to be able to change the serum parameters of OS evaluated. Higher values of FRAP, CAT and MDA were observed 96h after drug application ($P<0.01$). Lower pulsatility (PI) indices of the left and right uterine

arteries were observed 96 hours after EB application ($p<0.05$). Furthermore, greater endometrial blood flow was obtained 48 and 96 hours after EB application ($p<0.01$). Under the conditions of the present study, the exogenous estrogen administration was able to alter the systemic oxidative status and uterine hemodynamics of female horses in anestrus. However, similar behavior was not observed in mares submitted to intrauterine ozone therapy.

Keywords: Antioxidant activity. Doppler. Equine. Endometritis. Ozone therapy.

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SÚMARIO

1. INTRODUÇÃO

A infertilidade e as perdas gestacionais são problemas frequentes na equideocultura. Por gerarem grandes prejuízos econômicos, ambas causam preocupação para os profissionais atuantes na área. Dentre as doenças reprodutivas que geram esta condição, a endometrite é a mais comum, provocando um impacto negativo significativo sobre os índices reprodutivos (SCHÖNIGER; SCHOON, 2020). Para que tal intercorrência seja evitada, uma gestão cuidadosa dos processos reprodutivos aplicados na prática deve ser priorizada, envolvendo rigorosa higiene, otimização dos efeitos imunológicos hormonais, correto diagnóstico e minimização de ações desnecessárias, como a aplicação de tratamentos inadequados.

As endometrites são tradicionalmente tratadas de forma multimodal, com a combinação de lavado uterino terapêutico e agentes ecbólicos, antiinflamatórios e antibacterianos. Todavia, algumas éguas não respondem bem a tais condutas, especialmente no que se refere à resolução de condições infecciosas (CANISSO, 2016). Nesses casos, é comum a administração empírica de antibacterianos, com base na experiência profissional prévia ou em estudos publicados na área, contrariando a necessidade de confirmação de cultura bacteriana positiva e teste de sensibilidade (SCOGGIN, 2016; DIAZ-BERTRANA et al., 2021). Tal condição tem levado à crescente incidência de bactérias resistentes, o que por si só favorece o interesse por novas alternativas terapêuticas (MORRIS et al., 2020), como a ozonioterapia.

Os poucos estudos publicados sugerem que a aplicação de ozônio por insuflação intrauterina apresente efeitos terapêuticos benéficos na resolução de endometrites (ÁVILA et al., 2022). Entretanto, seu impacto sobre parâmetros moleculares endometriais bem como sobre o estado oxidativo sistêmico dos indivíduos tratados ainda é pouco abordado. Além disso, não há estudos que avaliem o efeito do gás sobre a hemodinâmica uterina, altamente relacionada à fertilidade (FERREIRA et al., 2021).

De forma semelhante, o uso de protocolos hormonais envolvendo o estrógeno (E_2) no preparo de fêmeas para recebimento de terapias uterinas é comum na rotina da reprodução equina. O esteroide atua diretamente no aprimoramento do mecanismo de defesa uterina e aumento do fluxo sanguíneo para o órgão, melhorando assim, a resistência e o combate a infecções (WASHBURN et al., 1982; ESTELLER-VICO et al., 2016). Apesar disso, não há estudos que abordem o impacto da administração exógena de E_2 sobre parâmetros moleculares sistêmicos, como os de estresse oxidativo (EO).

Sendo assim, o objetivo do presente estudo foi caracterizar os efeitos da insuflação uterina com O_3 ou O_2, sobre biomarcadores de EO e fluxo sanguíneo endometrial. Ademais, buscou-

se verificar o impacto da aplicação de BE sobre parâmetros hemodinâmicos uterinos e de EO sistêmico.

2. REVISÃO DE LITERATURA

2.1. Endometrite Equina

O agronegócio do cavalo movimenta cerca de R$ 16 bilhões e é responsável pela ocupação direta e indireta superior a 3 milhões de pessoas (LIMA & CINTRA, 2016). Segundo Pesquisa da Pecuária Municipal (PPM) de 2021, o Brasil possui aproximadamente 6 milhões de equinos, o que confere ao país a quarta posição no ranking internacional do rebanho mundial, conforme dados da *Food and Agriculture Organization of the United Stated* (FAO, 2014). Nesse cenário, a reprodução equina apresenta importante papel por ser responsável pelo melhoramento genético da tropa nacional e, consequentemente, pela movimentação econômica gerada na forma de empregos diretos e indiretos, insumos e medicamentos.

Devido a seu alto impacto na capacidade reprodutiva com consequente redução na fertilidade de éguas, a endometrite é responsável pelas maiores perdas econômicas dentro do mercado da reprodução equina (TROEDSSON & NIELSEN, 2018). Tais perdas se relacionam diretamente às possíveis alterações hormonais, vasculares, imunológicas, linfáticas e estruturais geradas pela modificação do endométrio, bem como suas implicações fisiológicas. Falhas na concepção, perdas embrionárias precoces, abortamentos, placentites, septicemia neonatal, metrites puerperais e aumento no intervalo entre partos exemplificam as principais consequências geradas pela endometrite (LEBLANC & CAUSEY, 2009).

Além disso, estima-se que a afecção seja a principal causa de infertilidade em éguas (TROEDSSON, 1999; LIU & TROEDSSON, 2008; LEBLANC, 2010; TROEDSSON & WOODWARD, 2016; CANISSO et al. 2020), o que prejudica a aplicação de biotecnologias, como a transferência de embriões (TE) (SQUIRES et al., 2003; SQUIRES & HON 2009; JACOB et al., 2010). Segundo Squires et al. (1999), a inserção de éguas acometidas por endometrite nos programas de TE reduz em aproximadamente 40-45% as taxas de recuperação embrionária.

Éguas mantidas em programas de reprodução assistida, mesmo que portadoras de alterações uterinas que reduzem suas taxas de fertilidade, geralmente possuem alto valor financeiro e genético. Dessa forma, o controle dos fatores que culminam na inflamação/infecção endometrial é necessário e fundamental para que tais animais continuem a se reproduzir. A

melhoria do ambiente uterino para o recebimento dos espermatozoides e manutenção da viabilidade desses, seguida de um desenvolvimento endometrial saudável para a sobrevivência do embrião são fundamentais (ALVARENGA et al., 2014).

Nesse sentido, o estudo de estratégias, farmacológicas ou não, capazes de controlar, suprimir ou modular o processo inflamatório inerente à cobertura/IA ou aqueles cronicamente instaurados apresenta papel preponderante quando se trata de endometrite. (ALBIHN et al., 2003).

2.2. Ozonoterapia intrauterina

O ozônio (O_3) é uma molécula composta por três átomos de oxigênio, produzidos frente a descargas elétricas ou radiação ultravioleta sobre o oxigênio atmosférico (MANNING & HEDDEN, 2001). Devido à sua alta capacidade oxidante, a molécula exibe forte potencial na inativação de microrganismos (ELVIS & EKTA, 2011). A aplicação do gás pode ser realizada por meio de insuflação, *bagging* ou solução salina e óleo ozonizado (BHATT et al., 2016), sendo tais veículos os de maior utilização dentro da Medicina Veterinária.

Sua ação terapêutica está diretamente relacionada a capacidade do O_3 em atuar como um estressor terapêutico, por meio da ativação de espécies reativas de oxigênio (ROS) (SAGAI & BOCCI, 2011). Estas últimas, operam diretamente em diversos processos biológicos, como a defesa do organismo e ativação do sistema antioxidante enzimático (KIRSCHVINK, MOFFARTS, LEKEUX, 2008; SAGAI & BOCCI, 2011).

A aplicação da ozonioterapia na reprodução animal ainda é incipiente e parece apresentar efeito benéfico sobre a fertilidade, especialmente na espécie bovina (ZOBEL et al., 2014; ESCADÓN et al., 2020) e humana (HE et al., 2015). Em éguas, os recentes estudos publicados sugerem que o tratamento intrauterino com O_3 foi efetivo no controle da inflamação endometrial (ÁVILA et al., 2022), bem como na promoção da angiogênese local sem indução de dano tecidual (FERREIRA et al., 2021).

Apesar dos efeitos benéficos obtidos com a terapia, seu impacto sobre o tecido endometrial, bem como sobre o estado oxidativo ainda é controverso. Em estudo de Almeida e colaboradores (2021), a insuflação uterina com o gás (39 μg O_3 mL^{-1}) em éguas hígidas foi capaz de gerar uma resposta oxidativa sistêmica, com aumento das concentrações séricas de metabólitos associados a capacidade oxidante total e redução da capacidade antioxidante.

2.3. Ultrassonografia Doppler

A ultrassonografia modo Doppler tem sido cada vez mais difundida nos últimos anos, em especial no estudo e acompanhamento da hemodinâmica dos órgãos reprodutivos de grandes animais (GINTHER, 2014). A técnica apresenta a vantagem de ser não invasiva e permite, além da avaliação estrutural, a observação quanto à funcionalidade do sistema em função do grau de vascularização tecidual.

Os primeiros registros de sua aplicação na teriogenologia equina foram realizados por Bollwein e colaboradores (1997), a partir da verificação de mudanças no fluxo sanguíneo da artéria uterina durante todo o ciclo estral. Anos depois, Ginther e colaboradores (GINTHER, 2014) passaram a utilizar a técnica no estudo da dinâmica folicular, onde grandes avanços e descobertas a respeito da vascularização do folículo foram alcançadas (ACOSTA et al, 2004; GASTAL et al., 2006b; GINTHER et al., 2007).

Além da análise de estruturas e dinâmica ovarianas, o exame se mostra como uma importante ferramenta para a avaliação da viabilidade endometrial na espécie equina. Sabe-se que a degeneração de vasos e uma consequente pobre perfusão dos tecidos uterinos é associada à infertilidade em éguas (FERREIRA et al., 2008). Nesse sentido, a técnica tende a ser promissora no acompanhamento de resposta a terapias uterinas (SÁ, 2017).

2.3.1. Obtenção de Imagens Doppler

A tecnologia se baseia na frequência Doppler, gerada a partir da emissão de ondas sonoras pelo aparelho e sua consecutiva interpretação. Esta última, se fundamenta na mudança da frequência de ondas emitidas entre objeto estacionário (transdutor) e objetos refletores (hemácias). Quando a diferença obtida entres estes últimos é positiva, a interpretação tomada é de que as hemácias se direcionam no sentido do transdutor, ao passo de que se frequência negativa for acusada, o sentido dessas células é contrário ao objeto estacionário (BOLLWEIN, 2016). A direção e velocidade dos objetos refletores é representada na forma de diferentes cores, intensidade de pixels ou graficamente (FERREIRA et al., 2011).

De acordo com a interpretação e representação do fluxo sanguíneo realizada pelo aparelho, três modos podem ser distinguidos: *Color-flow*, *Power-flow* ou espectral. Na imagem *Color-flow*, a orientação do fluxo em relação ao transdutor e a pixelagem gerada são codificadas na mesma cor. A área de insonância se torna visível como um local colorido sobre a imagem modo B. Tradicionalmente, imagens com de vermelha representam fluxos que vão de encontro ao transdutor, enquanto azuis, fluxos que se distanciam do mesmo. Cores mais claras representam fluxos mais rápidos enquanto tonalidades mais escuras representam fluxos mais

lentos (GINTHER, 2007). Entretanto, o quadro de cores pode ser alterado pela configuração do aparelho.

