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Estradiol cypionate aided treatment for experimentally induced ascending placentitis in mares

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ABSTRACT

The overall goal of this study was to assess the efficacy of various therapeutic combinations of estradiol cypionate (ECP, a long-acting estrogen) and altrenogest (ALT, a long-acting progestin) in addition to basic treatment for placentitis with trimethoprim-sulfamethoxazole (TMS) and flunixin meglumine (FM). Specific outcomes measured in this experiment were (i) time from induction of bacterial placentitis to delivery, and foal parameters (high-risk, survival, and birth weight); and (ii) serum steroid concentrations (progesterone, 17α-hydroxyprogesterone, 17β-estradiol, and cortisol) in response to treatment. Pregnant mares (~300 days gestation, n = 46) were randomly assigned into healthy mares (control group, CONT, n = 8) and mares with experimentally induced ascending placentitis (n = 38). Placentitis was induced via intracervical inoculation of *Streptococcus equi subspecies zooepidemicus*. Thereafter, placentitis induced mares were randomly assigned into: (1) basic treatment, TMS+FM (n = 8); (2) basic treatment with ALT supplementation, TMS+FM+ALT (n = 8); (3) basic treatment with ECP supplementation, TMS+FM+ECP (n = 6); (4) basic treatment with ALT and ECP supplementation TMS+FM+ALT+ECP (n = 6); and (5) no treatment (INOC, n = 10). Treatments were started 48 h after bacterial inoculation and carried out for ten consecutive days. Blood samples were collected daily, and mares were assessed for signs of placentitis until the mare delivered, or for ten consecutive days after onset of treatment. Steroids were analyzed via RIA. Continuous data were analyzed by ANOVA, and categorical data analyzed by Fisher's exact test. Significance was set at $p < 0.05$. Foal survival at parturition and seven days post-delivery were similar across treated groups (66.7–100%), and to the CONT group. Similar to CONT group, mares in the TMS+FM+ECP group had no high-risk foals while mares in the other groups had higher incidences (50–75%) ($p < 0.05$). The inclusion of ECP in the treatments resulted in foals with body weight similar to CONT group ($p > 0.05$). There were no group effects or time by group interactions on concentrations of steroids assessed herein ($p > 0.05$). In conclusion, in addition to basic treatment TMS+FM, mares with experimentally induced ascending placentitis benefited from ECP supplementation. Conversely, ALT did not appear to make a difference in outcomes. The immunoassays used for measurements of steroid concentrations did not appear useful to assess treatment response.

1. Introduction

Ascending placentitis is an important cause of abortion, stillbirth and premature delivery of weak foals [1–3]. While there are regional variations in the bacterial and fungal agents associated with ascending placentitis in mares, β-hemolytic streptococci (*Streptococcus equi* subspecies *zooepidemicus* and *Streptococcus*

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equisimilis), and coliforms such as *Escherichia coli* are the predominant microbial isolates worldwide [1,4–6].

In ascending placentitis infection begins at the caudal placental pole (cervical star region), then bacteria spread cranial-ventrally towards the uterine body segments of the chorioallantois and gain access to the fetus either by migrating through umbilical vessels or through fetal fluids [6–8], consequently, reaching the fetus and becoming a potential cause of septicemia and fetal morbidity and mortality.

Placentitis is characterized by the production of pro-inflammatory cytokines such as IL-6 and IL-8, and prostaglandins (PGF2α and PGE2) [9,10]. Prostaglandin release increases uterine contractility and consequently increases the risk of premature delivery [11]. Inflammation and infection of the fetoplacental unit can induce premature activation of the fetal hypothalamic-pituitary-adrenal axis, thus accelerating fetal maturation before parturition [6,11,12]. Thus, early fetal maturation likely counterbalances premature delivery and may help improve the odds for foal survival [6,11]. It is worth noting that among farm animals, maturation of the equine fetus occurs latest in gestation [13]. This implies that any event that interferes with the normal function of the fetal-maternal unit such as placentitis or maternal disease could be devastating to the newborn foal.

Clinical diagnosis of ascending placentitis is based on the presence of clinical signs such premature udder development (with/without lactation), purulent/serosanguinous vulvar discharge, and ultrasonographic evidence of thickening and edema of the chorioallantois, and chorioallantois detachment from the endometrium at the caudal placental pole [14,15]. While overt clinical cases of ascending placentitis can be easily diagnosed, subtle and early cases can be missed using standard diagnostic means [6]. Recently, several molecular markers, including serum amyloid A, haptoglobin, 17β-estradiol, and alpha-fetoprotein have been identified as useful diagnostic tests for experimentally induced placentitis [16–21]. Some of these molecules are also suitable markers for spontaneous placentitis [20].

The equine fetoplacental unit is an intricate system involving the mare endometrium, the fetus, and the fetal membranes, where large quantities of steroids (estrogens, progestogens, and androgens) are produced and metabolized [18,22,23]. While the function of most equine fetoplacental steroids remains unknown, several studies have evaluated their concentrations to assess fetal well-being and placental health [12,18,24–26]. However, limited work has been carried out to determine the validity of using these steroid hormones as prognostic indicators in response to treatment of placentitis. Douglas [24] suggested that mares (from 100 d gestation to term) with low serum estrogen concentrations (<700 pg/mL), as determined by a commercial assay called "total-estrogens," were prone to abortion, whereas serum estrogen concentrations >1000 pg/mL resulted in the delivery of a live foal. In mares with experimentally induced ascending placentitis, progesterone concentrations were remarkably reduced in mares aborting in less than seven days compared to mares sustaining the pregnancy more than eight days post induction [25].

Treatment for bacterial placentitis is aimed at (i) eliminating or reducing the spread of microorganisms through the fetal membranes and fetus, (ii) keeping the uterus quiescent, and (iii) reducing the inflammatory response [6,27]. It has been suggested that these goals can be accomplished by treating mares with antimicrobials (i.e. a combination of penicillin and gentamicin, or trimethoprim-sulfamethoxazole), progestins (altrenogest or progesterone), and anti-inflammatories (flunixin meglumine, phenylbutazone, acetylsalicylic acid, pentoxifylline) [6,27–29]. If the treatment goals are accomplished, the gestation length of mares affected with placentitis should be similar to the expected normal duration of pregnancy (~330–340 days) and result in a live, well-developed foal with minimal health issues [6].

