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Dissertação

Histomorfometria microcotiledonar da placenta de éguas com placentite ascendente induzida submetidas a diferentes tratamentos

Letícia da Silva Souza

Pelotas, 2020

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Dedico este trabalho aos meus pais

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''O mais corajoso dos atos ainda é pensar com a própria cabeça''
Coco Chanel

Resumo

SOUZA, Letícia da Silva. **Histomorfometria microcotiledonar da placenta de éguas com placentite ascendente induzida submetidas a diferentes tratamentos.** 2020. 49f. Dissertação (Mestrado em Ciências) - Programa de Pós-Graduação em Veterinária, Faculdade de Veterinária, Universidade Federal de Pelotas, Pelotas, 2020.

A saúde fetal é influenciada pelas funções da placenta, órgão epiteliocorial, difuso e microcotiledonar. Patologias, como a placentite, podem levar ao desprendimento da união útero-placenta, acarretando em quadros de hipóxia fetal e distúrbios na produção hormonal. O objetivo do presente estudo foi investigar os efeitos do estradiol sobre os microcotilédones da placenta de éguas com placentite induzida submetidas a diferentes tratamentos, com o auxílio da análise digital de imagens. Foram utilizadas 30 placentas de éguas mestiças Crioulas durante as temporadas reprodutivas de 2012, 2013 e 2014. A indução da placentite foi realizada através da infusão intra-cervical de Streptococcus equi subespécie zooepidemicus entre os dias 280-295 de gestação. A hormonioterapia juntamente com avaliação clínica teve início 48h após a indução e as éguas foram divididas em grupos de acordo com a terapia administrada: G1 (n=5) éguas não induzidas; G2 (n=5) éguas com placentite induzida tratadas com antibioticoterapia e anti-inflamatório (sulfametoxazol e trimetoprim -SMT + Flunexin Meglumine -FM); G3 (n=5) éguas com placentite induzida tratadas com SMT+FM acrescido de Cipionato de estradiol (ECP); G4 (n=5) éguas com placentite induzida tratadas com SMT+FM acrescido de ECP e Altrenogest; G5 (n=5) éguas com placentite induzida tratadas com SMT+FM acrescido de Altrenogest; G6 (n=5) éguas não tratadas. Após o parto assistido, amostras histológicas foram obtidas a partir de quatro pontos de cada placenta referente a porção da estrela cervical; corno gravídico; corno não gravídico e corpo uterino. As lâminas foram avaliadas por microscopia óptica, digitalizadas e processadas pelo software NIH ImageJ 1.48r. A área capilar microcotiledonar média da porção referente a estrela cervical e do corno não gravídico do G1 e G3 foram superiores dos demais grupos ($p < 0,05$). Enquanto a área capilar microcotiledonar média do corpo uterino foi maior no G1, comparada aos demais grupos. Quando analisada a área microcotiledonar do corno gravídico do G1 e G3 foram maiores que a observada dos demais grupos ($p < 0,05$). A área microcotiledonar do corno não gravídico do G1 e G3 foram superiores aos demais grupos. Sendo assim, o grupo de éguas tratadas com ECP apresentou uma melhor resposta microcotiledonar, frente aos demais tratamentos.

Palavras-chave: obstetrícia; gestação; estradiol; equinos

Abstract

SOUZA, Letícia da Silva. **Microcotyledon histomorphometry of the placenta of mares with induced ascending placentitis submitted to different treatments.** 2020. 49f. Dissertation (Master degree in Sciences) - Programa de Pós-Graduação em Veterinária, Faculdade de Veterinária, Universidade Federal de Pelotas, Pelotas, 2020.

Fetal health is influenced by the functions of the placenta, epithelochoral, diffuse and microcotylated organ. Pathologies, such as placentitis, can lead to the detachment of the uterus-placenta union, resulting in pictures of fetal hypoxia and disturbances in hormonal production. The aim of the present study was to investigate the effects of estradiol on the microcotyledons of the placenta of mares with induced placentitis submitted to different treatments with the aid of digital image analysis. Thirty Crioulas mares were used during the breeding seasons of 2012, 2013 and 2014. Placentitis was induced by intra-cervical infusion of Streptococcus equi subspecies zooepidemicus between days 280-295 of gestation. Hormone therapy together with clinical evaluation started 48 hours after induction and the mares were divided into groups according to the therapy administered: G1 (n = 5) non-induced mares; G2 (n = 5) mares with induced placentitis treated with antibiotic therapy and anti-inflammatory (sulfamethoxazole and trimethoprim -SMT + Flunexin Meglumine -FM); G3 (n = 5) mares with induced placentitis treated with SMT + FM plus estradiol cypionate (ECP); G4 (n = 5) mares with induced placentitis treated with SMT + FM plus ECP and Altrenogest; G5 (n = 5) mares with induced placentitis treated with SMT + FM plus Altrenogest; G6 (n = 5) untreated mares. After assisted delivery, histological samples were obtained from four points on each placenta for the portion of the cervical star; pregnant horn; non-pregnant horn and uterine body. The slides were evaluated by optical microscopy, scanned and processed using NIH ImageJ 1.48r software. The mean microcotyledon capillary area of the portion referring to the cervical star and the non-pregnant horn of G1 and G3 were higher than the other groups ($p < 0.05$). While the mean microcotyledon capillary area of the uterine body was larger in G1, compared to the other groups. When the microcotyledon area of the pregnant horn of G1 and G3 was analyzed, they were larger than that observed in the other groups ($p < 0.05$). The microcotyledon area of the non-pregnant horn of G1 and G3 was superior to the other groups. Thus, the group of mares treated with ECP showed a better microcotyledon response, compared to other treatments.

