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Acute endometritis induced by *Escherichia coli* in mares evaluated through color doppler ultrasonography

[*Endometrite aguda induzida por* Escherichia coli *em éguas avaliadas por ultrassonografia com Doppler colorido*]

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ABSTRACT

The objectives of this study were to characterize the endometritis induced in mares using color Doppler ultrasonography and traditional exams. Experiment 1. Mares (n=20) were submitted to intrauterine inoculation with *Escherichia coli*. Uterine evaluation was performed at M0 and M1. Experiment 2. Animals were divided into two groups: control group (n=10), and treated group (n=10) using phytotherapeutic solution. In both groups, the uterine evaluation was performed at time T1, T2, and T3. Experiment 3: Uterine evaluation was compared after antibiotic therapy, phytotherapy, and M0. For statistical analysis, the Tukey test, t Student, and Anova test were applied. Experiment 1. The mean values of vascularization at M1 were significantly higher than those obtained at M0 ($P<0.05$). Bacterial growth was observed in all samples collected. Experiment 2. The mean value of vascularization at time T1 in both groups was significantly higher ($P<0.05$) compared to M2 and M3. Experiment 3. After antibiotic therapy, the vascularization of the body and uterine horns was not equivalent to the vascularization presented at M0. We can conclude that it was not possible to correlate results obtained by color Doppler ultrasonography with the traditional findings for the diagnosis of endometritis.

Keywords: Doppler ultrasonography, endometritis, *Escherichia coli*, diagnostic methods, vascular perfusion

RESUMO

Os objetivos deste estudo foram caracterizar a endometrite induzida em éguas utilizando-se a ultrassonografia com Doppler colorido e exames tradicionais. Experimento 1: as éguas (n=20) foram submetidas à inoculação intrauterina com Escherichia coli. *A avaliação uterina foi realizada em M0 e M1. Experimento 2: os animais foram divididos em dois grupos: grupo controle (n=10) e grupo tratado (n=10), sendo usada solução fitoterápica. Nos dois grupos, a avaliação uterina ocorreu nos momentos T1, T2 e T3. Experimento 3: a avaliação uterina foi comparada após antibioticoterapia, fitoterapia e M0. Para análise estatística, foram aplicados os testes de Tukey, t de Student e ANOVA. Experimento 1: os valores médios de vascularização em M1 foram significativamente maiores que os obtidos no M0 (P<0,05). Houve crescimento bacteriano em todas as amostras coletadas. Experimento 2: o valor médio da vascularização no tempo T1 nos dois grupos foi significativamente maior (P<0,05) do que o obtido em M2 e M3. Experimento 3: após antibioticoterapia, a vascularização do corpo e dos cornos uterinos não era equivalente à vascularização apresentada em M0. Pode-se concluir que não foi possível correlacionar os resultados obtidos pela ultrassonografia com Doppler colorido com os achados tradicionais para o diagnóstico de endometrite.*

Palavras-chave: ultrassonografia Doppler, endometrite, Escherichia coli, *métodos de diagnóstico, perfusão vascular*

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INTRODUCTION

Endometritis is a major cause of mare infertility. In the last decade, much of the efforts of practitioners and researchers working in the equine breeding industry have been directed to comprehend the pathophysiological mechanisms underlying poor reproductive performance in mares. The improvement in gestation rates in mares requires early detection through gynecological exam (transrectal palpation, ultrasound, and/or endometrial cytology), and opportune intervention to solve the persistent endometrial inflammation.

Temporary post-breeding endometritis is a local inflammatory response necessary to remove the excess sperm and bacteria introduced into the uterus (Troedsson, 2006). In healthy mares, endometritis is eliminated in 36h to 48h, leaving the uterus free of inflammation (Leblanc, 2003). Endometritis can be divided into acute infection, chronic infection (endometriosis), and persistent post-breeding (Leblanc, 2010). Clinical, ultrasound, and laboratory signs of endometritis can vary widely. Some microorganisms are associated with the influx of neutrophils and fluid from the uterine lumen while others are associated with large amount of cellular debris in cytological samples. The identification of the cause may require more than an endometrial swab. In order to obtain more details at the moment of endometritis diagnosis, the color Doppler ultrasound may be applied.