A configuração *Power*, por sua vez, apresenta apenas uma escala de cor, que varia de intensidade de acordo com a velocidade do sangue nos vasos. O modo apresenta maior sensibilidade para fluxos de baixa intensidade, bem como independe do ângulo de insonação (GINTHER, 2007). Este último, representa o ângulo de interseção entre o pulso de ondas emitido pela probe e as hemácias, que deve sempre estar entre 30 e 60 graus (GINTHER & MATTHEW, 2004). A característica de independência do *Power* Doppler a este, atrelada a sua maior sensibilidade, o tornam ideal para avaliação uterina, bem como o eximem de possíveis artefatos de imagem (FERREIRA et al., 2011). Em estudo de Abdelnaby e colaboradores (2016), seu uso foi correlacionado com uma melhor e mais precisa avaliação uterina durante todo o ciclo estral, sendo superior ao modo *Color-Flow*.

A avaliação de imagens nos modos *Color-Flow* e *Power-Flow* pode ser realizada de forma objetiva ou subjetiva. Na avaliação objetiva, uma imagem com vascularização máxima é escolhida e os pixels coloridos contabilizados em programas de computador. Por trabalhar com apenas uma imagem, a análise é sujeita à influência do momento e pode não representar suficiente área de amostragem (GINTHER, 2007). Por sua vez, a avaliação subjetiva gradua a sobreposição de pontos coloridos Doppler à imagem ultrassonográfica em modo-B utilizando escores. A atribuição destes é realizada em tempo real, facilitando a identificação do perfil e a análise estatística durante um período e entre os grupos experimentais. Todavia, o método está sujeito ao efeito da subjetividade atribuída ao técnico avaliador (GINTHER, 2007; FERREIRA et al., 2011).

O modo espectral fornece valores exatos de velocidades de fluxo sanguíneo e índices Doppler teciduais (GINTHER & MATTHEW, 2004). Este modo permite quantificar o fluxo sanguíneo de um vaso específico, através do posicionamento de um cursor (gate) no lúmen do vaso escolhido, em uma imagem modo-B ou Doppler colorido. As alterações na velocidade do fluxo sanguíneo são exibidas na forma de gráfico, denominado espectro.

A correta formação do gráfico espectral é altamente dependente do ângulo de insonação (GINTHER & MATTHEW, 2004), o que faz com que a utilização desse modo seja dificilmente aplicada na reprodução animal, devido à tortuosidade dos vasos do sistema reprodutivo. Além disso, o movimento dos órgãos adjacentes ao sistema reprodutivo facilita a formação de artefatos.

Dentre as informações obtidas pelo modo espectral, encontram-se a velocidade de pico sistólico (PSV), velocidade de pico diastólico (ESV) e velocidade máxima média (TAMV). Os índices de resistência (RI) e pulsatilidade (PI) são uma alternativa para minimizar os erros de mensuração das velocidades do fluxo sanguíneo, por não dependerem do ângulo de insonação (GINTHER, 2004). Esses excluem a necessidade do posicionamento supracitado, sendo assim indicados para a avaliação da hemodinâmica do útero e vasos anexos (SILVA et al., 2005). RI e PI arterial apresentam correlação negativa com a perfusão vascular do tecido ou órgão irrigado pelo vaso avaliado (GINTHER, 2007).

2.3.2. Anatomia Vascular do Trato Geniturinário de éguas

Na espécie equina, os órgãos reprodutivos abdominais correspondem ao útero, tubas uterinas e ovários e estão unidos às paredes pélvicas e abdominais pelo ligamento largo (SISSON, 2008). Esse ligamento é constituído por três áreas, que se aderem às estruturas supracitadas e dão origem ao mesométrio, mesossalpinge e mesovário. As porções ligamentosas têm origem lombar e se unem à superfície dorsal dos órgãos do trato genitourinário (GINTHER, 2007). O conhecimento de tais anexos é de suma importância na avaliação por meio de ultrassonografia modo Doppler, uma vez que albergam os leitos vasculares para os órgãos em questão.

O suprimento sanguíneo alocado para útero, tubas uterinas e vagina é realizado por seis ramos arteriais: artéria (a.) ovariana, a. ilíaca externa, a. ilíaca interna, a. uterina, a. pudenda externa e a. vaginal. As artérias ovarianas, ilíaca externa e ilíaca interna se originam diretamente da aorta abdominal, e se ramificam para a formação da a. uterina e a. pudenda externa.

A artéria ovariana está localizada na face dorsal da parede abdominal e se liga cranialmente ao mesovário. Tal vaso é relativamente reto e localizado a uma pequena distância da veia útero-ovária. Além dos ovários, por meio de seu ramo uterino, a artéria fornece o suprimento sanguíneo para as extremidades dos cornos (GINTHER, 2007).

Por sua vez, a artéria vaginal é originada da artéria pudenda interna, um ramo da artéria ilíaca interna. Esta, além de fornecer suprimento sanguíneo para toda extensão da vagina e porção da bexiga, através de seu ramo uterino, irriga cérvix e corpo do útero (GINTHER, 2007).

O suprimento sanguíneo do útero ocorre pela artéria uterina. Esta, é uma ramificação da a. ilíaca externa, que se divide ao adentrar a porção ligamentosa mesometrial (SISSON, 2008). Adicionalmente, como supracitado, o útero também recebe aporte sanguíneo do ramo uterino da artéria ovariana e do ramo uterino da artéria vaginal. Estas três encontram-se interligadas e sua extensão varia individualmente.

2.3.2.1. Arteria Uterina

A artéria uterina se divide em ramos dorsal e caudal. Cranialmente a artéria apresenta menor calibre e é responsável pela vascularização da tuba uterina e do segmento cranial dos cornos. Tal porção sofre anastomose com o ramo uterino da artéria ovariana e com seu ramo caudal. Já caudalmente, forma anastomose com o ramo uterino da a. vaginal e supre o segmento caudal dos cornos. Ambas porções cursam ao longo da borda antimesometrial e dão origem a numerosas ramificações tortuosas, que correm transversalmente ao longo das faces lateral e medial dos cornos uterinos (GINTHER, 2007).

Sua identificação no exame ultrassonográfico deve ser realizada a partir do posicionamento da probe sobre a a. ilíaca externa. Esta última é facilmente visualizada, devido seu maior calibre e disposição longitudinal em relação a aorta. Duas variações individuais quanto à localização da artéria uterina são descritas, podendo apresentar posicionamento posterioir ou anterior à artéria circunflexa profunda. Diâmetros de 2-6mm são descritos em éguas não gestantes com idade entre 6-13 anos, ao passo que 6-7mm são esperados na mensuração da artéria circunflexa profunda nos mesmos animais (GINTHER, 2007).

Apesar de seu pequeno diâmetro, cortes transversais da artéria uterina podem ser obtidos em todo o mesométrio. Em animais idosos uma maior tortuosidade associada ao afrouxamento do ligamento largo é descrita, ao passo que em éguas nulíparas é observada maior dificuldade na sua visualização (GINTHER, 2007).

2.4. Estresse Oxidativo

O estado oxidativo pode ser entendido como o produto da relação entre quantidade de pró-oxidantes e antioxidantes que ocorrem nas células e/ou tecidos. Tal estado está diretamente relacionado à capacidade do sistema biológico desintoxicar de maneira rápida e eficiente os intermediários reativos (espécies reativas de oxigênio- EROS) produzidos pelo metabolismo em atividade. Quando incompetente, um desequilíbrio entre os fatores oxidantes e antioxidantes é gerado, o que culmina no intitulado estresse oxidativo (EO). Esse conduz à oxidação de biomoléculas com consequente perda de suas funções biológicas e desequilíbrio homeostático (BARBOSA et al., 2010).

Os mecanismos endógenos fisiológicos de geração de EROS ocorrem, em sua maioria, nas mitocôndrias, membranas celulares e no citoplasma. A mitocôndria, por meio da cadeia transportadora de elétrons, é a principal fonte geradora de EROS (GREEN et al., 2004). Nessa organela, via enzima citocromo oxidase, o O_2 sofre reduções tetravalentes, dando origem a uma

molécula de água. Tal reação controla a formação de compostos oxidantes, impedindo sua geração em excesso. Entretanto, cerca de 2% a 5% do oxigênio metabolizado é desviado para outra via metabólica e reduzido de forma univalente, dando origem à formação das EROS (FERREIRA & MATSUBARA, 1997).

EROS são moléculas eletricamente instáveis, com elétrons desemparelhados, e altamente reativas. Sua produção apresenta importante papel biológico, por estar diretamente relacionada a diversos processos, como a defesa do organismo. Entretanto, quando em abundância, EROS são capazes de provocar danos a proteínas, lipídios e ao material genético (JONES, 2006, BONI et al., 2022). As principais EROS incluem o radical superóxido (O_2^-), a hidroxila (OH^-), o peroxila (ROO^-) e alcoxila (RO^-). As espécies derivadas do oxigênio não apresentam radicais livres e abrangem: o peróxido de hidrogênio (H_2O_2), o ozônio (O_3), o oxigênio singleto ($^1O_2$) e o hidroperóxido lipídico (LOOH).

O radical OH^- desempenha um fundamental papel na peroxidação lipídica. Através da retirada de um átomo de hidrogênio dos ácidos graxos poli-insaturados da membrana, atua como precursor do processo. Ademais, por possuir a capacidade de alterar qualquer estrutura celular próxima, é reconhecido como o mais reativo dos radicais (BARBOSA et al., 2010).

A peroxidação lipídica (LPO) é uma das importantes causas de lesão e morte celular desencadeada pelo estresse oxidativo. A LPO ocorre a partir de reações em cadeia que levam ao colapso das membranas plasmáticas e à perda da permeabilidade seletiva (BONI et al., 2022). Os principais derivados das reações envolvidas na LPO são o radical hidroxila (OH^-), os radicais peroxinitrito (NO2), hidroperóxidos lipídicos ($LOOH^-$), radial alcoxila (LO^-), aldeídos (MDA), isoprostanos e ácidos graxos insaturados. Todas essas moléculas e radicais podem se difundir em outros compartimentos celulares, danificar proteínas e o DNA, e causar a inibição das enzimas (JONES, 2006). Em vista dos danos gerados pelo EO, o organismo dispõe de uma vasta gama de antioxidantes (KIRSCHVINK; MOFFARTS; LEKEUX, 2008).

Os antioxidantes possuem elevada estabilidade oxidativa em função de sua estrutura molecular e desempenham papel fundamental na prevenção da oxidação resultante da ação dos radicais livres. Eles são classificados conforme sua origem, em enzimáticos e não enzimáticos. As enzimas antioxidantes são superóxido dismutase (SOD), catalase (CAT), glutationa peroxidase (GPx) e Glutationa- S- Transferase. Já os antioxidantes não enzimáticos são compostos por substâncias de origem endógena, como a glutationa, ubiquinona e ácido úrico, ou de origem dietética, como as vitaminas E e C (FLETCHER et al., 2013). O dano oxidativo é detectável sob condições fisiológicas normais em indivíduos saudáveis, o que sugere que as

eficácias dos mecanismos antioxidantes e de reparo não podem evitar completamente a reação de oxidação mediada pelas EROS (JONES, 2006).

A enzima SOD está presente no ambiente mitocondrial (SOD2), intracitoplasmático (SOD1) e extracelular (SOD3). Em conjunto, atua na dismutação do ânion superóxido em H_2O_2 e O_2. Algumas, como SOD1 e SOD2 são dependentes de intermediários, como cobre, zinco (Cu/Zn-SOD1) e manganês (Mn-SOD2) (BARBOSA et al., 2010).