Keywords: obstetrics; gestation; estradiol; equine

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Lista de Abreviaturas e Siglas

ALT	Altrenogest
CAPES	Coordination for the Improvement of Higher Education Personnel
CNPq	Scientific and Technological Development
cm	Centímetros
CTUP	Combined thickness of uterus and placenta
ECP	Cipionato de estradiol
FM	Flunixin meglumine
Kg	Kilogramas
m	Metros
MHz	Megahertz
mm^2	Milímetros quadrados
SEM	Erro padrão da média
UFPel	Universidade Federal de Pelotas
µm	Micrômetro
μm^2	Micrômetro ao quadrado
TSM	Trimethoprim-sulfamethoxazole

Lista de Símbolos

<	Menor
>	Maior
+	Mais
±	Mais ou menos
=	Igual
%	Porcentagem
α	Alfa
x	Multiplicação
β	Beta
p	Valor-p; probabilidade estatística

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1 Introdução

A placenta equina é um órgão transitório composto por tecidos maternos e fetais, responsável pelo transporte de diversas substâncias entre estes organismos. Realiza a síntese, secreção e absorção de hormônios, fatores de crescimento, enzimas, proteínas e carboidratos, todos essenciais ao desenvolvimento do feto (LE BLANC, 2004).

Longas redes capilares se desenvolvem nos lados materno e fetal (ABDELNAEIM, 2006) e as vilosidades alantocorionares e seus sulcos endometriais tornam-se cada vez mais ramificados e sub-ramificados, para criar a complexa estrutura microcotiledonar placentária por volta dos 150 dias de gestação. O desenvolvimento microcotiledonar, assim como sua ramificação, continuam durante o restante da gestação (MACDONALD, 2000) para aumentar a área de contato epitelial feto materna e, assim, atender as crescentes demandas nutricionais, gasosas e hemotróficas do feto em crescimento acelerado. Isto porque, a maior taxa de crescimento e ramificação acontece no terço final de gestação (MACDONALD, 2000).

A avaliação placentária no pós-parto imediato pode ser útil para o reconhecimento de alterações não observadas durante a gestação (LE BLANC, 2010). Em contrapartida, a histopatologia da placenta pode revelar transtornos não diagnosticados durante a gestação ou durante a sua avaliação macroscópica (MACPHERSON, 2008). Vários autores destacam a eficiência da análise digital na avaliação da placenta humana (CROSS, 1994; ARORA, 2011), já que a área da interface feto materna funcional é o fator mais importante no estabelecimento de troca placentária adequada. O método mostra-se confiável e eficiente em segmentação, cálculo, extração de delineamento de vilosidades.

Alterações placentárias podem levar ao aborto, parto prematuro ou nascimento de neonatos não viáveis (LE BLANC, 2010). Uma das afecções mais comuns no terço final da gestação é a placentite, responsável por quase um terço dos abortos tardios e mortalidade fetal no primeiro dia de vida dos neonatos (GILES et al., 1993). É mais comumente causada por bactérias que ganham acesso a cérvix

através da vagina da égua e rompem a barreira cervical (HONG et al., 1993). Em consequência, as membranas fetais se espessam em resposta à contaminação bacteriana, podendo se separar do útero da mãe no local da infecção (HONG et al., 1993). O Streptococcus equi subsp zooepidemicus é o agente patológico isolado no maior número de casos (WHITWELL, 1988).

Com base em lesões morfológicas e na sua patogênese, quatro tipos diferentes de placentite foram identificados: placentite ascendente; placentite mucoide focal; placentite difusa e placentite multifocal. Na maioria dos casos, a infecção da placenta resulta em infecção subsequente do feto e na liberação de prostaglandinas da placenta inflamada (LE BLANC, 2010). Os potros afetados podem experimentar uma maturação fetal acelerada em resposta ao ambiente patológico. Além disso, o quadro infeccioso pode levar a distúrbios relacionados à produção hormonal indispensável à manutenção adequada da gestação (LE BLANC et al., 2002).

O manejo de éguas de risco, devido à placentite, é direcionado ao prolongamento da gestação, ao combate da infecção, a redução da inflamação e o controle da atividade miometrial (MACPHERSON, 2006). Sendo assim, buscando formas de tratamento, muitos foram os regimes terapêuticos extrapolados de outras espécies, principalmente de seres humanos. O tratamento da placentite sugerido tem como base o uso de antimicrobianos, por meio de uma combinação de (penicilina e gentamicina ou trimetoprim sulfametoxazol), progestinas (altrenogest ou progesterona) e anti-inflamatórios (flunixina meglumina , fenilbutazona, pentoxifilina, ácido acetilsalicílico) (CANISSO et al., 2015).

Se os objetivos do tratamento forem atingidos, a duração da gestação da égua afetada pela placentite deve ser semelhante à duração normal e resultar em um potro hígido e bem desenvolvido. As progestinas foram incluídas como parte do tratamento em vários estudos com placentite. Já a terapia com estrogênio tem sido defendida como um tratamento necessário para a placentite equina para reduzir o risco de aborto e nascimento de potros fracos (BAILEY, 2012).

Os hormônios envolvidos na gestação equina podem ser amplamente divididos em três grupos, com base em sua função fisiológica. Primeiro são os

hormônios que regulam a quiescência miometrial. Em segundo lugar estão os hormônios que estimulam a atividade uterina e causam contrações miométricas regulares e rítmicas, resultando no rápido fornecimento do potro e das membranas fetais. Em terceiro lugar estão os hormônios que induzem a maturação fetal (MACPHERSON, 2008).