Regarding the exams using color Doppler, vascularization may be graduated through the quantification of the number and size of dots or colored areas (objective evaluation), as well as a score evaluation (1 to 4, for example, indicating minimum and maximum vascularization; subjective evaluation). The main advantage of this kind of evaluation is the estimation of vascular features directly in a structure. Consequently, the evaluation may be targeted to a specific area, allowing systematic evaluation of the desired location, rather than evaluating just one focus (for example, a single blood vessel during the spectral exam). Furthermore, the movement of bowels and/or the animal itself may frequently hinder the maintenance of the cursor in spectral exam mode, making it more difficult and time-consuming. The main disadvantages of this exam are the use of selected images rather than a real-time exam (during objective analysis) and/or the subjectivity in the assignment of scores among evaluators (subjective analysis) (Ginther and Utt, 2004; Ginther, 2007).

Due to its multifactorial aspect, as well as numerous variables regarding the affected mare, treatment is usually difficult and should not be standardized (Hurtgen, 2006). Thus, the following are proposed examples of treatment to avoid endometritis: surgical correction of anatomical defects such as abnormal perineal conformation and vulvar abnormalities, cervix laceration, and recto-vestibular; post-breeding therapies or artificial insemination to create a favorable uterine environment for the development of gestation; uterine flush, administration of ecbolic drugs; and immunomodulators, nitric oxide inhibitors, among others (Christoffersen *et al*., 2012b; Canisso *et al.*, 2016; Scoggin, 2016; Khan *et al.*, 2017).

In spite of the advantages of uterine infusion with antibiotics, analysis of endometrial biopsies demonstrates that the *Streptococcus zooepidemicus* was present in the tissue of infertile mares (Petersen *et al.*, 2009, 2015). In these cases, treatments limited to intrauterine infusions may not be successful, especially if an adequate concentration of antibiotics to eliminate the microorganism does not reach deep tissues (Dascanio, 2011). In addition, the use of repeated intrauterine infusions of antibiotics may lead to vaginal flora disorder, predisposing the mare to fungal endometritis (Canisso *et al*., 2016).

Uterine flush is another therapeutic option, which is a useful procedure in managing endometritis in susceptible mares. The objective is to use uterine clearance to help eliminate inflammatory cells (Scoggin, 2016). The uterine flush can remove biofilm, a protection strategy developed by bacteria against antibiotics (Livini *et al*., 2013; Ferris, 2016).

Phytotherapy has been increasingly used and is considered by many to be an alternative therapy. However, it is not a medical specialty and fits with natural medicine (Cândido, 2001). Due to its low cost and easy access, phytotherapy is considered a more accessible method for cure and prevention. However, studies are necessary

to prove the effectiveness of phytotherapy so there is no damage to the patient's health (Rezende and Cocco, 2002). Commercially available in Brazil, phytotherapy compost (extracts of Calendula, Barbatimão, Comfrey, Burdock, Guaçatonga, Aloe Vera, and Propolis) has been empirically used in equine reproductive clinics through intrauterine infusion to treat endometritis, and studies are needed to prove its effectiveness.

Therefore, the objectives of the present study were to: 1) evaluate if the color Doppler ultrasound exam is able to identify acute endometritis in mares; 2) evaluate the uterine response 24h and 48h after the treatments of uterine infusion in mares presenting bacterial endometritis and compare the effectiveness of uterine phytotherapy and Ringer's lactate solution; and 3) describe any correlation between the findings in the uterine laboratory exams (cytology, culture, and antibiogram) and color Doppler.

MATERIAL AND METHODS

All the experimental procedures were conducted in accordance with the ethical principles adopted by the National Council of Animal Experimentation with definitive authorization from the Ethics Committee on Animal Use of the University Center of Barra Mansa under protocol no. 010/2016.

The work was carried out during working hours in the city of Barra Mansa (Latitude: 22°32′27.164″S, Longitude: 44° 10′ 42.092″ O) and the uterine samples obtained were sent to the Laboratory of Reproduction Pathology/UFRRJ. A total of 20 mixed-breed and Mangalarga Marchador mares were used, free of endometritis (confirmed through uterine cytology, uterine culture, and B mode ultrasound), ranging from 6-20 years of age. All mares weighed between 350 and 450 kg and were kept in pickets with grass and fresh water *ad libitum*, supplemented with mineral salt and concentrated feed, 1.0% LW daily.