A number of controlled studies have shown the value of antimicrobial drugs, highlighting differences in drug selection (ability to cross membranes, the spectrum of activity, and potential toxicity to the fetus), duration of therapy, as well as immunomodulators in the treatment of experimentally induced ascending placentitis in mares [28–32]. However, the role of steroids hormone supplementation (estrogens and progestins) in the treatment of placentitis is poorly defined. Progestins have been included as a part of treatment in multiple placentitis studies [26,29–32], and one report failed to achieve an improvement in foal survival with the addition of altrenogest treatment [30]. However, it remains to be determined if progestins are beneficial for the treatment of placentitis. Estrogen therapy has been advocated as a necessary treatment for equine placentitis to reduce the risk of abortion [24]. Despite its anecdotal use in equine practice for years [24], to date, the treatment of placentitis with estrogens has not been critically evaluated under controlled experimental conditions.

The overall goal of this study was to assess the efficacy of various therapeutic combinations of a long-acting estrogen (estradiol cypionate; ECP) and a long-acting progestin (altrenogest; ALT) in addition to a basic treatment for placentitis with trimethoprim-sulfamethoxazole and flunixin meglumine (TMS+FM). Specific outcomes evaluated in this experiment were (i) time from induction of placentitis to delivery, gestational length, and foal parameters (high-risk, survival, and birth weight); and (ii) serum steroid concentrations (progesterone, 17α-hydroxyprogesterone, 17β-estradiol, and cortisol) in response to treatment. Our primary hypothesis was that the different treatment combinations (in particular ECP) would affect pregnancy outcomes and newborn foal parameters. Our secondary hypothesis was that measuring progestagens (progesterone and 17α-hydroxyprogesterone), 17β-estradiol, and cortisol could be used to assess response to treatment for experimentally induced ascending placentitis in mares. It was our expectation that the information obtained from the present study would enhance our understanding of the efficacy of various drugs and drug combinations in the treatment of equine placentitis.

2. Materials and methods

2.1. Mares and animal husbandry

All procedures carried out in the present study were approved by the Ethical Committee on Animal Experimentation of the Universidade Federal de Pelotas (UFPel) under protocol # 4750. Animal procedures carried out herein followed the guidelines of the European Union Directive (2010/63/EU) for animal experimentation. The mares were housed at Palma Farm of the UFPel, Capão do Leão, Rio Grande do Sul, Brazil. Forty-six pregnancies from 27 multiparous Criollo and Criollo-type mares (age 10 ± 2 years; parity 3 ± 0.5; body weight 437 ± 22 kg) were used in the experiment. None of the mares enrolled in this study had a history of subfertility or late-term pregnancy abnormality. Ovulation was determined by transrectal palpation and ultrasonography examinations performed every other day. All mares were bred via artificial insemination with fresh semen from a single fertile Criollo stallion (1.72 breeding/conception). Mares were maintained on pasture and supplemented with commercial concentrate pellets and water *ad libitum*. Before foaling, mares were kept in individual stalls at night and on pasture during the day. This study was carried out during the natural breeding season of the Southern Hemisphere from September–December for the years of 2012, 2013, and 2014.

2.2. Study design and therapeutic regimens

By 300 days of gestation, mares carrying normal pregnancies (, mean 301.7 ± 2.7, range 295–303 days) were randomly divided into healthy mares (control group, CONT, n = 8); and mares with experimentally induced ascending placentitis (n = 38). Before the beginning of the study, all mares had reproductive examinations, and transrectal ultrasonography of the caudal placental pole performed [6]. Since none of the mares presented any alteration on the ultrasound or overt clinical signs associated with pregnancy abnormalities, all mares were enrolled in this study [6]. Mares with experimentally induced placentitis were randomly assigned to treatment groups as follows: (1) TMS+FM (n = 8); (2) TMS+FM+ALT (n = 8); (3) TMS+FM+ALT+ECP (n = 6); (4) TMS+FM+ECP (n = 6); and (5) no treatment (INOC, n = 10). Treatment was started at 48 h post experimental induction of ascending placentitis [30] and carried out for ten consecutive days. The duration of treatment was chosen based on the protocol applied in the authors' practice and after the recommendations published elsewhere [27,28,30]. Detailed therapeutic regimens are described below (Table 1).

2.3. Experimental induction of ascending placentitis

Ascending placentitis was experimentally induced via intracervical inoculation of 10^7 colony forming units *of Streptococcus equi* subspecies *zooepidemicus (S. zooepidemicus)* as described by Mays and collaborators [10] and successfully used by others [16,17,29–32]. The *S. zooepidemicus* strain used in the present study was isolated from the chorioallantois of a Thoroughbred mare exhibiting typical clinical and pathological signs of ascending placentitis [1,27]. The isolate was identified using standard microbiological techniques (i.e., β hemolysis on blood agar, Gram-staining, and biochemical characteristics) [33].

The inoculum was aerobically cultured in brain heart infusion medium (BD Diagnostics Systems, Sparks, MD, USA) for 24 h at 37 °C. After culture, glycerol was added up to a final concentration of 10%, and 1.5 mL aliquots were prepared and cryopreserved in liquid nitrogen until use. Approximately, 48 h preceding the induction of placentitis, an aliquot of frozen bacteria (the stock isolate) was thawed and plated in ovine blood agar for 24 h to confirm the purity of *S. zooepidemicus*. On the day of induction, an inoculum containing 10^7 colony forming units in 1 mL of 0.9% saline solution was prepared by the McFarland turbidity standard method for bacterial suspensions (McFarland Turbidity Standard No 0.5, BD Diagnostics Diagnostic Systems, Sparks, MD, USA). The inoculum was deposited midway through the cervix with an equine artificial insemination pipette, using digital guidance. After cervical inoculation, bacterial viability was confirmed by re-culturing the content of each vial.

2.4. Blood sampling and monitoring

Blood samples were collected by jugular venipuncture from all mares immediately before induction of placentitis and then daily for ten consecutive days, or until premature delivery (i.e. blood sampling was discontinued after delivery). Blood samples were allowed to clot and then centrifuged at 600 ×*g* for 10 min. Serum was harvested and preserved at −20 °C until further analysis.

Transrectal ultrasonography of the caudal placental pole was performed daily to measure the combined thickness of uterus and placenta (CTUP) and to assess for signs of chorioallantois separation from the endometrium [6,15,34,35]. All mares were assessed daily for the presence of mammary gland development and vulvar discharge.

2.5. Foaling management and post-partum mare and foal care

After enrollment in the study mares were maintained in paddocks nearby the foaling barn. When imminent signs of parturition were observed, the mares had the tail wrapped, vulva washed and brought inside foaling stalls (6 × 6 m) for assisted vaginal delivery. During the second stage of labor, a subset of mares (n = 30) had amniotic fluid collected aseptically via aspiration with a sterile needle and syringe. Amniotic fluid was aerobically cultured as described above. All mares were closely monitored until the passage of the fetal membranes. Immediately after placental release and by 24 h post-delivery, a subset of mares (n = 26) had uterine swabs aseptically collected for aerobic cultures as aforementioned (subheading 2.3) [33].