O nascimento de um potro viável a termo depende da coordenação desses eventos endócrinos, para garantir que haja o adequado desenvolvimento fetal. A alteração na secreção estrogênica placentária durante a gestação pode caracterizar um parto com contrações miométricas de baixa intensidade. A severa interrupção da produção de estrogênio na gestação tardia, embora não abortiva, claramente impede o crescimento normal e desenvolvimento do feto, a partir resultado da redução da vasculogênese na placenta. Os potros maduros ou não, oriundos de gestações deficientes em estrógeno podem apresentar flacidez grave da musculatura corporal (WILSHER, 2005).

O benefício do tratamento com estradiol foi evidente em potros nascidos de éguas tratadas com ECP (Cipionato de Estradiol) que apresentavam peso normal ao nascimento, sem morte e sem evidência de dismaturidade ou doença clínica, (MULLER et al., 2018). Em éguas não gestantes, o estrogênio demonstrou ser um vasodilatador uterino. Alguns autores sugeriram que as concentrações de estrogênio nas éguas tratadas são provavelmente suficientes para manter o fluxo sanguíneo uterino ou que o estrogênio pode alterar a vascularização da placenta sem alterar a hemodinâmica da artéria uterina (CURCIO et al., 2017).

Portanto, hipotetizamos que o uso do estrogênio de longa ação no tratamento de éguas com placentite poderá estimular a vasculogênese placentária assim como a melhora no fluxo sanguíneo uterino. Assim, o objetivo do presente estudo foi investigar os efeitos do estradiol sobre os microcotilédones da placenta de éguas com placentite induzida submetidas a diferentes tratamentos, com o auxílio da análise digital de imagens.

2 Objetivos

2.1 Objetivo Geral

Avaliar o efeito do ECP sobre a estrutura morfométrica microcodiledonária, por meio da análise digital de imagem, da placenta no pós-parto de éguas com placentite induzida, submetidas a diferentes terapias hormonais.

2.2 Objetivos específicos

Verificar a capacidade da terapia medicamentosa em recuperar/preservar a área capilar dos microcotilédones, após a ocorrência de placentite ascendente induzida.

Avaliar a área total e as ramificações das vilosidades presentes nos microcotilédones, levando-se em consideração as diferentes terapias instituídas após a ocorrência de placentite ascendente induzida.

3 Artigo

Application of Digital Pathology to Assess the Effect of Hormone Treatment on Placental Histomorphometry in Mares with Induced Ascending Placentitis

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Abstract

This study aimed to evaluate the effects of hormonal treatments with altrenogest (ALT) and estradiol cypionate (ECP) on placental histomorphometry in mares with experimentally induced ascending placentitis, using digital pathology as a quantitative tool. Thirty Criollo-type mares were assigned to five groups: healthy controls (n = 5); basic treatment (n = 5), treated with Trimethoprim-sulfamethoxazole (TSM), and flunixin meglumine (FM); ALT

group (n = 5) + basic treatment; ECP group (n = 5) + basic treatment, ALT+ ECP group (n=5) add to basic treatment; and an untreated placentitis group (n = 5). Following parturition, chorioallantoic samples were collected from the cervical star, uterine body, and both horns. Digital images were analyzed using NIH ImageJ software to quantify microcotyledonary and capillary areas per field. Gestation length in the ECP group was comparable to that of the healthy controls, while placental weight and expulsion time did not differ significantly among groups. Histomorphometric analysis revealed that mares in the ECP group exhibited significantly increased capillary area and total microcotyledonary area compared to other placentitis groups. These results suggest that ECP enhances placental vascularization and microstructure in mares with ascending placentitis. The findings support the therapeutic potential of ECP in promoting placental recovery and maintaining uterine homeostasis. Additionally, this study highlights the utility of digital pathology in the quantitative assessment of equine placental morphology, providing new perspectives for improving therapeutic strategies in the management of placentitis.

Keywords: Estradiol cypionate; Equine; Microcotyledons; Placenta.

1. Introduction

Placentitis represents the most frequent cause of miscarriages and stillbirths, accounting for more than 30% of premature births and neonatal deaths within the first 24 hours of life (Canisso et al., 2015). The feto-placental unit comprises the maternal endometrium, the fetus, and the fetal membranes, and is responsible for metabolic exchanges between dam and fetus, as well as for the production and metabolism of steroid hormones (Ousey, 2005). Ascending microbial infections, typically with the cervix serving as the primary gateway, are the primary cause for placentitis. These infections compromise the feto-

placental unit by inducing hypoxemia or direct infection, thereby reducing the supply of nutrients and oxygen to the fetus and placenta (Rossdale, 2004).

Infectious conditions leading to placental inflammation cause detachment of the uterus-placenta interface, reducing the maternal-fetal exchange area. This reduction leads to fetal hypoxia and disturbances in hormone production, jeopardizing the maintenance of pregnancy. Additionally, the inflammatory process triggers the release of prostaglandin, which reduces the uterine quiescence, thereby increasing the likelihood of miscarriage (Le Blanc et al., 2002; Le Blanc, 2010). Placental insufficiency resulted from placentitis, impairs metabolic and gas exchange between mother and fetus. The extent of fetal impairment depending on nature, duration, severity and the stage of pregnancy at which it occurs, are also factors to be considered (Le Blanc, 2010).