As enzimas CAT e GPx atuam com o mesmo desígnio, impedindo o acúmulo de H_2O_2. A CAT é uma molécula com quatro cadeias de polipeptídios idênticas, cada uma composta por mais de 500 aminoácidos em subunidades, com grupos heme porfirínicos e NADPH. Em altas concentrações é responsável por catalisar a formação de água e oxigênio molecular a partir da molécula de H_2O_2 (FLETCHER et al., 2013). Por sua vez, a GPx realiza a conversão de peróxido de hidrogênio em água sob duas formas: dependente e independente de selênio. A enzima pode se apresentar no citoplasma ou no compartimento mitocondrial e sua ação depende da manutenção do ciclo redox da glutationa, por meio do controle da relação entre glutationa reduzida (GSH) e oxidada (GSSG) (FERREIRA & MATSUBARA, 1997).

Glutationas S-Transferases atuam em rotas de excreção de substâncias endo e xenobióticas, protegendo as células de toxicidade química e estresse, em diferentes tecidos. Essas proteínas, encontradas sob diferentes formas de isoenzimas, são capazes de aumentar a solubilidade de moléculas tóxicas em água, o que facilita a excreção desse conteúdo pela célula (SALINAS & WONG, 1999).

Na reprodução, as EROS possuem importante papel pois atuam na maturação do oócito, ovulação, fertilização, desenvolvimento embrionário e gestação. Elas também têm sido associadas a doenças ovarianas, uterinas e embrionárias, que culminam na queda do desempenho reprodutivo, como as endometrites (KIRSCHVINK; MOFFARTS; LEKEUX, 2008; HANAFI et al., 2008; ABDELNABY, et al., 2020). O estado oxidativo envolvido nas doenças reprodutivas tem sido estudado em animais e humanos, apesar de pouco abordado na espécie equina.

Nas endometrites, o sistema imunológico ativado pode levar a excessiva produção de radicais livres pelas células imunológicas e epiteliais do endométrio (SOLEILHAVOUP et al., 2016). Em trabalho de Abdelnaby e colaboradores (2020), maiores concentrações malondialdeido (MDA) foram encontradas em éguas acometidas pela doença. A desproporção entre o desafio oxidativo e a eficiência da defesa antioxidante nas éguas do estudo é evidenciada pelas menores taxas de capacidade antioxidante total (TAC) detectadas nas éguas acometidas

pela doença. Os biomarcadores do estresse oxidativo mais utilizados na espécie equina são o NO, SOD, CAT, glutationa (GSH), e potencial redox (KIRSCHVINK; MOFFARTS; LEKEUX, 2008).

2.4.1. Éstrogeno e Estresse Oxidativo

Além de atuarem como hormônios sexuais esteroidais, os estrógenos (E_2) são reconhecidamente associados à atividade antioxidante e redução do estresse oxidativo (TANG et al., 1996; AL-GOBORY et al., 2008; UNFER et al., 2014). Seu potencial antioxidante resulta, em parte, de sua estrutura hidroxifenólica, capaz de realizar a remoção de grupos OH^- (SUGIOKA et al., 1987), e de seu efeito estimulador sobre o sistema enzimático antioxidante natural (UNFER et al., 2014; ARIAS-LOZA et al., 2013). Diversos estudos, principalmente voltados para área médica humana, têm sido conduzidos com o objetivo de se delinear métodos capazes de aumentar a capacidade antioxidante, controlando assim a atividade deletéria das EROS. Nesse sentido, diversas evidências de que o estrógeno atua diretamente sobre o status oxidativo de diferentes espécies, com efeito significante sobre enzimas antioxidantes, são disponíveis (STREHLOW et al., 2003; ZHANG et al., 2007; LAGRANHA et al., 2010; ARIAS-LOZA et al., 2013).

Estudos conduzidos em mulheres no período pós-menopausa sugerem que os baixos níveis de E_2 endógenos estão relacionados a maiores concentrações séricas de EROS, danos a lipídios de membrana e menor capacidade antioxidante total (BEDNAREK-TUPIKOWSKA et al., 2004; SÁNCHEZ-RODRIGUES et al., 2012). Nesses estudos, a administração exógena do esteroide resultou no aumento da atividade de enzimas antioxidantes e redução dos metabólitos resultantes de lipoperoxidação. Recentemente, em estudos conduzidos por Vernier e colaboradores (2020) e Tsialtas e colaboradores (2021), uma ligação molecular direta entre a atividade de isoformas de receptores ligados ao E_2 e o controle da produção de antioxidantes *in vivo* foi demonstrada.

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ARTIGO I

How the intrauterine ozone gas treatment can impact on uterine blood flow, local and systemic oxidative stress in mares

Artigo redigido de acordo com as normas do periódico científico *Theriogenology:*

Highlights

1. Intrauterine insufflation with O_2-O_3 gas mixture gas did not modify the systemic oxidative status.

2. Intrauterine insufflation with O_2-O_3 gas mixture promoted a local oxidative stress response evidenced by increase in Nitric Oxide levels in low-volume uterine flush samples.

3. Despite increasing local Nitric Oxide concentrations, intrauterine insufflation with O_2-O_3 gas mixture did not influence endometrial blood flow.

HOW THE INTRAUTERINE OZONE GAS TREATMENT CAN IMPACT ON UTERINE BLOOD FLOW, LOCAL AND SYSTEMIC OXIDATIVE STRESS IN MARES.

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Declaration of Interest: none

Abstract

This study aimed to investigate the impact of the uterine insufflation with ozone gas (O_3) on endometrial blood flow and evaluate its impacts on oxidative stress (OS) biomarkers in samples of blood serum, low-volume uterine lavage (LVL) and endometrial biopsy from treated mares. Nineteen cyclic mares were submitted to 3 sessions of uterine insufflation with gas mixtures containing 44 μg O_3 mL^{-1} (treated group) and 0 μg O_3 mL^{-1} (control group) every 48 hours. Endometrial tissue samples were obtained through uterine biopsy before and after the experimental protocols; LVL's were performed immediately before each session of the treatments and after the last. Blood samples were collected previously the first session until 7 days after treatments finished. The samples were submitted to quantification of superoxide dismutase-SOD, catalase-CAT, malondialdehyde-MDA, total antioxidant capacity-FRAP, total protein-PT, and nitric oxide-NO. Immediately before the beginning and every day after uterine insufflation, the mares were subjected to trans-rectal Doppler ultrasonography to analyze endometrial blood flow. Mares in treated group had a higher concentration of NO in all LVL samples collected after the beginning of the protocol ($P<0.05$). However, no changes between groups were observed in the OS biomarkers obtained from serum ($P>0.05$) and endometrial tissue ($P>0.05$). No differences in the endometrial blood flow measured by Doppler ultrasonography were found ($P>0.05$). In conclusion, uterine insufflation with O_3 gas was not able to induce systemic changes in the oxidative status of mares at the studied concentration but there was alteration on the local OS biomarkers. These findings would be of great value for the development of alternative therapies for infertility in mares.

Keywords: Antioxidants; Total antioxidant capacity; Equine; Nitric oxide; Uterus.

1. Introduction

Infertility and gestational losses are important problems observed in equine breeding, and generate large losses, which causes concern for professionals working in the area. Endometritis is defined as inflammation of the endometrium and ‘it is observed in 25 to 60% of mares considered sub-fertile [1]. In such situations, the choice of the appropriate therapeutic approach is essential for the resolution of the disease.

Endometritis is traditionally treated with a combination of therapeutic uterine lavage, ecbolic drugs, anti-inflammatories and antibacterial agents. However, some mares do not respond to such treatments [2] especially when it comes to the resolution of infectious conditions. In these cases, the empirical administration of antibacterial drugs, based on previous professional experience or on published studies in the area is a common procedure, contradicting the need for confirmation of a positive bacterial culture and sensitivity test. This condition has led to the increasing incidence of microorganisms resistant to antibacterial drugs [3], a fact that justifies the need for developing new therapeutic alternatives [4]. In this sense, according to studies carried out with mares [5] and cows [6], the intrauterine treatment with ozone (O_3) has shown to be somewhat promising.

This technique is simple to apply, has a low cost and presents an antimicrobial and anti-inflammatory response over the endometrium [5]. Moreover, the uterine insufflation of mares with O_3 gas was shown to be effective in promoting endometrial angiogeneis [7]. However, currently, no study had systematically evaluated blood flow changes resulting from ozone therapy in mare’s uterus.

Beside its local effects in the uterus when used in low concentrations, O_3 can act as a therapeutic stressor [9]. Such performance is directly related to its activity on the production and activation of reactive oxygen and nitrogen species (ROS and RNS, respectively) [10]. The production of ROS has an important biological role, since they are necessary for the maintenance of cellular homeostasis. However, when in abundance, they are responsible for

disturbing the oxidative status, culminating in the oxidative stress (OS). This leads to the oxidation of biomolecules with consequent loss of their biological functions and homeostatic imbalance [11].

Lipid peroxidation (LPO) is one of the main causes of cell injury and death triggered by OS. The LPO occurs through chain reactions that lead to the collapse of plasma membranes and loss of the selective permeability, rising well-defined chain end products, as Malondialdehyde (MDA) [12]. OS can also affect proteins and DNA, leading to enzyme malfunction and mutations [12,13]. To avoid oxidative damage caused by ROS the cells have enzymatic systems, as superoxide dismutase (SOD), catalase (CAT) and glutathione S-transferase (GST), that act preventing or minimizing the oxidative damage [14]. The oscillations in this antioxidative defense system and in target molecules that have undergone oxidation, can be used to evaluate OS in tissues [14], as uterus. Despite the potential therapeutic effects of intrauterine ozone therapy already described [5,7], only one study about the effects of intrauterine ozone therapy on OS biomarkers in the equine species has been described [15].

In view of the scarcity of studies evaluating the impact of uterine insufflation with O_3 gas on OS biomarkers and uterine blood flow, we aimed: (1) to evaluate the effects of uterine insufflation in mares with O_3 gas or Oxygen (O_2) on OS biomarkers obtained from blood serum, low-volume uterine lavage (LVL) and endometrial biopsy samples, and (2) to evaluate the effect of uterine gas insufflation on the endometrial blood flow through power-Doppler ultrasonography.

2. Material and Methods

1.1.Ethics Approval

This study was conducted in accordance with the Ethics Committee for the Use of Animals of the Federal University of Viçosa (CEUA-UFV, protocol n. 59/2021).

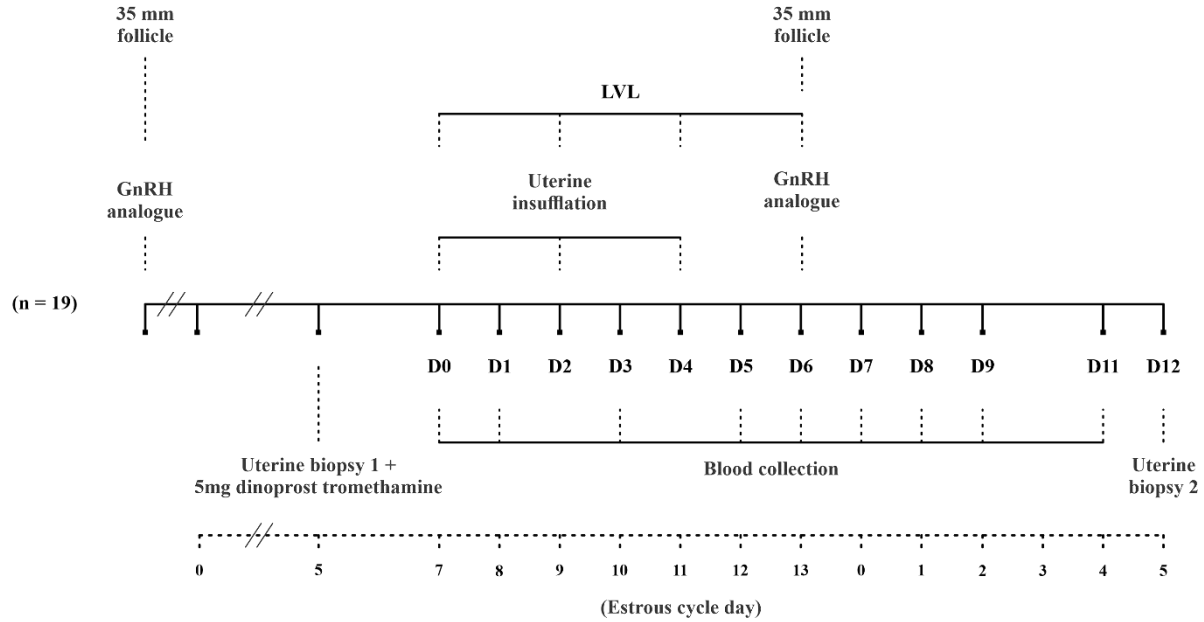

Figure 1. Schematic view of the experimental design. Upon ultrasound contact of a follicle ≥35mm, 250 µg of GnRH analogue was administered. Five days after ovulation (Day 5 of the cycle), the first biopsy was performed (Biopsy 1). By the same time, 5 mg of dinoprost tromethamine i.m. were administrated. Two days later, (day 7 of the cycle), uterine insufflations treatments were performed. Low-volume uterine lavages (LVL) were carried out immediately before treatments (D0=baseline, D2, and D4) and on M6. Doppler evaluations were taken immediately before and every day after insufflations. On D6, another dose of GnRH analogue was administered. The second uterine biopsy was taken five says after the second ovulation (Biopsy 2). Days of blood collection are present (D0= baseline).