Immediately after delivery, all foals had a full physical examination performed and birth weight recorded. Within 15 min of parturition, foals delivered alive had blood collected by venipuncture of the jugular vein, for determination of leukocyte counts (reference 5.3–16.8 × 10^3 cell/μL, neutrophil: lymphocyte ratio <2:1), and fibrinogen concentration (reference ≤ 400 mg/dL) [36]. Total leukocyte counts were determined using a commercial cell counter (Sysmex pocH-1000V™ Hematology-Analyzer, Sysmex Brazil), and smears were prepared for differential leukocyte counts. Fibrinogen was determined by heat precipitation. Foal attitude and demeanor was carefully assessed immediately after delivery. Foals that demonstrated the ability to breathe without assistance (<2 min), assume sternal recumbence (<5 min), exhibit normal suckling reflex (<20 min) and stood with no or minimal assistance (<1 h) were classified as low-risk (apparently healthy) at birth [36]. Foals classified as high-risk required major assistance and showed clinical signs of immaturity (silky hair-coats, floppy ears, delayed sucking, difficulty standing without support, and an abnormal neutrophil: lymphocyte ratio), or had evidence of sepsis (injected mucous membranes, hypo- (<36.6 °C), or hyperthermia (>38.8 °C), depression, mucous membranes petechial hemorrhage, abnormal total leukocyte counts, increased immature neutrophils, and hyperfibrinogenemia). Classification of high-risk or low-risk was carried out immediately after parturition, and all foals clearly fell into one category or the other.

Foals classified as high-risk received Ampicillin (20 mg/kg, IV, q 8 h; Ampicilina Veterinária® Vetnil, São Paulo, Brazil), flunixin meglumine (0.5 mg/kg, IV, q 12 h; Desflan® Ouro Fino Saude Animal, São Paulo, Brazil) and intravenous fluid therapy as needed for seven days post-delivery. All foals had a full physical examination performed daily for the first seven days post-delivery, and survival rates were recorded and used for comparisons among groups.

Table 1
Therapeutic armamentarium used to treat mares with experimentally induced ascending placentitis.

Drugs	Dose	Route	Manufacturer
Trimethoprim-sulfamethoxazole	30 mg/kg	IV, q12 for 10d	Trissulfim®, Ouro Fino Saude Animal, São Paulo, Brazil
Flunixin meglumine	1.1 mg/kg	IV, q24h for 10d	Desflan®, Ouro Fino Saude Animal, São Paulo, Brazil
Altrenogest (long action)	0.088 mg/kg	IM, q 7d for 2 treatments	Altrenogest®, Botupharma, São Paulo, Brazil
Estradiol cypionate	10 mg/mare	IM, q 3d for 3 treatments	E.C.P.®, Zoetis, São Paulo, Brazil

Stillborn animals were accounted for as high-risk foals for effects of comparisons among the different groups.

2.6. Placental pathology and microbiology

Following the passage of fetal membranes from all mares, the weights of the fetal membranes were recorded and gross examination performed as described elsewhere [1,37]. Specimens (full thickness duplicates of 3 × 3 cm) were collected from chorioallantois (cervical star area, and segments corresponding to the cranial uterine body, pregnant horn, and non-pregnant horn), amnion, and umbilical cord, and were submitted for histopathology and aerobic culture. Placental cultures were carried out as described above (subheading 2.3) to demonstrate the presence of *S. zooepidemicus*. Any grossly abnormal placental region of the fetal membranes, other than specified above, were also fixed in formalin for further histological evaluations. Stillborn foals and foals that died after delivery had a standard necropsy performed which included tissue (spleen, liver, lungs, heart, kidneys, and brain) for histopathological and microbiological evaluations. Gross and histological evaluations were conducted to confirm the clinical diagnosis of ascending placentitis and to assess the severity of lesions.

Histological sections (3- to 5-μm thick) from various tissues were mounted on glass slides and stained with standard hematoxylin and eosin. Histological evaluations were carried out by an experienced pathologist blinded to treatment groups. Based on the histological assessment, fetal membranes were classified as having no significant lesions, or lesions consistent with acute or chronic placentitis [1]. The presence or absence of bacteria or lack of any lesions were recorded, and results are described below.

2.7. Hormonal analyses

Serum concentrations of progesterone, 17α-hydroxyprogesterone, 17β-estradiol and cortisol were measured via tube-coated radioimmunoassays (MP Biomedicals, Solon, OH, USA (Table 2).

According to the manufacturer, the reported cross-reactivity for antiserum used for the progesterone assay: progesterone (100%), 17α-hydroxyprogesterone (2.5%), 20α- hydroxyprogesterone (0.1%), 20β-hydroxyprogesterone (0.1%), dihydrotestosterone (0.1%), testosterone (0.06%), 17β-estradiol, and cortisol (<0.01%). For the 17α-hydroxyprogesterone, assay, cross-reactivity was: 17α-hydroxyprogesterone (100%), 17α-hydroxypregnenolone (2.2%), progesterone (0.49%) and pregnenolone, pregnenolone sulfate, 20α-hydroxyprogesterone, dehydroepiandrosterone (DHEA), 17β-estradiol and cortisol (<0.01%). For the estradiol assay, known cross-reactivity was: 17β-estradiol (100%), estrone (6%), estriol (1.45%), DHEA, DHEA-sulfate, 20α-hydroxyprogesterone, 5α-hydroxyprogesterone, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, pregnenolone, progesterone, and testosterone (<0.01%). For the cortisol assay, known cross-reactivity was: cortisol (100%) prednisolone (45.6%), 11-desoxycortisol (12.3%), corticosterone (5.5%), prednisone (2.7%), cortisone (2.1%), 17α-hydroxyprogesterone (1.0%), progesterone (0.25%) and testosterone (<0.01%).

2.8. Statistical analyses

All analyses were performed using Statistix 9.0 software (Analytical Software, Tallahassee, Florida, USA). Normality was assessed by the Shapiro-Wilk test. Continuous data (time from induction of placentitis to delivery, gestational length, foal weight, placental weight and its time for release) were analyzed by ANOVA one way. Hormone concentrations were analyzed by ANOVA repeated measures. When significant, posthoc comparisons were made by the least-significant difference test. Fisher's exact test was used to analyze categorical data [clinical signs, premature chorioallantois separation from the endometrium, dystocia, fetal membranes pathological features, microbiology (uterus, fetal membranes, and amniotic fluid), high-risk foals at parturition, and foal survival]. Statistical significance was set at $p < 0.05$, whereas statistical tendency was defined as $0.05 \leq p < 0.1$. Continuous results were expressed as mean ± SEM, whereas categorical results were represented as percentage and proportions to facilitate interpretation.