Several studies have proposed the use of single or serial evaluations of blood markers for the diagnosis of placentitis in mares. Among these, steroids derived from the feto-placental unit have shown a strong correlation with placentitis. Chronic placentitis is associated with increased peripheral progestogens, whereas acute placentitis results in reduced concentrations. Additionally, plasma levels of estradiol-17β (in both free and conjugated forms) are markedly diminished in mares with placentitis (Canisso et al., 2020; Shikichi et al., 2017). The treatment of placentitis has three main objectives: to inhibit bacterial proliferation, maintain uterine quiescence, and reduce the production of inflammatory cytokines (Le Blanc, 2010). Alongside antibiotics and anti-inflammatory drugs, hormone therapy is employed to maintain pregnancy, support fetal development, and prevent uterine contractility. This includes administering synthetic progestogens and estrogens (Bailey, 2010; Curcio et al., 2017; Ousey, 2005).

In horses, the fetal growth rate depends on the gross area, density, complexity, and depth of the micro-cotyledons. In addition to macroscopic assessment, histolopathological

evaluation of the placenta can provide valuable information about pregnancy.0 However, traditional histological methods only allow three-dimensional structures to be assessed in two dimensions, limiting their ability to quantify microscopic characteristics (Allen et al., 2002). Digital histological tools, such as ImageJ and QuPath, enable comprehensive structural assessments and are invaluable for the quantitative morphometric description of diverse tissues (Ferreira, 2011). These methods are particularly effective for assessing the morphological changes to the placenta during the gestational period in a range of species (Salazar-Petres et al., 2022). Histomorphometric analyses are well-established tools for assessing the feto-maternal relationship in humans, mice, and horses (Salazar-Petres et al., 2022; Bianco et al., 2014; Veronesi et al., 2010).

This study aimed to evaluate the effects of different hormonal treatments on the histomorphometry of term placentas in mares with experimentally induced ascending placentitis, using digital pathology for quantitative image analysis.

2. Materials and Methods

All procedures in this study were approved by the Ethical Committee on Animal Experimentation of the Universidade Federal de Pelotas (UFPel) under protocol #3891. The mares were housed at the Palma Farm of the UFPel, located in Capão do Leão, Rio Grande do Sul, Brazil. A total of 30 multiparous Criollo and Criollo-type mares (mean age 8.9 ± 1 year; mean parity 3 ± 0.5; mean body weight 437 ± 22 kg) were used. None of the mares enrolled in this study had a history of subfertility or late-term pregnancy abnormality. Mares were maintained on improved native pasture with ryegrass planting and supplemented with commercial concentrate pellets (2% of body weight/day) and water provided *ad libitum*.

2.1 Design of the study and therapeutic regimens

The mares were randomly divided into the following groups: a control group of healthy mares (n=5), a second group of mares with healthy pregnancies that were not subjected to placentitis induction; and a third group of mares with placentitis (n=25), which was further divided into five groups according to treatment. The TSM+FM group (n=5) consisted of mares treated with sulfamethaxazole trimethoprim and flunixin meglumine. The ECP+TSM+FM group (n=5) consisted of mares treated with estradiol cypionate. The ALT+ECP+TSM+FM group (n= Group 5) consisted of mares treated with the addition of estradiol cypionate and altrenogest. Group ALT+TSM+FM (n=5) comprised mares with induced placentitis treated with the addition of altrenogest. No Treatment group (n = 5) consisted of mares with induced placentitis who did not receive any medication.

All pregnant mares underwent monthly clinical and ultrasound assessments from the 150^{th} day of gestation onwards. From the 250^{th} day of gestation, evaluations were conducted weekly until placentitis was induced. The ultrasound assessment was conducted transrectally with a 5-7.5 MHz linear transducer to measure the combined thickness of uterus and placenta (CTUP), as well as to evaluate fluid characteristics and the detachment of the fetal membranes.

Ascending placentitis was experimentally induced via intracervical inoculation of 10^7 colony forming units of *Streptococcus equi* subspecies *zooepidemicus*, between 295 and 300 days of gestation, as described in our parallel study (Curcio et al., 2017). Subsequently, clinical (for the presence of mammary gland development and vulvar discharge) and ultrasound assessments were conducted daily following the bacterial inoculation.

The treatment was initiated at 48 hours post-experimental induction of ascending placentitis and continued for a period of ten consecutive days. Mares with induced placentitis received the standard treatment for placentitis, comprising Trimethoprim-sulfamethoxazole and flunixin meglumine (TSM+FM), in combination with different therapeutic regimens of

Drugs	Dose	Route	Brand
Trimethoprim-sulfamethoxazole	30mg/kg	IV, q12 for 10d	Trissulfim®, Ouro Fino Saúde Animal, São Paulo, Brasil
Flunixin meglumine	1.1mg/kg	IV, q24h for 10d	Desflan®, Ouro Fino Saúde Animal, São Paulo, Brasil
Altrenogest (Long action)	0.088 mg/kg	IM, q7 d, for 2 applications	Altrenogest®, Botupharma, São Paulo, Brasil

estradiol cypionate (ECP) and long-acting altrenogest (ALT). The therapeutic regimens are detailed in Table 1.

Table 1. Description of the therapeutic regimens* used to treat mares with ascending placentitis experimentally induced by intracervical infusion of *Streptococcus equi subs zooepidemicus*.

Estradiol Cypionate	10mg/mare	IM, q 3d for 3 applications	E.C.P.®, Zoetis, São Paulo, Brasil

*Adapted from our parallel study Curcio et al., 2017.

2.2 Monitoring and foaling management

By 300 days of gestation all mares were observed daily to assess softening of the perineal region, udder development, and the pH of mammary secretion to identify signs of impending parturition. The mares were kept in paddocks near the foaling barn. Upon the rupture of the chorioallantois, the mares were brought into foaling stalls (6 x 6 m) for assisted vaginal delivery.

All mares were closely monitored until the release of the fetal membranes. The placentas were weighed on a digital scale, and the time taken to eliminate it was recorded in minutes. Immediately after delivery, all the foals underwent a full physical examination performed, and their birth weight were recorded.