The experiment was conducted during the reproductive season of 2016/2017. Mares were submitted to a transrectal B mode ultrasound to verify the presence or absence of endometrial edema. The animals without endometrial edema were subjected to the administration of 5.0mg Estradiol Benzoates (Gonadiol®, Zoetis, São Paulo, Brazil), intramuscularly (IM) in order to enable the collection of samples for cytology, endometrial culture, and antibiogram. The uterine edema was graduated according to (Brinsko *et al*., 2011), as follows: 0 (no edema), 1 (light), 2 (moderate), and 3 (advanced).

In order to carry out a color Doppler ultrasound, the Mindray Z5-VET (DPS, China) device was used, equipped with a 6.5 MHz linear transducer. The exam was carried out through a slow scan of the entire uterine extension, obtaining 30s videos for later analysis. The vascular uterine perfusion was analyzed subjectively considering the percentage of color Doppler signs present in the mesometrium, myometrium, and endometrium, in a longitudinal cut of the uterine body and transversal cut of uterine horns (adapted from Ferreira *et al.*, 2008). The classification used was graduated as a percentage. All analysis and image capture were conducted by the same operator. The objective evaluation was carried out through analysis of color and intensity of pixels presented by the frames selected from the films taken. For this evaluation we used the ImageJ 1.46r® (NIH *Image*) program.

Experiment 1: A total of 20 mares free of endometritis, previously evaluated through B mode ultrasound, cytology, and uterine culture, were used. Mares were submitted to intrauterine inoculation using 10ml of the *Escherichia coli* (1.5×10^9 UFC/ml) solution, derived from equine uterus. A transcervical inoculation was performed with an artificial insemination pipette.

The ultrasound exams (B mode and color Doppler), culture, and uterine cytology were used on all mares at moments M0 (immediately before the intrauterine inoculation procedure with *Escherichia coli*), M1 (24h after the intrauterine bacterial inoculation with *Escherichia coli*), M2 (48h after uterine infusion with phytotherapy and Ringer's lactate solution) and M3 (72h after uterine infusion with phytotherapy and Ringer's lactate solution).

Experiment 2: To compare the efficiency of treatments using uterine wash and phytotherapy solution after the intrauterine inoculation with *Escherichia coli*, the animals were divided into two groups: the RL group (G1, n=10) and the

FITO group FITO (G2, n=10). At moment M1 (24h after bacterial inoculation), mares in both groups were submitted to treatment with wash and uterine infusion. In the RL group, they were submitted to uterine wash using 1.0L Ringer's lactate solution, with immediate draining of this content. Subsequently, the uterus was infused with 100ml of Ringer's lactate solution and the liquid was not drained. The mares in the FITO group were submitted to uterine wash with 1.0L of phytotherapy solution 1 (950ml Ringer's lactate solution + 50ml Fitoclean® (Organnact Saúde Animal, Paraná, Brazil)). The previous content was immediately drained and then the uterus was infused with 100ml of phytotherapy solution 2 (60ml Ringer's lactate solution + 40ml Fitoclean®). The solution was not drained.

Experiment 3: In order to compare the vascular perfusion after phytotherapy and antibiotic therapy, mares in which pathogenic agents were identified in the samples collected 10 days after the end of stage 2 were submitted to antibiotic therapy, according to the sensibility shown in the antibiogram. Seven days after antibiotic therapy, a new mode B ultrasound, uterine culture and antibiogram, endometrial cytology, and color Doppler were performed.

The strain of *Escherichia coli* was obtained from the uterus of a mare which presented clinical signs of endometritis; for this reason, the collection of material was submitted for endometrial culture, which confirmed the presence of the pathogen. After isolation, the bacteria were cultivated for 24h in an enrichment medium of brain heart infusion (BHI). Glycerol was added to the BHI, stored in 2ml sterilized cryotubes and maintained at -20 ºC. Before infusion, the bacteria were defrosted and incubated at 37ºC for 24h in BHI agar plates in order to promote bacterial growth. For intrauterine inoculation, the colonies were suspended in 10ml of saline solution at a final concentration of 1.5 x 10^9 UFC/ml, using the MacFarland scale (Neflobac®, Probac do Brasil Ltda, São Paulo, Brazil). The isolation, cultivation, and inoculation were performed according to the procedure established by (Camozzato *et al.*, 2014).

Before the collection of the sample for bacterial culture, the tail of the mare was bandaged, and the vulva and perineal region were carefully washed with running water and degerming povidone and dried with a paper towel. The sample for bacterial culture was collected through a protected sterile swab, which was introduced into the uterine body with the aid of an adequate gynecological clamp for equine use, made of sterile stainless steel. The swab was only exposed to the uterine lumen to obtain the endometrial sample, and circular movements were used. Additionally, it was removed and stored in identified tubes with the *Stuart* culture medium and transported to the lab under refrigeration for incubation and later bacterial identification.