1.1.1. Animals

A total of 19 cyclic healthy mares of different breeds, with 3-22 years (8.5 ± 3) and weighting 290-570kg (398 ± 36 kg) were used. The animals were kept in Tifton (*Cynodon spp*.) paddocks and supplemented with adequate feed for the category twice a day. Mineral salt and good quality water were offered *ad libitum*. The study was conducted in the horse breeding center of the Federal University of Viçosa at Florestal, Minas Gerais, Brazil (Latitude: 19° 53' 12" South, Longitude: 44° 25' 56" West), during the breeding season (November to December) of 2021. The body score condition of all animals remained constant [16] throughout the study.

1.1.2. Experimental design

The reproductive tracts of the mares were scanned every 48 hours and at the presence of a follicle ≥35 mm of diameter and uterine edema ≥3 (scored from 1 to 4) the ovulation induction with 250 μg of histrelin (Strelin®, Botupharma, Botucatu, SP, Brazil) was performed. Five days after the ovulation detection (day five of the cycle), a uterine biopsy was collected (Biopsy 1, Figure 1) and the mares received 5 mg of dinoprost tromethamine i.m. (Lutalyse®, Zoetis Brasil, Campinas, SP, Brazil). Forty-eight hours later, at day seven of the cycle, the experimental protocols were initiated (D0, Figure 1): one group of 10 mares received the uterine insufflation procedure with 44 μg mL^{-1} (treated group) and another group, with 9 mares, received the uterine insufflation with 0 μg O_3 mL^{-1} (control group). The intrauterine therapy with O_3-O_2 mixture gas was performed every 48 hours from D0 to D4 (Figure 1) and the mares were randomly distributed into the experimental groups.

Before the insufflation procedure, it was performed LVL with 0.9% NaCl solution with a uterine catheter (Bivona, Minitub©, Porto Alegre, Rio Grande do Sul, Brazil) and the uterus was massaged for 60 seconds transrectally. The recovered fluid was used for later analysis of OS biomarkers dosage. At the end of the LVL, with the uterine catheter already positioned the uterine insufflation with O_3-O_2 mixture gas was carried out. The insufflation was conducted until complete uterine distention, verified by transrectal palpation. At the end of the procedure, the uterus was massaged transrectally, and 20 IU of oxytocin was administered intravenously, in order to guarantee that no gas remained in uterine cavity.

After the last LVL (D4, figure 1) and at the presence of a follicle ≥35 mm of diameter and uterine edema ≥3 on ultrasound evaluation, all mares were submitted to ovulation induction with 250 μg of histrelin intramuscularly. Five days after the ovulation detection the females were assigned to collect a second sample of endometrial tissue through uterine biopsy (B2). Blood collections were performed immediately before starting the protocol (Baseline, D0 - Figure 1) and on days 1, 3, 5, 6, 7, 8, 9 and 11 (Figure 1) for later analysis of systemic OS

biomarkers dosage. Doppler assessment of endometrial blood flow was performed on D0 and on the days after insufflations.

1.2.Sampling and Laboratory Evaluation

1.2.1. Blood Samples

Blood samples were obtained by jugular venipuncture. For this purpose, tubes with clot activator were used. For clot retraction and better serum collection, the samples were stored at room temperature for 30 minutes. Subsequently, samples were centrifuged for 10 minutes at 1,500 x g and the serum obtained stored at -20º C for future analysis.

1.2.2. Low Volume Uterine Lavages

Ten mL from the recovered volume of LVL were transferred to a sterile falcon tube and subjected to centrifugation at 400 x g for 10 minutes. 1% (100 µL) of Triton® X-100 was added to the centrifuge [18]. The samples were homogenized and then again centrifugated at 400 x g for 10 minutes. The supernatant obtained was aliquoted in microtubes and stored at -20 ºC prior to analysis.

1.2.3. Uterine Biopsy

Endometrial tissue fragments were collected with a biopsy forceps from the uterine bifurcation region, as previously described by Kenney and Doig [19]. Immediately after collection, tissue samples were stored at -80 ºC for further analysis.

1.2.4. Endometrial Blood Flow

The endometrial blood flow (EBF) was assessed via power-Doppler ultrasound scan (Z50-VET, Mindray, Shenzhen, China) equipped with a linear-array multifrequency transducer. All examinations were conducted at the same time of the day in order to avoid any interference. The routine scanning started at the caudal portion very close to the cervical internal *os* and the transducer was directed and focused on each horn. Then the longitudinal and cross-sectional planes of the uterine body and horns were scanned. Only the power signals that appeared to be

within the endometrial limits of the uterine body or horns were considered. Doppler gain and filter remained constant during data collection [20].

The images obtained were digitized in 30-second videos and then analyzed subjectively, considering the percentage of power-Doppler signals present in the endometrium of both uterine horns (left horn-LH; right horn-RH). Three evaluators analyzed the images and the average assigned by each one was considered in the present study. The classification used was graded into vascularity scores (0- no vascularity, 4- very vascularized), as suggested by Ginther [20].

1.2.5. OS Biomarkers

OS was evaluated by considering the quantification of the activity of antioxidant substances catalase (CAT), superoxide dismutase (SOD) and total antioxidant capacity (TAC) by the plasma iron reduction method (FRAP), and oxidants, by the quantification of malonaldehyde (MDA). Nitric oxide (NO) and total protein (PT) activity were also measured.

Serum, LVL and biopsy samples were thawed in a water/ice bath at 4 ºC. The endometrial tissue samples were weighed and homogenized with 1,000 µL of phosphate-saline buffer (1:1), aliquoted in microtubes and centrifuged at 1,000 x g at 4 ºC for 10 minutes. The pellets obtained were discarded and the supernatants used for analysis.

For CAT quantification, the hydrogen peroxide decomposition rate measured at time 0, 30 and 60 seconds, as defined by Aebi [21] was evaluated. For SOD, the method proposed by Dieterich [22] was used. TAC was determined through FRAP, in accordance with the methodology proposed by Benzie and Strain [23] and modified by Rubio et al [24]. The quantification of MDA was performed using TBARS solution, as proposed by Wallin et al [25]. For NO measurement, the Griess Reagent System was used [26]. For the calculation of PT in blood, LVL and biopsy samples, the Lowry method [27] was done. All reactions were evaluated at room temperature in a semi-automated microplate spectrophotometer (Multiskan FC©, Thermo Fisher Scientific Inc,Waltham, Massachusetts, USA).

2. Statistical Analysis

For data analyses the Statistical Analysis System (SAS On Demand) was used. Variables were submitted to Bartlett test and Kolmogorov-Smirnov test to verify homogeneity of variances and normality of errors, respectively. When necessary, variables were submitted to statistical transformations, but for clearance results were presented as original scale.

Quantitative variables were evaluated by linear mixed models (Mixed Procedure) according to the model: $Y_{ijk} = \mu + T_i + M_j + (TM)_{ij} + e_{ijk}$

Where: Y_{ijk}, response; μ, constant; T_i, effect of treatment; M_j, effect of the moment of evaluation; $(TM)_{ij}$, interaction; and e_{ijk}, error.

The LS-means were compared by Tukey test (LITTELL et al., 2006). The repeated measures factor was the day in each level of animal (subject).

3. Results

3.1.Effect of uterine insufflation on local OS biomarkers

No difference (P>0.05) between O_3 and O_2 groups was observed for CAT, SOD, MDA and PT quantified in LVL. However, in mares exposed to treatment with 44 µg O_3 mL^{-1}, a higher concentration of NO (P<0.05) was observed in all LVL samples collected after the beginning of the experimental protocol (Figure 2).

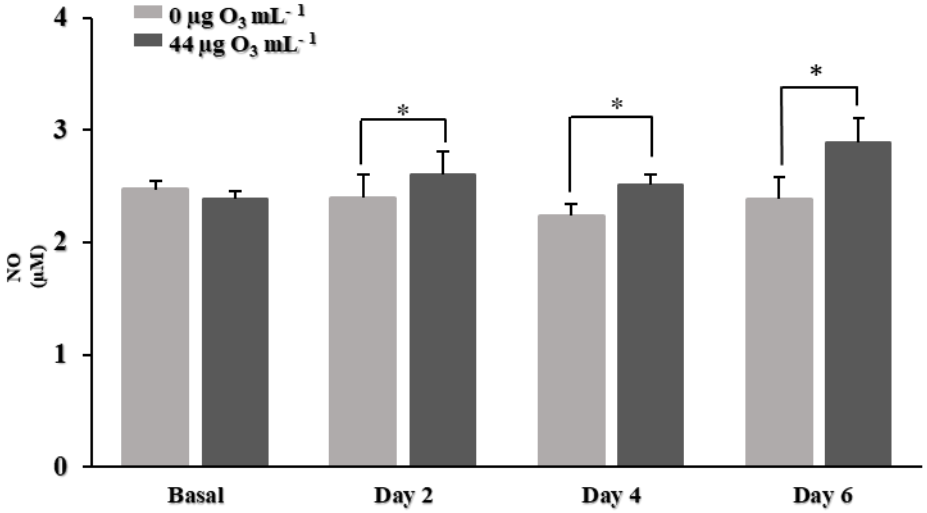

Figure 2. MEAN±SEM of nitric oxide (NO) concentrations in low-volume uterine lavage samples immediately before treatments (basal) and after uterine insufflation with 0 µg

O_3 mL^{-1} or 44 µg O_3 mL^{-1}(*n*=19). * Indicates statistically difference between groups within a same day ($P < 0.05$) by Tukey's test.

The treated and control groups presented higher concentrations ($P<0.05$) of CAT and FRAP quantified in biopsy 1 in relation to biopsy 2. However, no differences ($P>0.05$) in the OS biomarkers from uterine biopsy samples among groups were found (Figure 3).