2.3. Gross and histopathological evaluation of placenta

Following the weighing, the placentas were laid out in an "F" shape for gross evaluation of chorionic and allantoic surfaces, as previously described (Schalafer, 2004). This method facilitated for the observation of potential abnormalities such as changes in color, areas devoid of villi, thickened regions, and the presence of exudate.

Subsequently, fragments measuring 3×3cm were collected from four points of each placenta, corresponding to the regions of the cervical star, the gravid horn, the non-gravid

horn, and the uterine body. All samples were fixed in 10% formalin prior to processing for histology. Histological sections (5μm thickness) were mounted on glass slides and stained with hematoxylin and eosin. All slides were evaluated using light microscopy to identify the presence of placentitis and classify the occurrence of lesions compatible with acute or chronic placentitis (Hong et al., 1993).

Histomorphometry evaluation of the chorioallantoic microcotyledons was conducted for all mares. The procedures were performed randomly ten times in each section on four placental samples from each mare, corresponding to the uterine portions relating to the cervical star region, uterine body, gravid horn, and non-gravid horn, resulting in a total of 40 measurements per placenta. The digitized images (100 x magnification) were obtained using an Olympus DP72 high-resolution camera on an Olympus BX51 microscope (Olympus America, Center Valley, PA). The digital images were then processed using the open-source software NIH ImageJ 1.48r (US National Institute of Health, available at http://rsb.info.nih.gov/il/).

The histomorphometry involved the evaluation of the microcotyledon area and capillary area within the chorionic epithelium of the chorioallantois. Images were evaluated in RGB color with the total number of pixels calibrated to micrometers. Quantifications of the microcotyledonary and capillary area/field were performed with the "Color Threshold" macro, and the color range was adjusted by the RGB scale.

The differential absorption method was employed to analyze any differences in color intensity. If no erythrocytes were present in the capillaries, the capillary area/field measurements were corrected using the "Freehand" plug-in. The smallest diameter of the microcotyledonary network was measured by sampling 5 by 10 of each portion of the placental tissue evaluated. The field area (100 x magnification) to acquire digitalized images was 146.673 mm^2.

2.4. Statistical analysis

Statistical analyses were performed using the commercial software Statistix 10.0 (Analytical Software, Tallahassee, FL, USA). The Shapiro-Wilk test was used to assess the normality of the variables. For variables that did not follow a normal distribution, a log transformation was applied to enable one-way ANOVA and LSD tests for mean comparisons. Fisher's exact test was used to determine the prevalence of placentas with alterations

Groups (n)	Time from release of placenta (min)	Weight (kg)	Gestational length (days)

consistent with acute and chronic lesions. Statistical significance was set at $p < 0.05$, and results for quantitative variables are presented as mean ± SEM.

3. Results

The gestational lengths of mares receiving ECP as hormonal therapy for ascending placentitis were similar to those of the control group of healthy mares (Table 2). Mares that did not receive any treatment, those treated with TMS+FM alone, and those treated with ALT exhibited the shortest gestation length, with averages below the physiological length for the equine species (Table 2). Despite the numerical variations, no statistically significant differences were observed in the time from placental release to placental weight between the evaluated groups (Table 2).

Healthy (n=5)	34±7	4,5±0,6	332±6[a]
TSM+FM (n=5)	30±16	5,7±0,9	316±2[bc]
ECP+TSM+FM (n=5)	76±41	4,8±0,5	345±5[a]
ECP+ALT+ TSM+FM (n=5)	43±18	5,0±0,6	329±11[ab]
ALT+ TSM+FM (n=5)	38±15	5,2±0,3	316±2[bc]
Untreated (n=5)	120±64	6,2±1,0	304±4[c]

Table 2. Obstetric data from mares with experimentally induced placentitis and gestationally age matched healthy control mares (mean ± SEM).

Healthy: healthy mares-control group; TMS: trimethoprim-sulfamethoxazole; FM: flunixin meglumine; ALT: altrenogest; ECP: estradiol cypionate; No treatment: mares inoculated and did not receive treatment. Different letters within columns denote differences with LSD's ($p < 0.05$).

The frequency of gross lesions in fetal membranes was assessed across all placentitis groups (Table 3). Gross abnormalities were observed in 52% of cases (n=13/25) and included typical findings of ascending placentitis, such as thickening, edema, and purulent or serosanguineous exudate at the cervical star. In some cases, these lesions extended ventrally into the uterine body of the chorioallantois. Histologically, acute placentitis was characterized by the presence of inflammatory infiltrate composed mainly by neutrophils, especially in the region of the cervical star, the uterine body, and the gravid horn. In chronic placentitis, the cellular infiltrate was predominantly mononuclear cells with areas of necrosis, hypoplasia and/or atrophy of the microcotyledons. Histological evaluation revealed a higher percentage of mares with acute placentitis in the group receiving hormonal treatment with ALT alone (Table 3). In the "No treatment" group, 60% (n=3/5) did not exhibit histological changes

compatible with placentitis (Table 3). However, numerous bacterial colonies distributed throughout the chorioallantois confirmed the presence of the inoculating agent.

Table 3. Index of histologic classification of placentas from mares with experimentally induced ascending placentitis and gestational age-matched healthy control mares.