3. Results

3.1. Clinical signs of ascending placentitis

None of the healthy control mares showed premature mammary gland development or purulent vulvar discharge, whereas 89% (n = 34/38) of all inoculated mares started to show purulent vulvar discharge by 48 h post-intracervical inoculation (Table 3). It is worth noting that all four mares that failed to develop vulvar discharge after experimental induction of ascending placentitis were allocated to the INOC group. As anticipated [29], only 31% (n = 12/38) of the mares showed mammary gland development by 48 h post-inoculation, with no significant differences between groups (Table 3). However, for the INOC (i.e. group that did not receive treatment for placentitis), only one mare presented mammary gland development by 48 h post-inoculation (Table 3). Experimental induction of ascending placentitis resulted in increased CTUP values (92%, n = 35/38) and placental separation (95%, n = 36/38) across all groups, while gestationally age-matched control mares had CTUP values that remained within the reported ranges consistent with normal pregnancies [35] (Table 3). Three mares in the INOC group did not present any increase in CTUP values. Two of these mares with no increased CTUP and no placental separation aborted by 24 h and 48 h post-inoculation. One mare with no increase in CTUP, but with placental separation, delivered a premature septic foal by 48 h post-inoculation (at 297 days of gestation). This foal died 12 h post-delivery with sepsis.

Table 2
Reported antiserum sensitivity and limits of detection for the different assays used.

	CV (%)		Sensitivity	Standard curve (range)	Catalog#[a]
	Intra-assay	Inter-assay			
P4	4.9	6.5	0.02 ng/mL	0.15–80 ng/mL	07–270102
17-OHP	12	12	0.03 ng/mL	0.1–25 ng/mL	07–271102
17β estradiol	3.5	7.6	7.4 pg/mL	10-3000 pg/mL	07–238102
Cortisol	8.9	9.3	0.0017 μg/mL	0.01–1 μg/mL	07–221102

P4: progesterone, 17-OHP: 17α-hydroxyprogesterone.
[a] ImmuChem™ Coated Tube, MP Biomedicals, Solon, OH, USA.

Table 3
Vulvar discharge, premature mammary gland development, and ultrasonographic features of the caudal placental pole, at the onset and the end of treatment for experimentally-induced ascending placentitis. Treatment was initiated 48 h post induction of placentitis and continued for 10 consecutive days or until abortion. Gestationally age-matched (healthy control group, CONT) and mares receiving no treatment (inoculation, INOC) and no treatment were assessed at similar time points. All mares were enrolled in this experiment by 300 d of gestation and randomly assigned to the six groups.

Groups	Vulvar discharge				Mammary development				Increased CTUP >9 mm		Placental separation	
	48 h		10 d		48 h		10 d		48 h		48 h	
	(%)	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)	n
CONT (=8)	0^{b}	0	0^{b}	0	0	0	0	0	0^{b}	0	0^{b}	0
TMS+FM (n = 8)	100^{a}	8	75^{a}	6	25	2	25	2	100^{a}	8	100^{a}	8
TMS+FM+ALT (n = 8)	100^{a}	8	62.5^{a}	5	50	4	50	4	100^{a}	8	100^{a}	8
TMS+FM+ALT+ECP (n = 6)	100^{a}	6	33.3^{ab}	2	33.3	2	33.3	2	100^{a}	6	100^{a}	6
TMS+FM+ECP (n = 6)	100^{a}	6	0^{b}	0	50	3	50	3	100^{a}	6	100^{a}	6
INOC (n = 10)	60^{b}	6	60^{b}	6	10	1	10	1	70^{b}	7	80^{b}	8

TMS: trimethoprim-sulfamethoxazole; FM: flunixin meglumine; ALT: altrenogest; ECP: estradiol cypionate. CTUP: combined thickness of uterus and placenta. Different letters within columns denote statistical significance with Fisher's exact test.

3.2. Time from inoculation to delivery, gestation length, and occurrence of dystocia

Following intracervical inoculation, mares in the INOC group had the highest number of dystocias and premature parturitions (Table 4) ($p < 0.05$). The TMS+FM+ECP group had the longest time from inoculation to delivery. Gestation length of TMS+FM+ECP and TMS+FM+ALT+ECP groups were not significantly different than that of healthy CONT group (Table 4).

3.3. Foal survival

Foal survival at parturition and seven days post-delivery were not different for all groups of mares with experimentally induced ascending placentitis receiving any therapeutic regimen and not different than control mares ($p > 0.05$), but higher than mares in the INOC group ($p < 0.05$) (Table 5). Overall, 48% (n = 22/46) foals were classified as high-risk. Groups receiving the TMS+FM+ECP and TMS+FM therapeutic regimens had the lowest rates of foals classified as high-risk immediately after parturition (Table 5). There were nine stillborn foals; seven of them experienced dystocia (INOC group), whereas the other two animals did not experience problems with the delivery (TMS+FM+ALT; TMS+FM+ALT+ECP). Foal clinical findings are not reported here as it was outside the scope of this manuscript.

3.4. Fetal membranes expulsion and pathology

Placental weight and time for the release of the fetal membranes were not significantly different between healthy control mares and mares with experimentally induced ascending placentitis (Table 6). Two mares had retained fetal membranes for more than 3 h post foaling. One mare with retained fetal membranes for 6 h was allocated in the INOC group, while the second mare had retained fetal membranes for 4 h was assigned to the TMS+FM+ECP group.

There was a higher number of premature chorioallantois separations from the endometrium ("red-bag") in allocated in the INOC group ($p < 0.05$) (Table 4). Regardless of the treatment group, there was no significant difference in time for the release of fetal membranes between mares with evidence of "red-bag" (29.3 ± 10.7 min) or mares with no evidence (45.3 ± 9.3 min) at parturition.

Mares treated with TMS+FM and mares in the INOC group had the lowest frequency of gross lesions in the fetal membranes in comparison with the other placentitis groups ($p < 0.05$) (Table 6). Recorded gross abnormalities (55%, n = 21/38) were those typically reported in ascending placentitis, and consisted of thickening, edema, and purulent or serosanguineous exudate at the cervical star region [1]. Not infrequently, the lesions extended ventrally into the uterine body of the chorioallantois.

Histopathologic evaluations of the fetal membranes revealed that 45% (n = 17/38) of mares had acute placentitis, 18% (n = 7/38) had chronic placentitis, and 37% (n = 14/38) did not have significant lesions after experimental induction of placentitis (Table 6). Fetal membranes with acute placentitis had suppurative inflammation with intense neutrophilic infiltration in the chorioallantois at the cervical star region, and at the segments corresponding to the uterine body and pregnant horn (Fig. 1), whereas chronic placentitis was characterized by mononuclear inflammatory cells in

Table 4
Time from inoculation to delivery, gestation length, and occurrence of premature chorioallantois separation from the endometrium and dystocia for groups with experimentally induced ascending placentitis and gestationally age-matched healthy control group. All mares were enrolled in this experiment by 300 d of gestation and randomly assigned to the six groups.