The swabs were inoculated in Blood Agar and Chromagar plates (Laborclin®, Paraná, Brazil), for bacteriological culture and later incubated at 37°C in anaerobic conditions for 24h in order to observe the macroscopic characteristics of colonies. Only plates with more than five phenotypically similar colonies in the first 24h were considered, and those without bacterial growth stayed in the 37ºC incubation for another 24h. In sequence, the isolated colonies were submitted to coloration by the Gram method for evaluation of morpho-tinctorial characteristics. Microorganisms were identified through biochemical tests referring to each specie with inoculation of a small portion of the bacterial culture, well isolated in a series of culture media containing specific substrates and chemical indicators (Koneman *et al.*, 2008).

Tests for antimicrobial susceptibility (TSA) were carried out and interpreted according to standards set by CLSI (2013). Direct suspension was used in Mueller Hinton juice for 18h to 24h. After growth, the sample was distributed on Mueller Hinton Agar with the aid of a sterile swab. After three to five minutes, the simple diffusion in disk technique was applied. Plates were deposited in a heating chamber at 35ºC for 24h and the inhibition halos were then measured and interpreted. The antibiotic discs (SENSIFAR-CEFAR®, São Paulo, Brazil) used were Enrofloxacin (5µl), Gentamicin (10µl), Tetracycline (30µl); Sulfamethoxazole + trimethoprim (25µl), Penicillin (10UI); and Ceftiofur (30µl).

The sample for uterine cytology was obtained through scarification of the endometrium with circular movements using a sterile gynecological

brush for human use connected to a gynecological clamp for equines made of sterilized stainless steel. The cellular material from the gynecological brushes was distributed in three histological plates through imprint and fixed in trillimetan 0.1% (Solution 1 from Kit Fast Panoptic LB).

The plates were evaluated in an optical microscope (40x zoom). At least 10 fields in each plate were analyzed. The findings were classified according to the number of neutrophils. Cytology was considered normal in plates with up to two neutrophils per field, with moderate inflammation in the presence of two to five neutrophils per field, and severe inflammation in the presence of more than five neutrophils per field (Riddle *et al*., 2007).

For statistical analysis of the color and intensity variables, the variance analysis according to the Tukey test was followed. The t Student test was applied to compare the percentual averages of vascularization between M0 and M1, and ANOVA to compare the averages obtained in different periods among the treated groups and control. Chi Square was used to evaluate the effect of treatments on the results of exams in uterine culture, cytology, and color Doppler ultrasound. All analysis were performed at a 95% significance level. For correction of data in cytology, bacteriological isolation, and ultrasound findings, as well as for the results of experiment 3, a descriptive analysis was carried out.

RESULTS

Experiment 1: The average of color and intensity of pixels obtained through image analysis of all mares in the experiment at moments M0 and M1 can be found in Table 1.

One of the endometrial vascularization data at moment M1 was corrupted and could not be submitted for analysis. Thus, endometrial vascularization was evaluated for 19 mares. The average for subjective endometrial vascular perfusion for all mares before (M0) and after (M1) intrauterine inoculation with *Escherichia coli* can be found in Table 2.

Table 1. Average of color and intensity of pixels obtained through image analysis of all mixed-breed and Mangalarga Marchador mares in the experiment at moments M0 (moment immediately before intrauterine inoculation) and M1 (24h after intrauterine inoculation)

	Moments	Uterus		Left horn		Right horn	
		Color	Intensity	Color	Intensity	Color	Intensity
Average ± Standard Deviation	M0 (n=20)	3728.11± 2344.85[a*]	379012± 238969[a]	4549.37± 3192.55[a]	468544± 316354[a]	3885.56± 2155.81[a]	394074± 210043[a]
	M1 (n=19)	10125.5± 3814.47[b]	1045637± 392058[b]	7955± 4694,37[b]	837195± 466398[b]	10334.1± 6814.22[b]	897441± 401245[b]

*Different letters in the same column statistically differ. There was statistical difference (P<0.05).