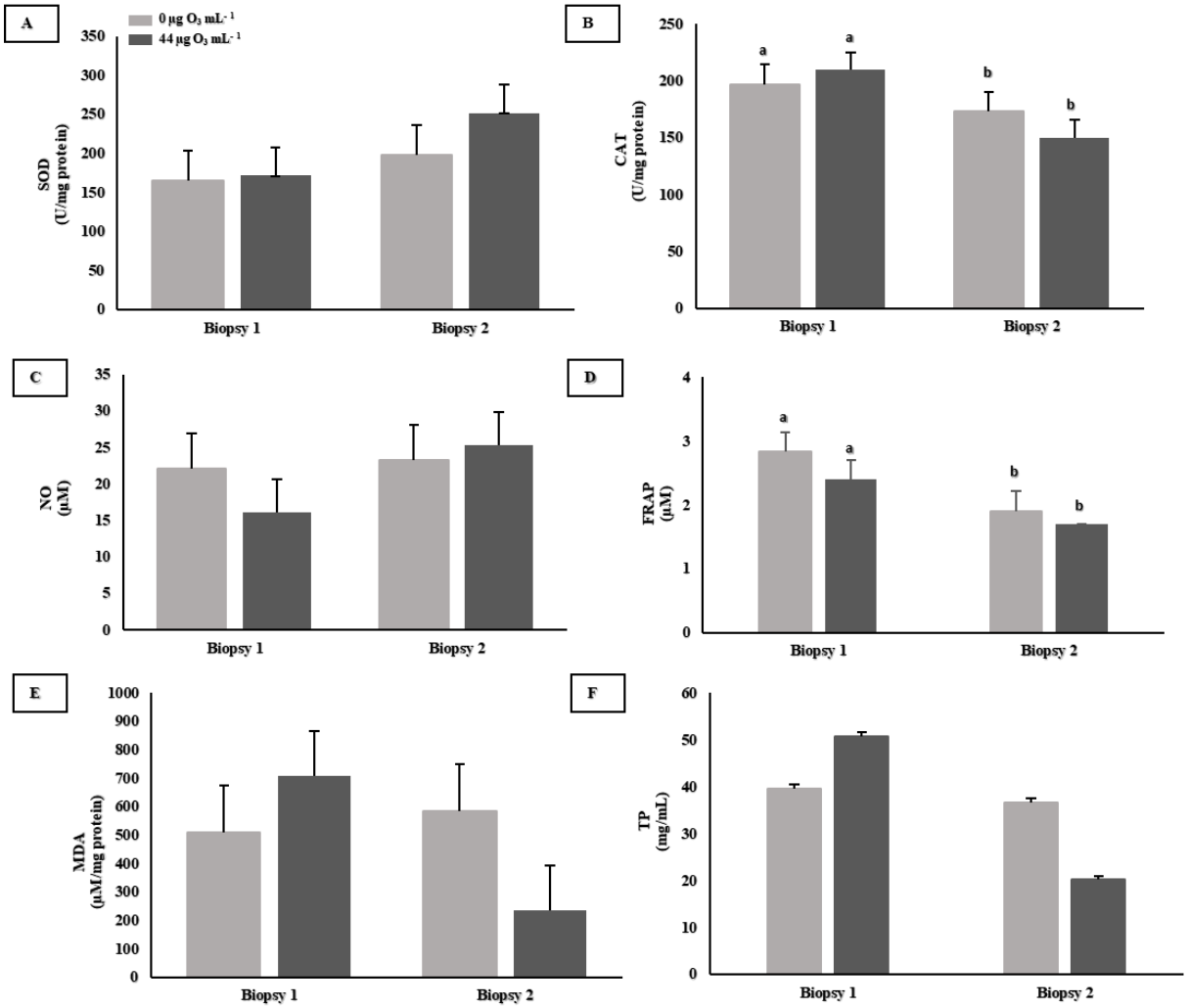

Figure 3. MEAN±SEM for endometrial oxidative stres biomarkers of cycling mares submitted to uterine insulation with 0 or 44 µg O_3 mL^{-1} (*n*=19). Oxidative biomarkers (A) Superoxide Dismutase (SOD), (B) Catalase (CAT), (C) Nitric Oxide (NO), (D) Total Antioxidant Capacity (FRAP), (E) Malonaldehyde (MDA), and (F) Total protein (TP) are shown in the graphs before

(Biopsy 1) and after (Biopsy 2) the insufflation protocol with0 µg O_3 mL^{-1} or .44 µg O_3 mL^{-1}. [a,b] The means with different letters within the same group are different ($P \leq 0.05$).

3.2.Effect of uterine insufflation on systemic OS biomarkers

The oxidative serum stress biomarkers were not affected (P>0.05) by the intrauterine treatments (Figure 4). Further, no differences in moments for each treatment or in the interaction between moments and treatments were found.

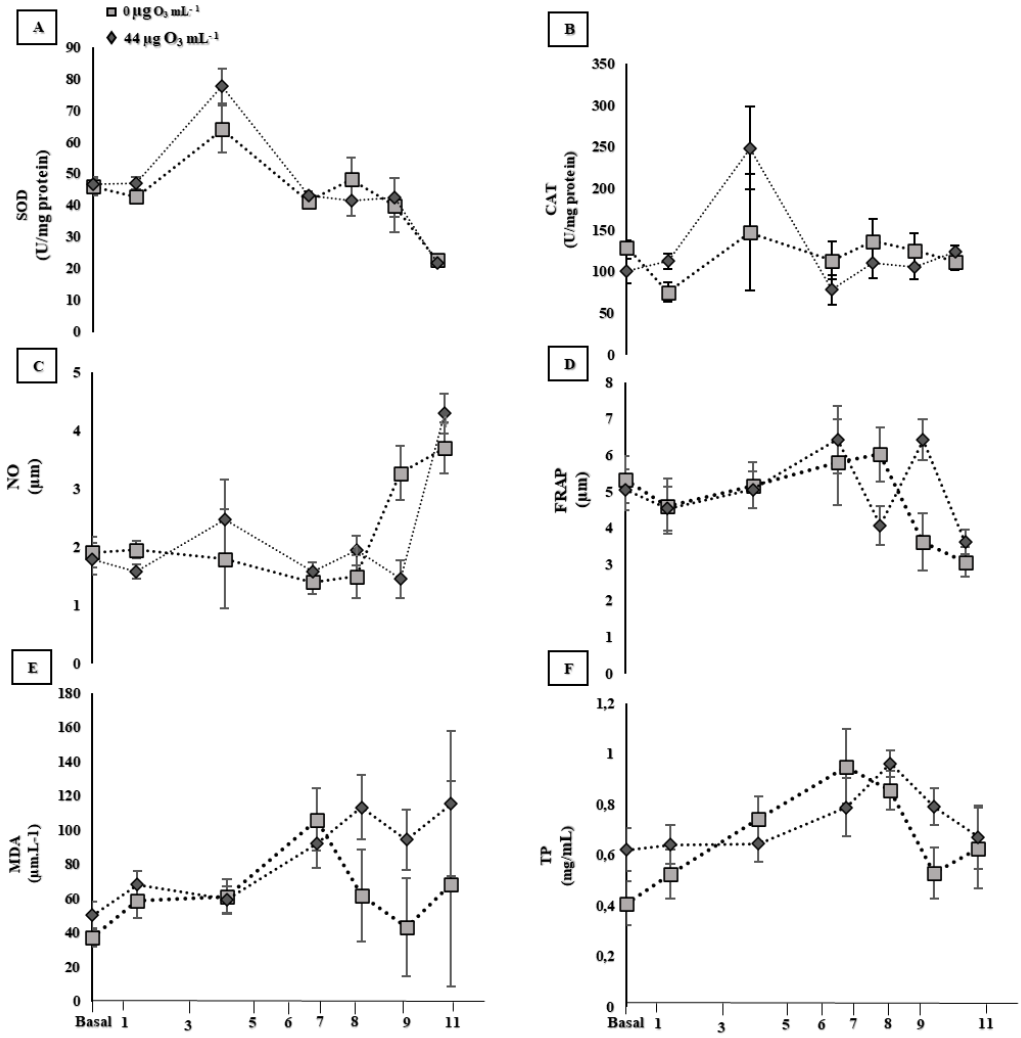

Figure 4. MEAN±SEM of serum oxidative stress biomarkers from mares in cyclic activity (*n*=19). The activity of (A) Superoxide dismutase (SOD), (B) Catalase (CAT), (C) Nitric Oxide, (D) Total antioxidant capacity (measured by FRAP), (E) Malonaldehyde (MDA), and (F) Total

protein (TP) are shown in graphs from moment 0 (D0-baseline) to day 11 after application of mixture containing 0 µg O_3 mL^{-1} or 44 µg O_3 mL^{-1} every 48h from D0 to D4.

4.3. Effect of uterine insufflation on endometrial blood flow

No differences in the endometrial blood flow between O_3 and O_2 mares, in moments, or in the interaction between groups and moments were found (P>0,05; Table 1).

Table 1. Mean ± SEM of endometrial blood flow in mares treated with intrauterine O_3 (n=10) or O_2 (n=9).

Moment	**O_3 (44 µg O_3 mL^{-1})**		**O_2 (0 µg O_3 mL^{-1})**	
	RH	LH	RH	LH
D0	1.4 ± 0.27	1.15 ± 0.28	1.56 ± 0.32	1.45 ± 0.33
D1	1.83 ± 0.27	1.68 ± 0.28	1.64 ± 0.32	1.66 ± 0.33
D3	1.37 ± 0.27	1.31 ± 0.28	1.43 ± 0.32	1.39 ± 0.33
D5	1.24 ± 0.27	1.06 ± 0.28	1.33 ± 0.32	1.39 ± 0.33

RH-Right horn; LF-Left horn.

Discussion

This is the first study to describe the effects of uterine insufflation with the O_3-O_2 mixture gas on local OS biomarkers. Under the current study conditions, the endometrial exposure to O_3 gas was able to induce an increase in the uterine NO. The greater NO activity verified after intrauterine treatment with O_3 gas is in accordance with the known regulatory action of it on genes associated with the expression of the enzyme nitric oxide synthase (NOs), responsible for catalyzing its synthesis [29]. NO is an inorganic free radical, which has, in addition to one unpaired electron, seven nitrogen electrons and eight oxygen electrons [30]. Its formation occurs from the oxidation of the essential amino acid L-arginine by the enzymatic activity of NOs [31]. In addition to acting as a vasodilator [32], in reproductive organs, is known to be involved in multiple functions, such as hemodynamics [33] and uterine contractility [34], vascularization of ovarian structures [35,36,37], follicular development, and ovulation

[38,39,40]. In the uterus, the control of NO activity is strongly related to the action of leptin, an important angiogenic factor [41]. Also, it is associated with greater vascularity of the organ when detected at high concentrations in the plasma of female horses [33]. Little is known about the endometrial angiogenic capacity of NO, however, *in vitro* studies in the equine species suggest that the radical may be a mediator in this process, through TNF and ovarian steroids [42]. In a recent report, Ferreira et al. [7] demonstrate a favorable effect of the infusion of a O_3-O_2 mixture containing 42µg $O_3.ml^{-1}$ on endometrial angiogenesis in healthy mares, however, the signaling pathways responsible for the angiogenic stimulus remain uncertain. Our results, yet, corroborate the hypothesis that pathways associated with the action of NO on the vascular system are responsible for what was observed by Ferreira et al. [7], since this was shown to be increased in the LVL of all mares on which ozone was used.

Despite higher concentrations of NO in the LVL samples collected from O_3 mares, no differences between groups were found in the analysis of uterine biopsy. We associate this finding with the moment when the tissue fragment was collected. Since the oxidative response associated with a beneficial application of O_3 therapy must be transient and controlled [43], elevations of the metabolite or others related to oxidative damage, as MDA, in endometrial tissue after an average of eight days between the last uterine insufflation were not expected. Also, no difference in endometrial blood flow between treated and control groups was observed. Taking in account the concentrations of NO in LVL samples, it conceivable that they may had been insufficient to generate a detectable increase in the blood flow by Doppler US.