Groups (n)	Time from inoculation-delivery (d)		Gestational length (d)		Premature chorioallantois separation from the endometrium		Dystocia	
	Mean ± SEM	Range	Mean ± SEM	Range	(%)	n	(%)	n
CONT (n = 8)	35 ± 4.9^{ab}	20–50	335 ± 5^{ab}	320–350	0^{c}	0	0^{b}	0
TMS+FM (n = 8)	27.6 ± 8.6^{b}	10–82	322 ± 6.5^{b}	310–353	62.5^{ab}	5	12.5^{b}	1
TMS+FM+ALT (n = 8)	21.3 ± 4.1^{b}	9–39	322 ± 3.8^{b}	312–339	12.5^{bc}	1	0^{b}	0
TMS+FM+ALT+ECP (n = 6)	22.2 ± 6.4^{b}	5–52	330 ± 11.2^{ab}	317–352	50^{ab}	3	16.7^{b}	1
TMS+FM+ECP (n = 6)	46 ± 4.2^{a}	36–65	346 ± 5.2^{a}	336–365	16.7^{bc}	1	0^{b}	0
INOC (n = 10)	3.5 ± 0.7^{c}	1–7	305 ± 2.3^{c}	296–307	80^{a}	8	70^{a}	7

CONT: healthy control group; TMS: Trimethoprim-sulfamethoxazole; FM: flunixin meglumine; ALT: altrenogest; ECP: estradiol cypionate; INOC: inoculation and no treatment. Different letters within columns denote differences with LSD's test (time from inoculation to delivery, and gestational length) or Fisher's exact test (premature chorioallantois separation from the endometrium and dystocia) ($p < 0.05$).

Table 5
Clinical parameters (survival rates, risk classification, and body weight) of foals born from mares with experimentally induced ascending placentitis and gestationally age-matched healthy control mares. All mares were enrolled in this experiment by 300 d of gestation and randomly assigned to the different groups.

Groups (n)	Survival at parturition		Foals classified as high-risk		Survival at 7 d delivery		Body weight at birth (kg)	
	(%)	n	(%)	n	(%)	n	Mean ± SEM	range
CONT (n = 8)	100[a]	8	0[b]	0	100[a]	8	39.2 ± 2.4[a]	29–45
TMS+FM (n = 8)	100[a]	8	75[a]	6	75[a]	6	31.1 ± 1.7[c]	26–40
TMS+FM+ALT (n = 8)	87.5[a]	7	50[a]	4	87.5[a]	7	28.4 ± 1.5[c]	21–32
TMS+FM+ALT+ECP (n = 6)	87.5[a]	5	50[a]	3	66.7[ab]	4	32.5 ± 2.7[bc]	25–39
TMS+FM+ECP (n = 6)	100[a]	6	0[b]	0	100[a]	6	36.4 ± 1[ab]	34–40
INOC (n = 10)	30[b]	3	90[a]	9	20[b]	2	29.4 ± 1.8[c]	25–39.5

CONT: healthy control group; TMS: trimethoprim-sulfamethoxazole; FM: flunixin meglumine; ALT: altrenogest; ECP: estradiol cypionate; INOC: inoculation and no treatment. Different letters within columns denote differences with Fisher's exact test (foal survival and risk classification), or with LSD's (body weight) ($p < 0.05$).

Table 6
Time from foaling until the expulsion of the fetal membranes and placental features from mares with experimentally induced ascending placentitis and gestationally age-matched healthy control mares. All mares were enrolled in this experiment by 300 d of gestation and randomly assigned to the different groups.

Groups	Fetal membranes features								
	Weight (kg)		Time from foaling until expulsion (min)		Gross lesions		Histopathology		
	Mean ± SEM	Range	Mean ± SEM	Range	(%)	n	acute	chronic	NSF
CONT (n = 8)	4.8 ± 0.3	2.8–5.6	32.4 ± 5.1	15–50	0[c]	0	0	0	8[a]
TMS+FM (n = 8)	5.1 ± 0.6	2.9–8.2	41.5 ± 13.9	1–110	50[b]	4	3	1	4[b]
TMS+FM+ALT (n = 8)	5.5 ± 0.4	4.0–7.9	34.5 ± 10.2	10–92	87.5[a]	7	6	1	1[b]
TMS+FM+ALT+ECP (n = 6)	4.7 ± 0.6	3.0–6.8	40.0 ± 15.0	15–107	66.7[a]	4	3	1	2[b]
TMS+FM+ECP (n = 6)	5.1 ± 0.5	3.3–6.8	66.3 ± 35.1	16–240	66.7[a]	4	0	4	2[b]
INOC (n = 10)	5.9 ± 0.6	3.5–9.5	83.4 ± 33.4	5–360	20[bc]	2	5	0	5[b*]

CONT: Healthy control group; TMS: trimethoprim-sulfamethoxazole; FM: flunixin meglumine; ALT: altrenogest; ECP: estradiol cypionate; INOC: inoculation and no treatment. Different letters within columns denote differences with LSD's test (placental weight and time to release), or Fisher's exact test (fetal membranes pathological features). *These fetal membranes had evidence of bacterial colonies in the chorioallantois, amnion, and umbilical cord.