Table 2. Subjective vascular endometrial perfusion average obtained in mixed-breed and Mangalarga Marchador mares at moments M0 (moment immediately before intrauterine inoculation with *E. coli*) and M1 (24h after intrauterine inoculation with *E. coli*)

Average (Standard deviation) *(%)*	Moments	Uterus (%)	Left horn (%)	Right horn (%)
	M0 (n=20)	13.74[a*] (12.00)	13.21[a](10,03)	10.28[a](8.62)
	M1 (n=19)	30.71[b](10.15)	23.84[b](11,44)	27.42[b](10.36)

*Averages of treatments with different letters in columns statistically differ. There was statistical difference (P<0.05).

It was verified that the average vascularization values at moment M1 were significantly superior (P<0.05) to those obtained at M0 for the three parts of the uterus in the analysis carried out using the ImageJ 1.46r® program as well as the subjective analysis, demonstrating an inflammation. Thus, in this experiment, it was possible to identify the inflammation of the equine endometrium through color Doppler ultrasound. Alongside the endometrial vascularization of 19 mares, it was possible to observe bacterial growth in all collected samples (Table 3).

All 20 mares submitted to intrauterine inoculation with 10ml of the solution containing 1.5 x 10^9 UFC/ml presented bacterial growth in the microbiological culture exam. Table 4 specifically shows the endometrial vascular perfusion in four animals where one of the exams performed (endometrial cytology or B mode ultrasound) did not identify infection. Despite the lack of diagnosis of endometritis in the exams, we observed through color Doppler the increase in vascularization, suggesting an infection situation, serving as an additional resource to endometritis diagnosis.

In the present study, 95% (19/20) presented positive cytology and bacterial growth (only one presented negative cytology). The bacteria isolated in the cultures performed during the diagnosis of endometritis 24h after inoculation are described in Table 5.

Table 3. Results of culture, endometrial cytology, and B mode ultrasound performed on mixed-breed and Mangalarga Marchador mares 24h after intrauterine inoculation with *Escherichia coli*

Nº of animals	Culture (bacterial growth)	Cytology (≥ 2 PMNs per field 400x)	US (presence of intrauterine fluid)
15	+	+	+
1	+	-	+
4	+	+	-

Nº - number. PMNs – polymorphonuclears. US – B mode ultrasound

Table 4. Subjective vascular endometrial perfusion of four mixed-breed and Mangalarga Marchador mares where it was not possible to identify endometritis in the exams performed (uterine cytology or B mode ultrasound) at moments M0 (moment immediately before intrauterine inoculation with *E. coli*) and M1 (24h after intrauterine inoculation with *E. coli*)

Mare		Vascularization (%)		
		Uterus	Left horn	Right horn
04	M0	1	5	3
	M1	30	80	65
06	M0	1	5	2
	M1	48	80	70
08	M0	5	5	5
	M1	26	70	90
15	M0	45	13	10
	M1	50	70	75
Average (Standard Deviation) (%)	M0	13.00 (0.21)	7.00 (0.04)	5.00 (0.04)
	M1	39.00 (0.11)	17 (0.06)	33 (0.12)

Table 5. Bacterial species isolated in the endometrial samples of mixed-breed and Mangalarga Marchador mares in M1 (24h after intrauterine inoculation with *E. coli*)

Bacteria	Nº of identified animals
Escherichia coli	n = 10
Klebsiella sp	n = 4
Streptococcus β hemoliticus	n = 3
Proteus mirabilis	n = 1
Serratia sp	n = 1
Providencia sp	n = 1
Staphylococcus sp	n = 1
Enterococcus sp	n = 1
Klebsiella sp	n = 1

In all mares there was bacterial growth in M1; however, in 50% (10/20) was *E. coli*. Three mares (15%, 3/20) presented mixed bacterial growth, meaning that the same uterine sample developed two bacteria. In the present study the presence of intrauterine fluid was identified in 80% (16/20) through B mode ultrasound in M1. This elevated percentage may be linked to the age of the animals used in the study: 75% (15/20) were over 10 years of age. Of these, 13 (13/15, 86.6%) accumulated intrauterine liquid.

Experiment 2. The evolution of endometrial vascular perfusion obtained at moments M1, M2, and M3 in the RL and FITO groups are displayed in Tables 6 (ImageJ 1.46r®) and 7 (subjective analysis).