Groups (n)	Gross lesions n (%)	Histology		
		Acute placentitis n (%)	Chronic Placentitis n (%)	NSF n (%)
Healthy (n=5)	0^{c}	0^{b}	0	5 (100%)
TSM+FM (n=5)	2^{b} (40%)	2^{b} (40%)	1 (20%)	2 (40%)
ECP+TSM+FM (n=5)	3^{ab} (60%)	0^{b}	3 (60%)	2 (40%)
ECP+ALT+ TSM+FM (n=5)	3^{ab} (60%)	3^{ab} (60%)	1 (20%)	2 (20%)
ALT+ TSM+FM (n=5)	4^{a} (80%)	4^{a} (80%)	1 (20%)	0
Untreated (n=5)	1^{bc} (20%)	2^{b} (40%)	0	3 (60%)

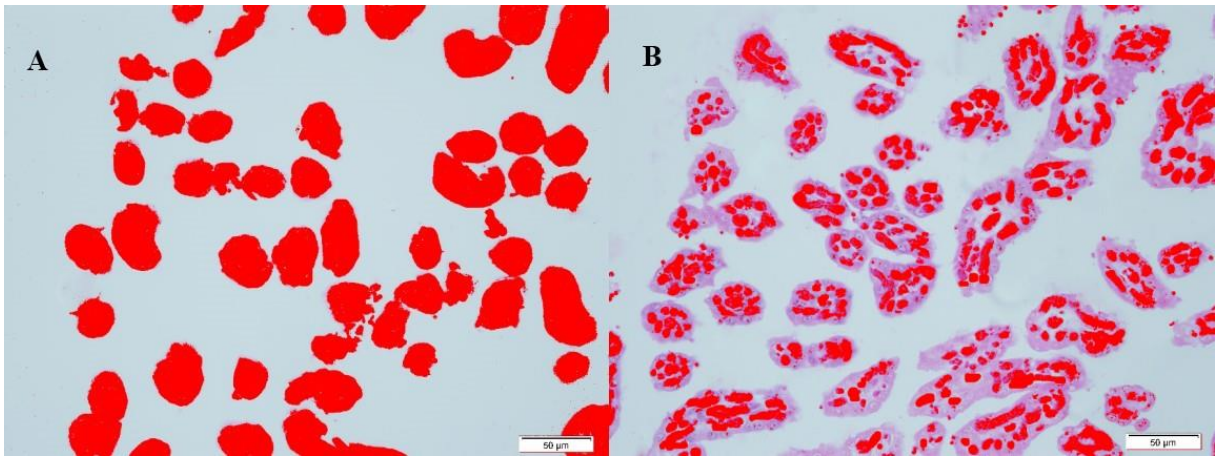
Healthy: healthy mares-control group; TMS: trimethoprim-sulfamethoxazole; FM: flunixin meglumine; ALT: altrenogest; ECP: estradiol cypionate; No treatment: mares inoculated and did not receive treatment. Different letters within columns denote differences with LSD's ($p < 0.05$).

In the histomorphometric evaluation of the placentas, the total field area of the images converted into pixels was 146.673 μm^2 for the quantification of total microcotyledonary tissue (Figure 1A) and capillary tissue (Figure 1B) using the "ColorThreshold" tool.

Figure 1. (A) Histomorphometric image of the microcotyledonary area at 50 μm magnification, evaluated in color with the "Color Threshold" macro, and the color range was adjusted using the RGB scale, with the total pixels calibrated to 146.673 μm2. (B) Histomorphometric image of the microcotyledon capillary area taken at 50 μm magnification,

evaluated in color with the Color Threshold macro, and the color range was adjusted by the RGB scale, with the total number of pixels calibrated to 146.673 μm2.

Portions of the placenta



Placentas from mares in the healthy group and the TMS+FM+ECP had the largest total areas of microcotyledons in the gravid horn and non-gravid horn portions (Table 4). Similarly, the capillary areas of the microcotyledons in placentas from the ECP-treated group exhibited values comparable to those observed in healthy mares in the cervical star, gravid horn, and non-gravid horn portions. However, in the gravid horn, the capillary area was only similar for the untreated and ALT-treated groups (Table 5).

Table 4. Total area/field* (μm^2) of microcotyledons (mean±SEM) in the chorioallantois of term placentas from mares with experimentally induced ascending placentitis and gestational age-matched healthy control mares.

Groups (n)	Cervical Star	Gravid horn	Non-gravid horn	Uterine body
Healthy (n=5)	38687±1688	88807±5283[a]	48457±1621[a]	106100 ±6611
TSM+FM (n=5)	32678±2301	58814±4523[c]	41713±1509[bc]	53295±4987
ECP+TSM+FM (n=5)	34814±1334	74922±6031[a]	50412±1549[a]	97411±7341
ECP+ALT+ TSM+FM (n=5)	34527±2827	58173±6184[c]	45586±2121[c]	73524±4328
ALT+ TSM+FM (n=5)	33437±1417	6669±6096[bc]	45330±1597[c]	71522±5312
Untreated (n=5)	36298±2044	62377±6794[bc]	46857±2054[ab]	93145±5819

Healthy: healthy mares-control group; TMS: trimethoprim-sulfamethoxazole; FM: flunixin meglumine; ALT: altrenogest; ECP: estradiol cypionate; No treatment: mares inoculated and did not receive treatment. Different letters within columns denote differences with LSD's ($p < 0.05$).

*The field area at 100x magnification.

Table 5. Capillary area/field* (μm^2) of microcotyledons (mean±SEM) in the chorioallantois of term placentas from mares with experimentally induced ascending placentitis and gestational age-matched healthy control mares.

Healthy: healthy mares-control group; TMS: trimethoprim-sulfamethoxazole; FM: flunixin meglumine; ALT: altrenogest; ECP: estradiol cypionate; No treatment: mares inoculated and did not receive treatment. Different letters within columns denote differences with LSD's ($p < 0.05$).