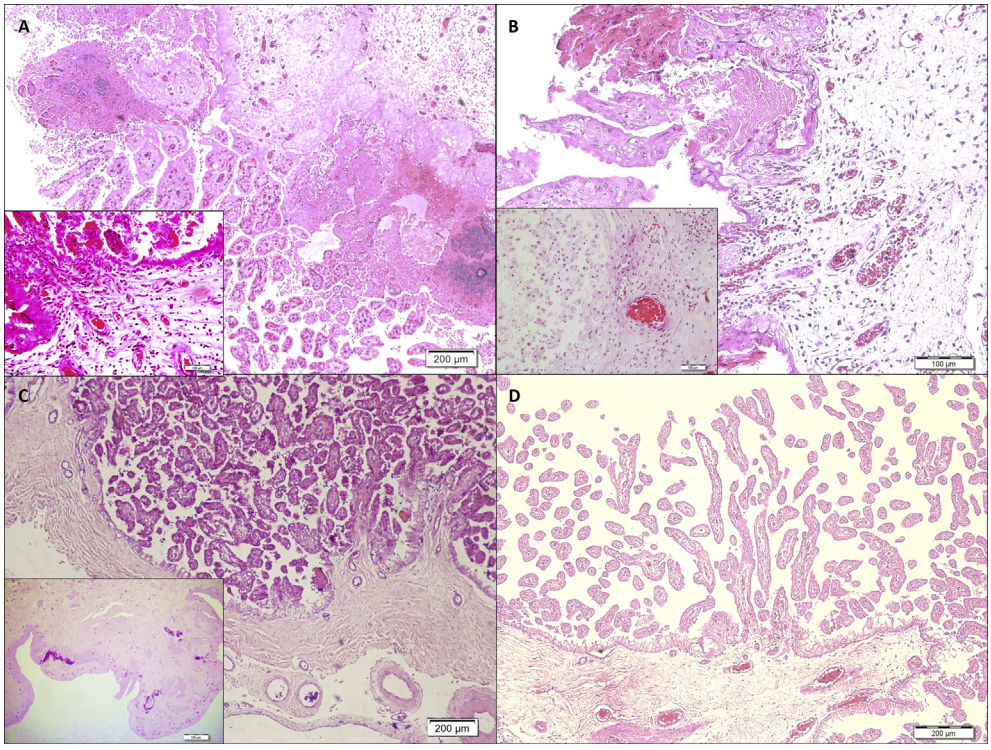

Fig. 1. Representative histological preparations from the chorioallantois from normal healthy mares and mares with experimentally induced placentitis. (A): Acute placentitis: placental edema and moderate neutrophilic infiltration can be observed; (B) Chronic placentitis, mononuclear inflammatory cells, and exudate can be noted in the chorionic epithelium; (C) Chorionic surface with no histological lesions, but bacterial colonies can be observed in the insert in this umbilical cord section. This presentation was typical for mares in the inoculated group that did not receive any. treatment after experimental induction of placentitis. (D) Healthy chorioallantois with no significant lesions. H&E stained.

the microcotyledonary trophoblast or chorionic stroma, and also by mild to moderate necrosis (Fig. 1). Mares in the INOC group had delivered prematurely shortly after intracervical inoculation of bacteria (typically within 48 h), with 50% of the mares not showing gross or histological lesions, despite the presence of bacterial colonies in the chorioallantois, amnion, and umbilical cord (Table 6).

Necropsy of stillborn and foals that died within the seven days of parturition revealed the following histological lesions: central nervous system (neuron chromatolysis, edema and vacuolization of microglial cells), lungs (atelectasis and presence of basophilic bacterial colonies), adrenal glands (diffuse to extensive hemorrhage), kidneys (necrosis, congestion, hemorrhage and mild tubular necrosis), liver (congestion, scant necrosis and hemorrhage), intestines (villous necrosis), spleen (hemorrhage), and heart (hemorrhage). Collectively, these findings were consistent with hypoxia and sepsis.

Data from the subset of samples described above (Table 7) were combined to assess the effects of isolating *S. zooepidemicus* in the fetal membranes, amniotic fluid on foal survival and being classified as high-risk. The presence of *S. zooepidemicus* in fetal membranes (7/9 dead vs. 2/9 live foals) tended to negatively affect foal survival at parturition ($p = 0.05$) and increased the percentage of foals classified as high-risk (12/17 high-risk vs. 5/17 low-risk foals; $p = 0.02$). Similarly, negative cultures for *S. zooepidemicus* in the amniotic fluid resulted in better foal survival at parturition (19/22 survived vs. 3/22 died; $p = 0.03$) and in a higher proportion of foals classified as low -risk (18/22 low-risk vs. 4/22 high-risk foals; $p = 0.01$).

3.5. Steroid concentrations

No significant differences were observed in plasma concentration of progesterone, 17α-hydroxyprogesterone, 17β-estradiol and cortisol between the groups and there was no time by group interaction (Fig. 2). Since our aim was to use concentrations of these steroids to assess response to treatments for experimentally induced ascending placentitis, we did not assay hormone concentrations for mares in INOC group.

4. Discussion

The findings in the present study appear to support the clinical impression by practicing veterinarians that therapeutic estrogen supplementation may aid recovery from ascending placentitis, as evidenced by normal gestation length and no pregnancy loss or premature foalings in TMS+FM+ECP group. Foal survival rates at parturition and seven days post-delivery were not different across groups receiving any therapeutic regimen or CONT foals. Notably, mares in the other groups had more foals classified as high-risk than healthy CONT and TMS+FM+ECP groups. This further supports our hypothesis that therapy with ECP, a long-acting estrogen, reduced complications of experimental induction of ascending placentitis. If we extrapolate our findings to spontaneously-occurring cases, adding estrogen to the therapeutic regime for mares with placentitis could be beneficial to mares and foals, having a long-lasting and positive economic impact. Foals born of mares with placentitis are typically underdeveloped, with clinical evidence of prematurity, dysmaturity, maladjustment syndrome, and most become septic [38]. Term foals from these mares are also at high-risk for perinatal diseases that require a high level of intensive care that results in exorbitant expenses to the owner despite the fair prognosis for recovery [2,38].

Estrogens appear to be essential for fetal development and maturation [39], but not necessary for pregnancy maintenance [40]. Surgical removal of the fetal gonads decreased DHEA and its conjugated form (DHEA-sulfate) in maternal circulation [39,41]. Since DHEA is used as a precursor for estrogens by the fetal membranes and mare's endometrium [18,42], fetal gonadectomy reduced serum concentrations of estrogen and PGF2α (which is intimately controlled by estrogen) and resulted in obstetrical complications due to delayed stage II of labor, and delivery of underdeveloped and immature foals [39,41]. A recent study using letrozole, a potent aromatase inhibitor, reduced peripheral estrogen concentration by 20% in mares carrying healthy pregnancies [40]. Foals born from letrozole-treated mares had lower birth weight than foals born from control mares. Interestingly, when ECP was included in the combination TMS+FM+ALT (as group TMS+FM+ALT+ECP of this study), foal weight at parturition increased. Collectively, these studies and the present study appear to support the use of estrogen supplementation for mares with placentitis to maintain normal gestation length and allow proper fetal development and maturation before delivery. While estrogens have been empirically advocated to treat equine placentitis in practice [24], it remains to be determined by case-control and cohort prospective studies whether recovery from spontaneous placentitis could be improved with estrogens.

Progestins supplementation has long been recommended as a standard therapeutic practice for women with pregnancy complications (e.g. short cervix, chorioamnionitis, and recurrent idiopathic miscarriage) [43]. In the horse, there is no evidence that inclusion of a progestin is necessary for management of placentitis [6]. In fact, a study in mares with experimental induction of placentitis treated with altrenogest, TMS, and different combinations of anti-inflammatories (acetylsalicylic, dexamethasone) concluded that TMS alone appeared to be a superior treatment [30]. However, it is important to mention that the study used a small dose of altrenogest (estimated in 11 mg/mare or 2 mg/50 kg of body weight), which is a fourth of the minimal recommended dosage (44

Table 7
The frequency of positive aerobic culture for *Streptococcus equi subspecies zooepidemicus* in swabs obtained from the uterus, fetal membranes, and amniotic fluid at the time of delivery from mares with experimentally induced ascending placentitis and gestationally age-matched healthy control mares.