In the present study, higher values of CAT and FRAP were detected in biopsy 1 when related to biopsy 2 in both groups. The CAT is a peroxidase that has the role of degrading hydrogen peroxide into oxygen and water, being responsible for the first line of cellular antioxidant defense. This enzyme is found in the cytoplasm and in high concentration in the peroxisomes of all animal tissues [21]. The FRAP, in other ways, indicates the binding capacity of ferritin to iron, indirectly implying the performance of the antioxidant system, once higher

amount of free iron can increase the generation of OH- radicals, and consequently, oxidative damage [45]. In their report, Ferreira et al. [7], also discuss the effect of intrauterine treatment with O_3 on endometrial tissue integrity in mares. The authors reported a discrete inflammatory infiltrate, characterized by the presence of mononuclear phagocytes, after the end of uterine insufflation in treated and control mares. Taking in account the intracellular occurrence of CAT, it seems plausible that, the decrease in the enzyme observed in both groups must be secondary to an endometrial reepithelization, since the inflammatory process, evidenced by mononuclear phagocytes infiltrate in the above-mentioned study, could lead to it [46]. Also, it's conceivable that the free iron concentration could be decreased by the reepithelization process, which could be responsible for the FRAP reduction.

No changes OS biomarkers quantified in blood serum were observed. In a study of Almeida et al.[15], an increase in total oxidant activity and a decrease in TAC (FRAP) after intrauterine O_3 treatment was reported. In their work, 5mg of Estradiol Benzoate were applied one day before the beginning of the intrauterine treatment with O_3. The effect of exogenous estrogen on uterine blood flow is well stablish [47,48,49,50]. In mares, the oral administration of estradiol benzoate is associate with increases in uterine blood flow [51]. In addition, the steroid is also correlated with increased endometrial microvascular permeability [50, 52]. Considering the physiological effects on the uterus generated by the administration of the hormone, it seems reasonable that in the previous study, the effect of the O_3-O_2 mixture on systemic OS was potentiated by the previous administration of Estradiol Benzoate, since this presumably leads to an increase in endometrial vascular permeability, and consequently, greater systemic diffusion of the therapeutic mixture. Despite the similar study design, the dose, and interval of application can be also attributed to the differences between results. Thus, the debate continues as to determine the true nature of ozone on mare's systemic OS.

Although being considered as one of the benefits of medical ozone therapy [43], OS is related to irreversible modification of proteins backbones, chain fragmentation, and oxidation

of amino acids, which could lead to cellular functionality and integrity damage [12,13,14,45]. Studies assessing OS biomarkers in horses undergoing ozone therapy are scarce, and no concrete conclusion about the benefits or harms of OS caused by its use are available, especially in the uterine treatment of mares. It should be noted that in the present study, intrauterine therapy with O_3 was not capable of modifying the biomarkers of tissue OS when compared to O_2 insufflation, nor did it demonstrate a significant impact on the systemic oxidative status. Thus, despite the lack of standardization for uterine ozone therapy in mares and taking in account the nature of OS, our protocol proved to be safe. In addition, the instituted protocol shows promise in terms of application in mares affected by endometritis, since in these, an oxidative process evidenced by higher concentrations of MDA and TAC reductions had been recently described [53]. Although promising, studies conducted with the gas therapy and aimed at evaluating the oxidative status in the presence of the pathological condition of reproductive organs are still necessary.

4. Conclusion

Under the conditions of the present study, no difference regarding the modification of the oxidative status was observed when compared to uterine insufflation with a gas mixture containing O_2-O_3 or only O_2. Likewise, no difference in endometrial blood flow was observed between groups. Although changes in blood serum were not stimulated by the application of the gaseous mixture, the treatment with intrauterine O_3 gas is capable of inducing a local increase in nitric oxide.

5. Conflict of Interests

The authors declare that they have no personal or financial relationship related to the content and publication of the manuscript.

6. Acknowledgments

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ARTIGO II

The impact of exogenous estrogen on systemic oxidative stress regulation and uterine blood flow in mares

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Highlights

- Estradiol benzoate acts on the systemic oxidative parameters in mares
- Estrogen therapy was associated with increased antioxidant defense system and uterine bold flow in anestrous mares
- Lipid peroxidation can be induced by exogenous estrogens

The impact of exogenous estrogen on systemic oxidative stress regulation and uterine blood flow in mares

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Abstract

The administration of exogenous estrogen (E_2) in mares is a common tool at equine reproduction routine but with no clear impacts that it may have on systemic parameters like oxidative stress regulators or on local conditions like uterine blood flow. The experiment aimed to study the effects of estradiol benzoate (EB) administration on uterine blood flow collected by doppler parameters and oxidative stress biomarkers in the serum of mares. For this purpose, 10 mg of EB were administered intramuscularly in 16 mares in anestrus. Blood serum samples were obtained every 48 hours from D0 to D4 (D0 = moment immediately before EB treatment), followed by the evaluation of endometrial blood flow (EBF) and middle uterine arteries indices (resistance index, RI and pulsatility index, PI). Oxidative stress biomarkers were assessed through the quantification of catalase (CAT), superoxide dismutase (SOD), total antioxidant capacity by the ferric reducing antioxidant power (FRAP) assay, malonaldehyde (MDA), total protein (TP) and nitric oxide (NO). Higher values of CAT, FRAP and MDA activity were observed in D4 after EB application compared to D0 ($P<0.01$). No differences were observed for SOD and NO ($P>0.05$). Lower PI of the left and right uterine arteries was observed in D4 after EB application ($P<0.05$). The assessed RI values showed lower averages for the right uterine artery at the same time ($P<0.05$). Furthermore, greater EBF was obtained on D2 and D4 ($P<0.01$). In conclusion, the exogenous administration of estrogen can boost the antioxidant enzymatic system and uterine blood flow of anestrous female mares, which may be beneficial, especially in controlling oxidative processes and reproductive diseases. Furthermore, this is the first known report to document the impact of E_2 on oxidative stress biomarkers in mares.

Keywords: Antioxidant system; Doppler ultrasonography; Horse; Steroid hormone.

1. Introduction

Oxidative stress is a disturb among oxidative and antioxidant agents (Sies, 1991), resulting from an imbalance between the amount of reactive oxygen species (ROS) produced by the metabolic processes and the organic capacity to detox the intermediates or the difficulty to repair the damage by an appropriate antioxidant defense system. In proteins, the oxidative damage can be reflected by the irreversible modification of the protein backbone, chain fragmentation and oxidation of amino acids side chains and lipoperoxidation (LPO), which leads to alterations of cellular membrane, damaging its functionality and integrity (Bergamini et al., 2004). Because of this potential harm, the body presents a wide range of antioxidants that aim reduce and inhibit the damage caused by the deleterious action of ROS (Kirschvink et al., 2008).

A combination of endogenous enzymes as superoxide dismutase (SOD), catalase (CAT) and gluthatione peroxidase (GP) and other substances like selenium, zinc, vitamin C and carotenoids can act together preventing or minimizing the oxidative damage (He et al., 2017). The regulation of antioxidant enzyme activity may be influenced by such factors as age, hormonal state and organ specificity. Since hormones regulate metabolic activities and metabolic activities need O_2, it is clear that any alteration at hormonal levels in aerobic cells might have some effect on the production of ROS.

Certain hormones are structurally disposed to act as antioxidants themselves or have impact upon the several enzymatic and nonenzymatic components of the antioxidant defense system (Chainy and Sahoo, 2020). There is evidence that estrogen have a deep influence on oxidant-antioxidant balance in many tissues and it is associated with antioxidant activity and reduced oxidative stress (Tang et al., 1996; Al-Gubory et al., 2008; Unfer et al., 2014). The antioxidant potential of estrogens is part from their hydroxyphenolic structure, capable of removing hydroxyl (OH) groups (Sugioka et al., 1987) and from its stimulating effect on natural antioxidant enzymatic system (Arias-Loza et al., 2013; Unfer et al., 2014).

Some biomarkers of oxidative stress can be invalidated with exogenous estrogen administration (Costa et al., 2015; Hao et al., 2016). In blood, menopause renewal induced oxidative stress marked by the transformation of estrogen redox state to normal levels establishing antioxidant behavior of oestradiol (Bellanti et al., 2013; Azizieh et al., 2019). Studies conducted in postmenopausal women suggest that low levels of endogenous estrogen are related to higher serum concentrations of ROS, more membrane lipids damages and weak antioxidant capacity (Bednarek-Tupikowska et al., 2004; Sánchez-Rodrigues et al., 2012). These studies showed that exogenous administration of the steroid culminated on the increased activity of antioxidant enzymes and reduction of metabolites from lipoperoxidation (LPO). Vernier et al. (2020) and Tsialtas et al. (2021) demonstrated a direct molecular link among the activity of estrogen-linked receptors isoforms and the control of antioxidant production *in vivo*.

In addition to its antioxidant effects, estrogen is related to increase uterine blood flow (UBF) (Greiss and Anderson, 1970; Ford and Chenault, 1981; Abdelnaby et al., 2016; Esteller-Vico et al., 2016). According to Bollwein et al. (2004), the application of 5 mg of estradiol benzoate in mares in the estrus period increased the UBF and in a study conducted by Esteller-Vico et al. (2016) was revealed that application of 17β-estradiol had a vasodilator effect in endometrial vessels. These finds are important because poor perfusion of uterine tissues is associated with infertility in mares (Bollwein et al., 2004).

Although the relationship between plasma levels of estrogen and UBF during estrous cycle is well established in the equine species (Bollwein et al., 2002; Abdelnaby et al., 2016), there are still few studies that evaluate the effect of its exogenous administration on uterine hemodynamic parameters in mares. Considering also the antioxidant effect obtained with the exogenous administration of estrogen, the purpose of the present study was to describe the uterine hemodynamic parameters and serum oxidative stress biomarkers of mares treated with estradiol benzoate.

2. Material and Methods

2.1. Ethical approval

This study was carried out in accordance with the Ethics Committee for the Use of Animals of the Federal University of Viçosa (CEUA-UFV, protocol n. 59/2021).

2.2. Animals

A total of 16 Mangalarga Marchador mares, clinically healthy, aged between 5 and 10 years old (7.5 ± 1.0 years) and weighting between 320 and 380 kg (351 ± 11 kg) were used. The study was carried out during the anestrus period at a breeding center located in Minas Gerais, Brazil (Latitude: 21° 1' 2" South, Longitude: 42° 50 ' 16" West). The mares were kept in Tifton paddocks with mineral salt and water *ad libitum*. The body score condition of all females was scored >7 throughout the experiment period (Henneke et al., 1983).

All females were evaluated by transrectal palpation and ultrasonography to establish their reproductive condition before the sample collection. Only mares without macroscopic abnormalities in the reproductive tract, no corpus luteum and follicle <20 mm in the ovaries were used.

2.3. Experimental design

All females underwent intramuscular administration of 10 mg of estradiol benzoate (EB; BER-Ric Be®, União Química Farmacêutica Nacional S/A, São Paulo, SP, Basil). The Middle Gluteal muscle was used as injection site. Blood sampling and Doppler exams were performed every 48 hours from D0 to D4 (D0 = moment immediately before EB treatment).

2.3.1. Oxidative stress assays

Blood samples were collected through jugular venipuncture using tubes with no additive. The aliquots obtained were centrifuged for 10 minutes at 3,000 *g* and the serum was stored at -20 ºC.

Activity of catalase (CAT), superoxide dismutase (SOD) and total antioxidant capacity (TAC) by the ferric reducing antioxidant power (FRAP) assay, and oxidants, through the quantification of malonaldehyde (MDA) were measured to evaluate oxidative stress. Nitric

oxide (NO) activity was also quantified. All reactions were evaluated at room temperature in a semi-automated microplate spectrophotometer (Multiskan FC©, Thermo Fisher Scientific Inc, Waltham, Massachusetts, USA).