*The field area at 100x magnification.

	Portions of the placenta			
Groups (n)	Cervical Star	Gravid horn	Non-gravid horn	Uterine body
Healthy (n=5)	49539±134[a]	96421 ±537[a]	45360±191[a]	108901±732[a]
TSM+FM (n=5)	41288±114[b]	67841±458[b]	44720±185[a]	75118±640[bc]
ECP+TSM+FM (n=5)	49947±140[a]	10064±398[a]	44752±167[a]	98135±636[b]
ECP+ALT+ TSM+FM (n=5)	37209±177[b]	55121±384[bc]	37954±174[b]	68596±380[c]
ALT+ TSM+FM (n=5)	40460±145[b]	71453±485[abc]	43582±145[b]	92302±373[b]
Untreated (n=5)	48787±189[b]	79396±581[ab]	42886±206[b]	85693±431[bc]

4. Discussion

The placentas of mares with induced ascending placentitis treated with ECP exhibited histomorphometric characteristics similar to those of healthy mares, as well as a comparable gestational length. Furthermore, this treatment group demonstrated a greater total microcotyledonary and capillary area than the other ascending placentitis treatment groups.

In specific regions of the placenta, mares in the healthy and ECP group mares exhibited the largest total area of microcotyledons in both the gravid horn and non-gravid horn portions. The assessment of capillary area in the microcotyledons revealed greater capillary areas in the portions corresponding to the non-gravid horn and the cervical star. This similarity between the healthy and ECP groups suggests that the benefits of ECP supplementation are reflected by the maintenance of microcotyledonary capillary vasculogenesis despite the injuries caused by placentitis.

Histomorphometric analysis is a well-established method to evaluate the interaction of the feto-placental unit with the uterus in humans (Higgins, et al., 2011) as well as in horses (Veronesi et al., 2010; Bianco et al., 2014). In this context, histomorphometry effectively demonstrates the response at the microcotyledon level promoted by ECP supplementation. Analog histopathological evaluation of fetal membranes using traditional light microscopy provides valuable information for equine practitioners, particularly when macroscopic lesions are identified during placental evaluation (Pozor 2015). Efforts to develop semi-quantitative histological scoring schemes for placental tissue have been described, particularly in the context of pre-eclampsia and eclampsia, and methods enhancing reliability via digitized ROI-based scoring (Donthi et al., 2020). In addition, recent advances in digital 3D morphometry, such as whole organ microcomputed tomography, offer promising digital pathology platforms in equine models. These platforms provide a non-destructive, quantitative assessment of gross morphology and vascular branching in the entire equine placenta using microCT (Laundon et al., 2024). Digital pathology further enhances histomorphometric analysis by enabling precise, automated, and reproducible quantifications of placental structures. High-resolution imaging combined with software tools like ImageJ provides detailed assessments of microcotyledonary and capillary areas, revealing subtle morphological changes that might be missed with conventional methods (Collins, 2007). This technology standardizes measurements, minimizes observer bias, and enhances the reliability of histomorphometric data. Given the diffuse, microcotyledonary, and epitheliochorial structure of the equine placenta, gas and metabolic exchanges are heavily dependent on placental vascularization (Chavatte-Palmer et al., 2021. The volume of these exchanges is dependent on the size of the microcotyledons, which increase their vascular development during pregnancy and are directly associated with fetal health and growth (Abd-Elnaeim et al., 2006). Previous studies have shown that placental estrogens primarily regulate placental

growth, differentiation, and function. Furthermore, the expression of estrogen receptors in fetal and maternal vascular tissues suggest their involvement in angiogenesis and the regulation of vascular functions (Abd-Elnaeim et al., 2008), supporting the mechanism by which therapeutic estrogen supplementation may aid in the recovery from ascending placentitis and help maintain gestation to term. The longest gestation was observed in the ECP supplementation group, which, together with the histological evaluation showing no changes in the placentas, confirms the maintenance of a healthy uterine environment and the complete development and maturation of the equine neonate. These findings suggest a potential association between the maintenance of total and capillary areas of microcotyledons and enhanced neonatal viability and endocrine responses in foals, as delineated in our parallel studies (Curcio et al., 2017; Muller et al., 2018). This outcome may be attributed to the incorporation of ECP in the management of induced ascending placentitis.

The treatment of bacterial placentitis aims to eliminate or reduce the spread of the microorganism across the fetal membranes (Le Blanc, 2010). When treatment successfully mantains the integrity of the utero-placental interaction, the gestation length of mares affected by placentitis should be like the expected normal gestation length (330 to 340 days), resulting in the birth of a healthy foal.

The typical histopathological pattern of ascending bacterial placentitis is characterized by acute lesions, predominantly featuring neutrophilic infiltrates and necrosis of the microcotyledons (Hong et al., 1993; Le Blanc, 2010). In addition to inflammatory infiltrates, expected histological changes in cases of placentitis include hypoplasia or atrophy of the microcotyledons of the chorioallantois (Laugier et al., 2011). These alterations were observed in this study, which were more frequent in the groups of mares receiving ALT as the sole hormonal therapy. However, such lesions were absent in the placentas of mares treated with ECP, which exhibited histological characteristics comparable to those of the healthy group.

Furthermore, the acute lesion pattern was less pronounced in the ECP-treated group, while chronic cases displayed focal or multifocal mononuclear cell infiltrates without microcotyledonary necrosis.

The groups of mares with no treatment, TMS+FM alone, or the addition of ALT had the shortest gestation length. These averages were lower than the physiological range described for the equine species of 320 to 360 days of gestation (Sauté et al., 2011), leading to the birth of premature foals, including those born at less than 320 days of gestation (Morresey et al., 2005). This finding confirms that uteroplacental interaction fails in induced placentitis in some treatment protocols in this study, resulting in potential alterations in nutritional, metabolic, and endocrine requirements that may lead to preterm birth and consequently reduced neonatal viability.