Groups	Uterine time 1		Uterine time 2		Fetal membranes		Amniotic fluid	
	(%)	n	(%)	n	(%)	n	(%)	n
CONT (n = 8)	0^{b}	0/7	0^{b}	0/7	12.5^{c}	(1/8)	0^{b}	0/6
TMS+FM (n = 8)	40^{a}	2/5	0^{a}	0/5	50^{ab}	(4/8)	0^{b}	0/3
TMS+FM+ALT (n = 8)	60^{a}	3/5	20^{a}	1/5	71.4^{ab}	(5/7)	16.7^{ab}	1/6
TMS+FM+ALT+ECP (n = 6)	33.3^{a}	1/3	33.3^{a}	1/3	16.7^{bc}	(1/6)	40^{a}	2/5
TMS+FM+ECP (n = 6)	50^{a}	2/4	50^{a}	2/4	33.3^{bc}	(2/6)	40^{a}	2/5
INOC (n = 10)	100^{a}	2/2	50^{a}	1/2	90^{a}	(9/10)	60^{a}	3/5

CONT: healthy control group; TMS: trimethoprim-sulfamethoxazole; FM: flunixin meglumine; ALT: altrenogest; ECP: estradiol cypionate; INOC: inoculation and no treatment. Uterine time 1: Swab collected immediately after the release of the expulsion of the fetal membranes. Uterine time 2: uterine swab collected by 24 h post-delivery. A subset of mares from each group had cultures performed. The proportion of samples with a positive culture for *Streptococcus equi subspecies zooepidemicus* are represented within brackets. Different letters within columns denote differences with Fisher's exact test ($p < 0.05$).

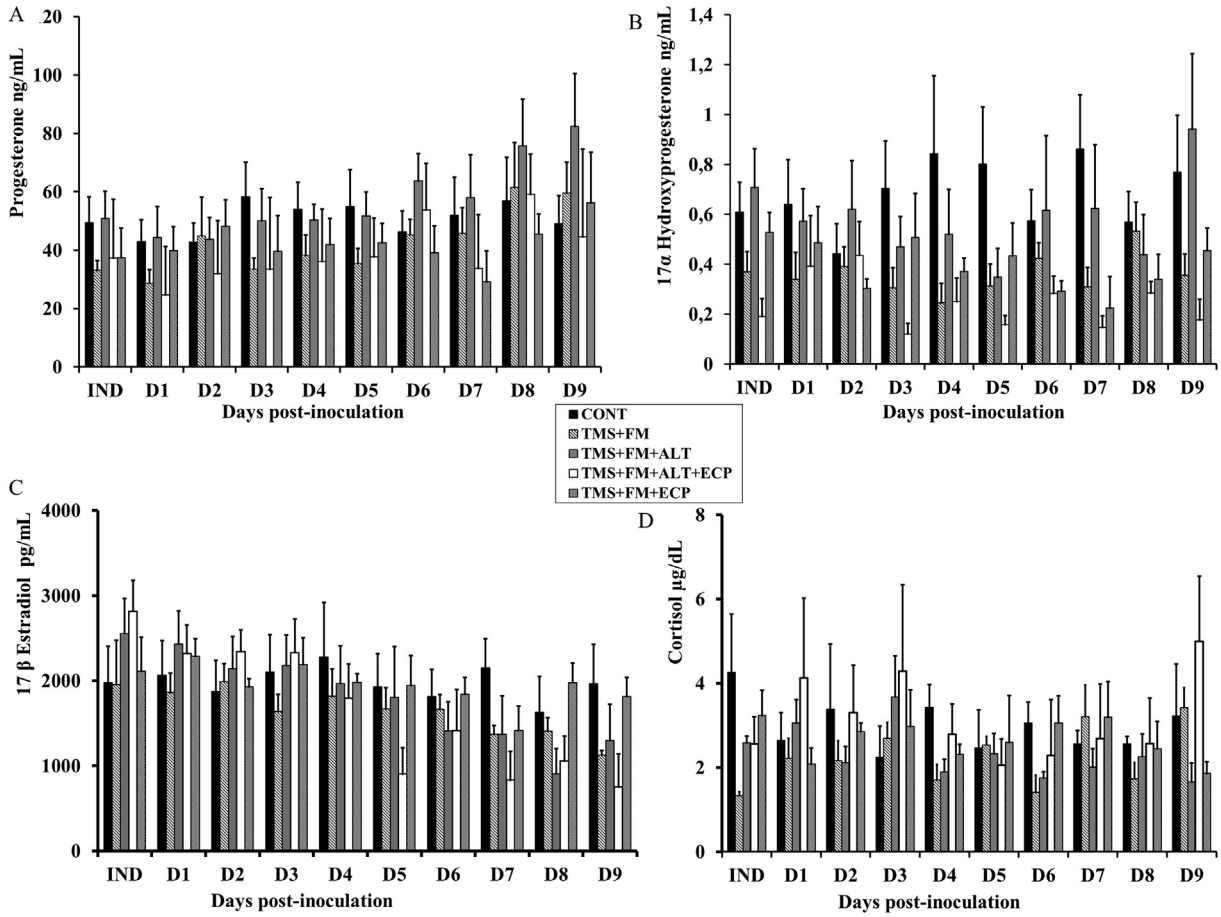

Fig. 2. Serum steroid concentrations in late-term pregnant mares with experimentally induced ascending placentitis and gestationally age-matched healthy control mares. (CONT, healthy control mares n = 6), TMS: sulfamethoxazole, FM: flunixin meglumine; ALT: altrenogest; ECP: estradiol cypionate. There was no effects of groups or time by group interaction for any of the hormones assay ($p < 0.05$).

mg/mare/day), and one eight of the presumed ideal dosage for placentitis (88 mg/mare/day). Our results appear to support the findings by Christensen et al. [30], in which a combination of antibiotics, progestins, and anti-inflammatory drugs did not improve mare or foal parameters in comparison to groups treatment with antimicrobial.

A study with a prostaglandin-induced model for the abortion of mares in the first trimester of gestation demonstrated that altrenogest supplementation (44 mg/day/mare) was superior to progesterone (300 mg/day/mare) at maintaining pregnancy and preventing generation of endogenous prostaglandin after daily treatment with cloprostenol [44]. Since placentitis increases prostaglandin concentrations in the fetal membranes and fluids [9], with a subsequent increase in uterine contractility and abortion [9,11], it has been assumed that inclusion of progestin is necessary to keep the uterus quiescent and prevent prostaglandin release [6]. Under this assumption, we included altrenogest in two treatment regimens to assess its efficacy. From our results, altrenogest did not appear to make a difference in outcome for mares with experimentally induced ascending placentitis. This should not be surprising as a recent large prospective study concluded that progesterone supplementation did not improve pregnancy outcome in women with a history of recurrent miscarriage [45]. However, it should be taken into consideration that horses and humans have different placentation and thus may require different management strategies.