2.3.1.1. Catalase Activity

Catalase activity was measured according to the method proposed by Aebi (1984). The reaction mixture contained 10 µL of sample, 1 mL of phosphate buffer (50 mmol/L, pH 7.0) and addition of 1 mL of H_2O_2. Posteriorly, the hydrogen peroxide decomposition rate was measured at 0, 30 and 60 seconds at 240 nm. A molar extinction coefficient of ε240 = 0.036 mmol/L cm was considered. The result was given in units (U) per mL.

2.3.1.2. Superoxide Dismutase Activity

The SOD activity was based on the reduction of the superoxide radical (O_2^-) and consequent decrease in the 50% auto-oxidation ratio of pyrogallol (Dieterich et al.,2000). Aliquots of 99 µl phosphate buffer (5 mmol/L, pH 7) and 15 µL pyrogallol (100 µmol/L) were added to 30 µL of the sample. The absorbance reading was done at 570 nm. The SOD unit was defined as the amount capable of inhibiting 50% self-oxidation of pyrogallol, with the final concentration calculated in U/mL

2.3.1.3. Total Antioxidant Capacity Determination

The evaluation of the total antioxidant capacity was performed based on the ferric reducing antioxidant power (FRAP) assay as proposed by Benzie and Strain (1996). In polystyrene microplates, 10 µL of sample was added to 220 µL of FRAP solution and then incubated for 30 minutes in the dark. Trolox solution (2 mM/L) was used as an oxidizing agent. The readings were performed at a wavelength of 593 nm and the concentrations related to the samples were expressed in nmol/mL.

2.3.1.4. Malondialdehyde determination / Thiobarbiturate Assay (TBARS)

The LPO rate was obtained considering malondialdehyde quantifications (Buege and Aust, 1978). A hundred µL of sample and 500 µL of TBARS solution (15% trichloroacetic acid,

0.38% thiobarbituric acid and 0.6% hydrochloric acid) were added into a 2 mL microtube. The mixtures were incubated in water bath at 100 °C for 40 min. Immediately after the water bath, the samples were immersed in ice for 10 minutes and 750 µL of butanol was added. The mixture was centrifuged at 3,000 *g* for 10 minutes and the supernatant was transferred to a polystyrene microplate (200 µL/well). The reading was performed at 535 nm and the results expressed in nmol/mL.

2.3.1.5. Production of Nitric Oxide

The Griess reagent method was used to quantify NO concentration. Fifty µL of sample and 50 µL of reagent (1% sulfanilamide, 0.1% ethylene naphthyl amide dihydrochloride and 2.5% phosphoric acid) were mixed and incubated in polystyrene microplates at room temperature for 10 min (Tsikas, 2007). Absorbance was measured at 540 nm. A standard curve of sodium nitrite (0-100 µM) was used to calculate de NO concentrations and the final unit was expressed in nmol/mL.

2.3.2. Doppler ultrasound exam

Mindray Z50-VET (DPS, China) equipped with a 6.5 MHz linear transducer was used to perform the Doppler ultrasound exam of the endometrium. Pulse range velocity, Doppler gain and filter, remained constant during data collection (Ginther, 2007). The exams were performed through slow scanning of the left and right uterine horns using power Doppler, obtaining 30 second videos for later analysis.

Endometrial blood flow (EBF) was analyzed subjectively, considering the extent of Doppler signals present in the cross-sectioning of the uterus during the recorded videos. Only color signals that appeared to be within the limits of the endometrium were considered. Three evaluators analyzed the images at different time and places, and the average score was considered. The classification was graded into vascularity scores (0- no vascularity, 4- very vascularized), as suggested by Ginther (2007).

Spectral assessment of Doppler indexes (pulsatility PI, and resistivity RI) were performed as described previously by Silva et al. (2005). To produce a spectral waveform, the sample-gate cursor was placed on each middle uterine artery. Spectral waveform, with a minimum of three cardiac cycles, was generated and one of the cycles was used to measure the the indexes. The procedure was repeated twice more, and the average was used for statistical analyses. The following settings were used: sample-gate cursor of 1 mm width, 25cm/s speed, and 100 Hz filter (Abdelnaby et al., 2020).

2.4. Statistical analysis

Data were analyzed using the statistical program IBM SPSS Statistics 27. The Shapiro Wilk test was applied with a 5% probability of error for data normality. Means were compared in relation to D0, through the t-test of paired samples when variables presented normal distribution and by Wilcoxon test when variables were considered non-parametrical or for qualitative category data. All analyzes were performed considering a 5% probability of error. Pearson's correlation was also used to determine correlation between the quantitative datas.

3. Results

3.1. Oxidative stress biomarkers

The activities of SOD, CAT, FRAP, MDA, PT and NO before and after EB application are shown (Figure 1). A positive effect of treatment was found for CAT, FRAP and MDA. Higher ($P<0.01$) enzymatic activity values were obtained for CAT (121.89 ± 60.59 U/mL) and

FRAP (5.25 ± 1.03 µmol/mL) after 96h (D4) of EB application in relation to D0. No statistical difference was observed between the compared times for SOD activity (P>0.05).

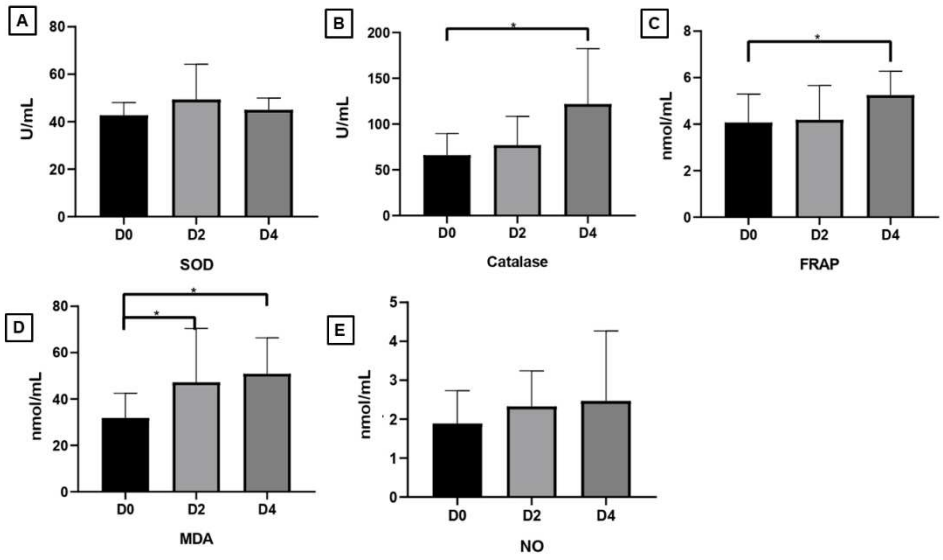

Figure 1. Serum oxidative stress markers from seasonal anestrus mares (n=16). Means (±S.E.M) of oxidative stress markers Superoxide dismutase (SOD), Catalase (CAT), Ferric reducing antioxidant power (FRAP), Malonaldehyde (MDA), and Nitric Oxide (NO), are shown in the graphs from day 0 (baseline) to D4 after application of 10 mg of Estradiol Benzoate. Means followed by * within the same treatment differ by the t-test (P≤0.05).

Higher MDA values were observed in D2 (47.22 ± 23.20 nmol/mL) and D4 (50.87 ± 15.52 nmol/mL) compared to D0 (31.85 ± 10.63 nmol/mL). The EB treatment did not affect (P>0.05) the serum concentration of NO of anestrous mares (Figure 1).

3.2. Uterine blood flow

Significant differences (P<0.01) were observed between the EBF's from D0 to D2 and D0 to D4 in both uterine horns (Figure 2). The uterine vascular perfusion was positively affected (P<0.01) by the EB treatment (Table 1).

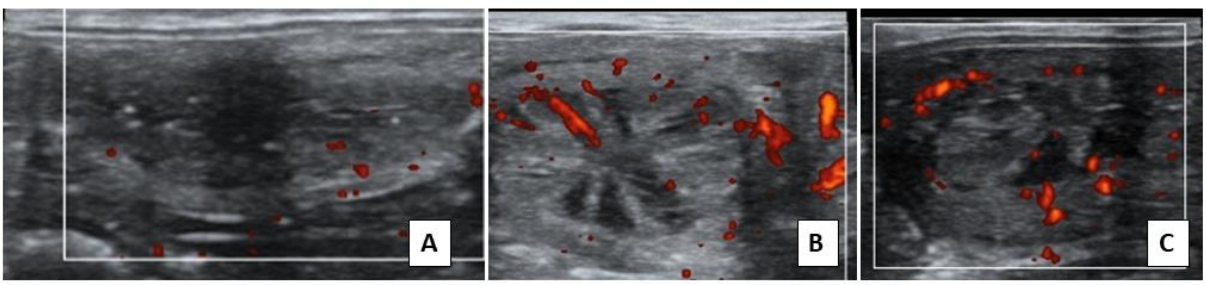

Figure 2. Power Doppler exams of a uterine horn on days 0, 2 and 4 (D0=moment immediately before EB treatment).

Lower RI were obtained (P<0.01) from the right uterine artery on D4 compared to D0 (Table 1). However, the RI from the left uterine artery was similar among the days (P>0.05; Table 1). The PI from the right and left uterine arteries significantly decreased between days D0 and D4 (P<0.05; Table 1).

Table 1. Mean (SD) of uterine vascular perfusion before and after exposure to 10 mg of estradiol benzoate.

Parameter*	D0	D2	D4	*P* value
RI left	0.67± 0.05	0.64 ± 0.66	0.68 ± 0.08	>0.05
RI right	0.68 ± 0.06 [a]	0.66 ± 0.08 [b]	0.64 ± 0.05 [a]	<0.05
PI left	1.59 ± 0.34[a]	1.40 ± 0.25[a]	1.42 ± 0.28[b]	<0.05
PI right	1.56 ± 0.28[a]	1.43 ± 0.32[a]	1.35 ± 0.24[b]	<0.05
EBF left	0.93 ± .41[A]	2.75 ± 0.62[B]	2.77 ± 0.44[B]	<0.01
EBF right	0.94 ± 0.52[A]	2.70 ± 0.72[B]	2.86 ± 0.44[B]	<0.01

[a, b] Means followed by different lowercase letters within the same parameter differ from each other by the paired-samples t-test in relation to D0 (P≤0.05).
[A, B] Means followed by different capital letters within the same parameter differ from each other by the Wilcoxon test in relation to D0 (P≤0.05).
*Resistivity Index (RI), Pulsatility Index (PI) and Endometrial Blood Flow (EBF).

4. Discussion

The present study provides for the first-time solid evidence of the role of estrogen in the regulation of antioxidant and oxidant activity in mares. Apparently, the administration of EB in anestrous mares induced an increase of serum CAT concentrations, which can be associated with a reduction of hydrogen peroxide (H_2O_2). This peroxidase degrades hydrogen peroxide into oxygen and water, being responsible for the first line of cellular antioxidant defense. Hydrogen peroxide (H_2O_2) is the main functional group of reactive oxygen species, responsible for LPO, DNA degradation (Halliwell and Aruoma, 1991) and separation of cytochrome C from

mitochondria, which culminates in death cell (Li and Nijihawan, 2000). Borras et al. (2010) and Song et al. (2009) also showed *in vitro* reductions in cellular H_2O_2 concentrations after treatment with exogenous estrogen. In this sense, CAT can play an important role in the antioxidant defense system by scavenge hydrogen peroxide, a precursor of more deleterious radical species (Ighodaro and Akinloye, 2018).