Mares in the untreated group in this study suddenly exhibited clinical findings characteristic of acute placentitis, such as galactorrhea, purulent vulvar discharge, detachment and thickening of the uteroplacental junction, 24-48 hours after bacterial inoculation. Nevertheless, 80% of the mares exhibited no gross lesions and 60% no histological lesions on the placentas during the postpartum period. Aside from the presence of numerous bacterial colonies distributed throughout the chorioallantois, which confirmed the inoculating agent. The authors propose that the absence of histological lesions can be attributed to the brief interval between inoculation and the onset of premature birth.

This study has several limitations. First, the sample size was relatively small, particularly within each treatment group, potentially limiting the generalizability of the findings. Second, the experimental induction of placentitis may not fully replicate naturally occurring conditions, which could affect the applicability of the results to clinical cases. Additionally, the short interval between bacterial inoculation and the onset of clinical signs in untreated mares may have limited the observation of long-term histological changes. Finally,

the study focused primarily on histomorphometric outcomes without a comprehensive evaluation of fetal viability or long-term neonatal health, which are critical aspects of placentitis management. However, this study offers significant insights into the therapeutic potential of estradiol cypionate in placentitis management, highlighting its capacity to improve placental histomorphometry and maintain gestation lengths comparable to healthy mares. The incorporation of digital pathology allowed for quantitative, scalable, and reproducible analyses of placental structures, enhancing the precision and reliability of the histological assessments. The use of robust histomorphometric analysis and a well-controlled experimental design provides valuable contributions to the understanding of equine placental recovery mechanisms.

5. Conclusion

Mares with induced ascending placentitis treated with estradiol cypionate exhibited increased capillary area and total microcotyledonary area compared to those treated with the addition of alternogest or without hormonal supplementation. This finding highlights the beneficial potential of estradiol cypionate for placental recovery and maintenance of uterine milieu in mares with induced ascending placentitis. Additionally, this study emphasizes the use of digital pathology for placental histomorphometric evaluation, offering valuable perspectives therapeutic strategies for managing placentitis and improving equine reproductive outcomes.

Author contributions

All authors have made substantial contributions to all of the following: B.R.C. and C.E.W.N. were responsible for the conceptualization and methodology of the study. Laboratory investigations were performed by L.S.S., G.C.S., F.M.P., A.S.V.J., and C.D.C. Resources were provided by B.R.C., A.S.V.J., C.D.C., and C.E.W.N. Data curation and formal analysis were conducted by B.R.C., R.S., F.M.P., and A.S.V.J., who also contributed to data visualization. The original draft of the manuscript was written by L.S.S., G.C.S., R.S., F.M.P., and B.R.C., and all authors participated in the review and editing process. Supervision and validation were carried out by B.R.C. and C.E.W.N., with project administration led by B.R.C. All authors have read and approved the final version of the manuscript.

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Competing interests

None of the authors have any conflict of interest to declare.

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4 Considerações Finais

A partir dos resultados encontrados no presente estudo, podemos sugerir que exista uma relação positiva entre a suplementação com o Cipionato de estradiol (ECP) em éguas com placentite induzida e a manutenção/recuperação da área/capilar microcotiledonar. Dada a importância central desta estrutura para o metabolismo placentário e fetal. Baseando-se em estudos anteriores que buscaram compreender e comprovar como a terapia hormonal age nos quadros de placentite e qual seu reflexo na maturação e viabilidade fetal tornou-se necessário desvendar os seus benefícios nas estruturas microcotiledonares da placenta.

Além das avaliações macroscópicas e histológicas placentárias, os métodos morfométricos podem ser utilizados como ferramentas para a obtenção de informações quantitativas acerca de estruturas microscópicas. Dentre os softwares para análise de imagens, o ImageJ demonstrou fácil aplicabilidade e caracterizou-se como um programa seguro.

Sendo assim os resultados encontrados são relevantes e possibilitam que novas investigações devam ser feitas na busca dos efeitos metabólicos e estruturais da suplementação com ECP na placenta de éguas com quadros de placentite.

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Anexos

Pelotas, 06 de Janeiro de 2014

De: Prof. Dr. Éverton Fagonde da Silva

Presidente da Comissão de Ética em Experimentação Animal (CEEA)

Para: Professor Carlos Eduardo Wayne Nogueira

Faculdade de Veterinária

Senhor Professor:

A *CEEA* analisou o projeto intitulado: **"Avaliação do efeito da hormonioterapia em éguas com placentite através da identificação de receptores na placenta e grau de perfusão sanguínea uterina e sua relação com a viabilidade do neonato",** processo n°23110.003891/2013-37, sendo de parecer **FAVORÁVEL** a sua execução, considerando ser o assunto pertinente e a metodologia compatível com os princípios éticos em experimentação animal e com os objetivos propostos.

Solicitamos, após tomar ciência do parecer, reenviar o processo à *CEEA*.

Salientamos também a necessidade deste projeto ser cadastrado junto ao Departamento de Pesquisa e Iniciação Científica para posterior registro no *COCEPE* (código para cadastro n° **CEEA 3891**).

Sendo o que tínhamos para o momento, subscrevemo-nos.

Atenciosamente,

Prof. Dr. Éverton Fagonde da Silva

Presidente da CEEA

Ciente em: 7 / 1 /2014

Assinatura do Professor Responsável:

Anexo A - Documento da Comissão de Ética e Experimentação Animal