Premature placental separation from the endometrium is one of the leading causes of perinatal asphyxia in foals [2]. Presumably, premature separation will result in oxygen deprivation prior to and during parturition [2]. As the mare has a long vagina if the chorioallantois is found unruptured protruding through the vulva, this means that a vast portion of the chorioallantois is detached from the endometrium, and the foal must be delivered immediately to prevent neonatal asphyxia. As expected, experimentally induced placentitis was associated with premature chorioallantois separation from the endometrium across groups, and absence of any treatment resulted in the highest occurrence of this condition (80%, n = 8/10). Regardless of group, treatment for placentitis reduced the occurrence of “red-bag”, when compared to the untreated group.

Dystocia is reported to occur ~10% of parturitions in light horse breeds [46,47]. Given this reported prevalence, experimentally induced placentitis with no treatment resulted in a seven-fold increase (70%) in dystocias. This is probably because most foals in this group were stillborn (78%, n = 7/9). It has been suggested that during the first to second stages of parturition, the foal's inner ear is involved and helps the foal to position itself to assume proper orientation and posture in the birth canal. This will not happen if the foal is dead, thus explaining the higher predisposition for dystocia with stillborn foals [48,49]. Interestingly, all nine cases of dystocia were due to malposture abnormalities (head and limbs), with a lateral/ventral deviation of the head and neck being observed in 66.7% (n = 6/9), and the types of dystocia found herein were consistent with the most prevalent types of dystocia reported

in referring hospitals [50].

Retained fetal membranes were observed in 4.3% of mares (n = 2/46); this is a very low occurrence of retained fetal membranes, especially given that 19% (n = 9/46) of mares experienced dystocia, which is a known risk factor for retained fetal membranes in mares [51]. The rate of retained fetal membranes appeared to be lower than two previous large retrospective studies involving Standardbred mares (3456 parturitions) in Canada [51], and Thoroughbreds (1432 parturitions) in Japan [52], that recorded 5.2–10% of foalings to have retained fetal membranes. Breed differences and environment can probable explain the low occurrence of retained fetal membranes observed in the present study. A study involving 270 parturitions of Thoroughbred mares in Brazil reported retained fetal membranes in 4.8% of foalings, suggesting that the incidence of retained membranes may be lower in southern Brazil [53].

Controversy exists with regards to the duration of the treatment for placentitis, with some authors suggesting that mares should be treated for a short period (6–15 days) [27,28,30], whereas others suggest that treatment should be continued until parturition [15,29,54]. In this study, mares were treated for ten consecutive days following the recommendations by Leblanc [27]. While we cannot assess whether treatment of mares for a prolonged period could have resulted in superior outcomes, our results are consistent with a previous publication for mares receiving treatment for an extended period (i.e. inoculation to parturition) [29]. This suggests that the duration of treatment used in the current study was adequate.

As aforementioned, the inclusion of antimicrobials is aimed at eliminating and reducing the spread of microorganisms through the fetal membranes and fetus [6,27]. However, there are limited therapeutic options for placentitis treatment, as only penicillin, gentamicin, and TMS are known to cross the placenta and to achieve high concentrations in the fetal fluids, and to be apparently safe for the fetus [28,55]. Since TMS is a cheap and broad spectrum antimicrobial, we elected to use this drug in the present study. For those reasons, this drug has been extensively used by previous authors for experimental placentitis [29,30,56] and spontaneous placentitis [15]. In fact, Christensen et al. [30], obtained apparently superior outcomes when mares were treated with TMS in comparison to other treatments containing other therapeutic drugs. While we cannot be certain whether the use of a different antimicrobial would have changed the outcome for various parameters assessed, this seems unlikely.

Recent evidence indicated that prolonged treatment for placentitis (from induction until parturition) suppressed bacterial growth, rather than eliminating bacteria, as mares treated for experimentally induced placentitis had a remarkable recovery of *S. zooepidemicus* in the uterus after delivery [29]. Similarly, despite treatment, we were still able to isolate *S. zooepidemicus* from the uterus, fetal membranes, and amniotic fluid from mares with experimentally induced ascending placentitis.

Contrary to our hypothesis, the four steroid hormones (progesterone, 17α-hydroxyprogesterone, 17β-estradiol, and cortisol) assessed in the present study did not change in response to treatment. Based on previous literature, we expected that progestins would be increased in mares maintaining pregnancy beyond seven days post-inoculation [25,26], and 17β-estradiol to be reduced following inoculation [18] as it has been suggested in mares with spontaneous cases of placentitis [24]. We expected that cortisol would be present in increasing concentrations in the peripheral serum of mares experimentally induced placentitis as a consequence of the disease process. However, concentrations of cortisol remained similar across time and groups. In addition, we cannot be certain whether steroids assessed here are not useful tools to evaluate response to treatment in mares with experimentally induced ascending placentitis, as it is possible that the lack of consistency observed here compared to previous studies might be due to assay sensitivities and cross-reactivity for the various tests used.

A recent study documented that following experimental induction of bacterial placentitis mares had a noticeable decline in 17 β-estradiol measured with a chemiluminescent immunoassay [18]. While estrogen concentrations were similar herein between groups, differences in cross-reactivity between assays can explain the discrepancies in results. For instance, the assay used here cross-reacted with estrone 6%, and estriol 1.45%, two steroid molecules present in high concentration in serum of pregnant mares [57]. On the other hand, the assay used by Canisso and others [18], cross-reacted with estrone at 2% and did not present any relevant cross-reactivity with other reproductive or adrenal steroids. Also, mares enrolled in that study did not receive any treatment after experimental placentitis induction [18].

As previously reported, treatment for placentitis prevents the remarkable spread of bacteria and lesion formation in experimentally induced models [29,31]. Since most mares in the current study received treatment, only 55% (n = 21/38) of the induced mares had macroscopic lesions, and 63% (n = 24/38) of them had histological lesions of placentitis. However, none of the untreated mares had macroscopic lesions, and only half of them had histologic lesions consistent with placentitis. This is likely because of inoculation of bacteria through the cervix, without treatment, resulted in a super acute infection with massive production of prostaglandins and abortion within 48 h.

In conclusion, mares with experimentally induced ascending placentitis benefited from estrogen supplementation, but progestin supplementation did not appear to make a difference in outcomes. The immunoassays used for measurements of steroid concentrations appeared not useful to assess treatment response.

Competing interests

None.

Acknowledgments

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