In addition to the increased CAT activity, mares treated with EB showed higher concentrations of FRAP. The total antioxidant capacity, measured by the FRAP assay, allows an evaluation of the entire performance of the antioxidant system and proves to be very useful in the analysis of the estrogen effect on oxidative stress. Higher FRAP indicates a higher binding capacity of ferritin to iron and, consequently, a lower amount of free iron, reducing the generation of OH- radicals, through the reactions of Fenton and Haber-Weiss (Welch et al., 2002). This fact can be considered an important mechanism of defense by estrogen role due to deleterious action of OH- radicals in the organism which have a small half-life and high reactivity. Because of the direct activity of the enzymatic system on physiological events, changes in oxidative status can indicate how an organ or system responds to adjustments in the endocrine environment under physiological conditions *in vivo* (Al-Gubory et al., 2008).

Beyond the increase observed in CAT and FRAP, higher concentrations of MDA were obtained 48 and 96 hours after the steroid application. Gómez-Zubeldia et al. (2001) reported that the treatment of female ovariectomized rats with different doses of 17β-estradiol, plasma E_2 levels <20 pg/mL or >200 pg/mL was associated with an increase in lipid peroxidation by measuring the values of MDA and, for plasma E_2 levels between 20 and 200 pg/mL there was a decrease in MDA concentration and, thus, a decline in lipid peroxidation. Despite contradictory actions of estrogen on lipid peroxidation it has been already described that dose (Markides et al., 1998), age (Schwenke, 1998), specificity of the organ or tissue (Nathan and Chaudhuri, 1998) and/or the chemical structure of the estrogen being used (Liehr and Roy, 1998) are influencing factors. Considering the half-life of estradiol benzoate (Greiss and

Anderson, 1970), it seems plausible that, in the present study, the higher systemic concentrations of MDA observed could be secondary to an increase in H_2O_2, that can be indicated in our work by an increase in the CAT value. Although most of the studies show that estrogens act as antioxidants in the lipid environment (Özden et al., 2001; Gómez e Mora, 2013), other studies have shown that E_2 can induce lipid peroxidation *in vivo* (Wang and Liehr, 1995). Thus, the debate continues as to determine the true oxidative nature of estrogens on lipids membrane integrity and H_2O_2 levels.

Similary to the reported by Azevedo et al (2001), the therapy with E_2 was not able to change the enzymatic activity of SOD. However, additional studies regarding the effect of the steroid on the oxidative stress of mares are necessary, since during the present work, only one high dose application of estrogen was defined. Unfer et al. (2014) carried out a study with women, in which increased values of SOD were observed after exogenous therapy with E_2, opting for continuous treatment for at least three months. Thus, our results may have been affected by the discontinuity of EB applications.

In equine reproduction routine, the exogenous administration of estrogen is a common tool, especially when it comes the treatment of endometritis. Endometritis is the major cause of infertility in mares, and associated with important economic losses to the horse industry (Troedsson and Nielsen, 2018; Schöniger and Schoon, 2020; Canisso et al., 2020). In a report discussing the impact of the disease in the oxidative stress biomarkers in mares, the authors postulated that mares with clinical endometritis undergo an oxidative process with a decrease in total antioxidant capacity (Abdelnaby et al., 2020). It's well stablished that E_2 play in enhancement of the uterine defense mechanism, and increased phagocytic activity in the peripheral circulation (Washburn et al., 1992). Our study shows that E_2 can also boost the antioxidant defense system, what can be very interesting in the control of systemic oxidative effect of endometritis. Despite that, the effect of the steroid in MDA concentration in mares that are already submitted to an oxidative process must be elucidated, once in our study, an elevation

in the biomarker were obtained after EB treatment. Further studies in this field involving the monitoring of different doses and types of estrogen in different reproductive status mares are necessary, and will facilitate more explorations of the possible E_2 effects in the treatment of reproductive diseases, as endometritis.

This is the first report of a positive effect of exogenous EB on the UBF in anestrus mares. Bolwein et al. (2004) reported a decreased PI and increased UBF increase 24 h after administration of estrogen in mares during the estrus phase. Specifically, higher levels of estradiol receptors in the endothelium of uterine vessels are found on the day of ovulation, during estrus (Aupperle et al., 2000). Similarly, anestrous mares showed decreased RI and PI associated with increased EBF between two and four days after treatment with EB.

Increase in E_2 concentrations shows elevated expressions of nitric oxide synthase (eNOS) in the uterine artery endothelium, which leads to higher levels of NO and relaxation of the vessel's smooth muscles responsible for endometrial vascularization (Rosenfeld et al., 2002). The mechanisms involved in uterine vasodilation in mares are unknown although pathways associated with NO have already been described for ewes (Rosenfeld et al., 2002). The UBF patterns observed in the present study after E_2 administration were similar to those previously published. Therefore, it seems plausible that in our study that the effect of the steroid was delayed due to refractoriness of the hormonal activity observed in the anestrus. Likewise, the long-lasting effect of the applied EB on UFB, observed up to D4, is expected, since it has a long plasmatic half-life (Greiss and Anderson, 1970).

In human's embryo transfer programs, women that presents higher levels of UBF previous to transfer had higher clinical pregnancy rates (Wang et al., 2010; Khan et al., 2016; Martins et al., 2019). Although the fertility rate of treated anestrus mares was not evaluated in our study, the application of 10mg of EB was able to induce a significant increase in UBF. This result is especially interesting when it comes the use of acyclic recipients in reproductive routine, once in those, protocols involving the exogenous application of E_2 are used with the

purpose to induce similar uterine changes to those which occur in cyclic mares that become pregnant. Despite this, the consequences of this practice have not yet been thoroughly investigated, particularly considering the uterine hemodynamics changes. More studies in the field are extremely necessary, in view of the fact that UBF, proved to be increased in anestrous mares by EB application, is positive correlated with pregnancy rates in other species.

Conclusion

Under the current study conditions, the exogenous administration of estrogen induced an increase in the uterine blood flow and serum concentrations of CAT, FRAP and MDA in anestrous mares. Our findings may contribute to elucidate the impact of the use of estrogen on the reproductive management of mares, especially considering the endometritis affected and embryo receptors. However, more studies may be conceived to better understand about the consequences of this practice.

Author Contribution Statement

V.K.C. Poltronieri: Methodology; Investigation; Writing – review and editing.

A.K.A. Jimenez: Methodology; Investigation.

R.A. Tavares: Methodology; Investigation.

P.P. Maitan: Writing – review and editing

L.L. Oliveira: Methodology; Investigation; Writing – review and editing.

J.D. Guimarães: Methodology; Investigation; Writing – review and editing.

B. Waddington: Conceptualization; Methodology; Statistical analysis; Investigation; Writing – review and editing; Project administration; Funding acquisition.

Competing Interests Statment

None of the authors has any other financial or personal relationships that could inappropriately influence or bias the content of this paper.

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4. CONCLUSÕES

A aplicação intrauterina da mistura gasosa O_3-O_2 na concentração de 44 µg O_3.ml^{-1} em éguas demonstrou-se segura, uma vez que não foram observadas alterações nos parâmetros de estresse oxidativo locais e sistêmicos compatíveis a citotoxicidade. Ademais, avaliando-se os resultados de NO nos LBV's entre os tratamentos e grupos, entende-se que o emprego da ozonioterapia seja possivelmente responsável por um incremento vascular local. No entanto, tal tendencia não foi observada em conjunto com o aumento densidade vascular endometrial avaliada mediante a ultrassonografia *power* Doppler.

Frente a análise dos resultados obtidos após a aplicação exógena de estrógeno, entende-se que esse atua diretamente sobre o controle hemodinâmico uterino e equilíbrio oxidante-antioxidante de éguas. Tais dados contribuem para a elucidação dos impactos do uso de estrógeno no manejo reprodutivo de éguas, o que é especialmente interessante pensando-se no uso de receptoras acíclicas e no preparo fêmeas para recebimento de terapias voltadas para o tratamento das endometrites.

5. ANEXO A

MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL DE VIÇOSA
PRÓ REITORIA DE PESQUISA E PÓS GRADUAÇÃO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA
Campus Universitário - Viçosa, MG - 36570-900- Telefone: (31) 3612 2315

Viçosa, 28 de setembro de 2021

Prof.
José Domingos Guimarães
Coordenador do projeto
DVT/UFV

Sr. Coordenador,

Após avaliação da Metodologia utilizada no Projeto de Pesquisa intitulado "**Efeitos da endometrite e da insulflacão uterina com ozônio sobre parâmetros hemodinâmica endometrias e de estresse oxidativo em éguas"**, aqui nomeado Processo 59/2021, a CEUA/UFV emite parecer favorável ao protocolo de utilização de animais proposto, tendo como base para análise a Legislação vigente (Lei Nº 11.794, de 08 de outubro de 2008), as Resoluções Normativas editadas pelo CONCEA/MCTIC, bem como a DBCA (Diretriz Brasileira de Prática para o Cuidado e a Utilização de Animais para Fins Científicos e Didáticos) e as Diretrizes da Prática de Eutanásia preconizadas pelo CONCEA/MCTIC.

Acresce a esse Parecer a exigência de Relatório Final de Atividades conforme itens a seguir:

RESUMO DOS RESULTADOS FINAIS OBTIDOS A PARTIR DOS EXPERIMENTOS ENVOLVENDO A ***UTILIZAÇÃO DE ANIMAIS*** *NO PROJETO DE PESQUISA*

1 Número do protocolo de submissão do projeto de pesquisa à CEUA/UFV:
2 Metodologia completa obrigatoriamente com:
- Local (is) Geral (is) e específico (s) oficial (is) onde ocorreu a experimentação;
- O nome científico do animal em questão;
- Número total de animais utilizados na pesquisa.

3 Resultados:
4 Nome do Coordenador do Projeto:
Assinatura:
5 Nome do Responsável Técnico:
Assinatura:
Inscrição em CRMV:

Profa. Mariella Bontempo Duca de Freitas
Coordenadora
Comissão de Ética no Uso de Animais – CEUA/UFV

CERTIFICADO

A Comissão de Ética no Uso de Animais - CEUA/UFV certifica que o processo nº 59/2021, intitulado **"Efeitos da endometrite e da insulflacão uterina com ozônio sobre parâmetros hemodinâmica endometrias e de estresse oxidativo em éguas",** coordenado pelo professor José Domingos Guimarães do Departamento de Veterinária, está de acordo com a Legislação vigente (Lei Nº 11.794, de 08 de outubro de 2008), as Resoluções Normativas editadas pelo CONCEA/MCTIC, a DBCA (Diretriz Brasileira de Prática para o Cuidado e a Utilização de Animais para Fins Científicos e Didáticos) e as Diretrizes da Prática de Eutanásia preconizadas pelo CONCEA/MCTIC, portanto sendo aprovado por esta Comissão em 28/09/2021, com validade de 12 meses.

CERTIFICATE

The Ethic Committee in Animal Use/UFV certify that the process number 59/2021, named **"Effects of endometritis and uterine ozone insufflation on endometrial hemodynamic parameters and oxidative stress in mares"**, is in agreement with the a actual Brazilian legislation (Lei Nº 11.794, 2008, Normative Resolutions edited by CONCEA/MCTIC, the DBCA (Brazilian Practice Guideline for the Care and Use of Animals for Scientific and the Guidelines of Practice the Euthanasia recommended by CONCEA/MCTIC therefore being approved by the Committee on September 28, 2021 valid for 12 months.

Profa. Mariella Bontempo Duca de Freitas
Coordenadora
Comissão de Ética no Uso de Animais – CEUA/UFV