Table 6. Pixel area and color intensity obtained through ImageJ 1.46r® software of the uterus, and the left and right uterine horns of mixed-breed and Mangalarga Marchador mares, at moments M1 (24h after intrauterine inoculation with *E. coli* and before starting the uterine infusion with phytotherapy and Ringer's lactate solution), M2 (48h after uterine infusion with phytotherapy and Ringer's lactate solution) and M3 (72h after uterine infusion with phytotherapy and Ringer's lactate solution) in the RL and FITO groups

Uterus				
	Pixel area		Color intensity	
Moments	RL Group	FITO Group	RL Group	FITO Group
M1	10899.9^{Aa*} (4955.1)	8797.2^{Aa}(3759.8)	1096886^{Aa}(493498)	931753^{Aa}(418759)
M2	6580^{Ab}(3205.2)	5513.1^{Aab}(2691.8)	678977^{Ab}(327300)	599066^{Aab}(286917)
M3	6510.6^{Ab}(3007.5)	2874.6^{Bb}(2209)	673603^{Ab}(311480)	311662^{Bb}(241153)
Left uterine horns				
	Pixel area		Color intensity	
Moments	RL Group	FITO Group	RL Group	FITO Group
M1	10899.9^{Aa*}(4955.2)	8797.2^{Aa}(3759.9)	1096886.1^{Aa}(493498)	931752.8^{Aa}(418759)
M2	6580.0^{Ab}(3205.2)	5513.1^{Aab}(2691.8)	678977.0^{Ab}(327300)	599065.8^{Aab}(286917)
M3	6510.6^{Ab}(3007.6)	2874.7^{Bb}(2209.0)	673601.7^{Ab}(311480)	311662.1^{Bb} (241153)
Right uterine horns				
	Pixel area		Color intensity	
Moment	RL Group	FITO Group	RL Group	FITO Group
M1	9349.6^{Aa}(3405.5)	11090.0^{Aa}(9428.8)	926283^{Aa}(362821)	851037.7^{Aa}(479787)
M2	6275.4^{Aa}(2417.6)	4828.0^{Ab}(2543)	644693.3^{Aa} (245861)	488270.4^{Aa} (268299)
M3	5258.3^{Aa}(2670.6)	3701.2^{Ab}(1973.6)	531582.2^{Aab}(271164)	392605^{Aab}(214568)

*Upper-case letters in lines and lower-case letters in columns with different letters differ significantly by the Tukey test ($P<0.05$).

The average values for subjective vascular perfusion, pixel area, and color intensity in the different segments (uterus and uterine horns), did not differ among the groups ($P>0.05$). We verified that the average value of the percentage of subjective vascular perfusion at M1 was superior ($P<0.05$) to that obtained at M2 and M3 in the regions of the uterus and uterine horns (except the left uterine horn – RL group). In the objective analysis, M1 was superior ($P<0.05$) in the regions of the uterus and uterine horns (except the right uterine horn – RL group). The largest vascular perfusion in M1 was observed in most of the analyses carried out and is probably due to the vasodilation characteristic of the acute stage of endometritis occurring up to 24h after intrauterine inoculation. The region of the right uterine horn did not present differences ($P>0.05$) among the three moments in G1, diverging from the subjective analysis.

In both groups, a significant reduction in vascular perfusion (subjective and objective evaluation) was observed at M2 and M2 compared to M1. However, bacterial growth and inflammatory cells were identified in exams carried out in both groups. In the RL group, 50% (5/10) of mares still presented bacterial growth, and 100% (10/10) presented positive cytology at M2; at M3, only one mare presented negative cytology.

In the FITO group, 80% (8/10) of uterine samples presented bacterial growth and positive cytology at M2; at M3, 80% (8/10) presented bacterial growth and 100% (10/10) presented positive cytology. Thus, the vascular perfusion identified did not present a correlation with the uterine laboratory exams. The results of the culture, endometrial cytology, and B mode ultrasonography performed in both groups are given in Table 8.

There was no statistical difference ($P=0.2$) between the RL and FITO groups regarding the culture, uterine cytology, and B mode ultrasound results. Thus, it was not possible to identify the most effective treatment of those used in this experiment because none was able to eliminate the infection-causing agent.

Table 7. Average subjective endometrial vascular perfusion of the uterus of mixed-breed and Mangalarga Marchador mares, and left and right uterine horns, at moments M1 (24h after intrauterine inoculation with *E. coli* and before starting the uterine infusion with phytotherapy and Ringer's lactate solution), M2 (48h after uterine infusion with phytotherapy and Ringer's lactate solution) and M3 (72h after uterine infusion with phytotherapy and Ringer's lactate solution) in the RL and FITO groups

Uterus (%)		
Moments	RL Group	FITO Group
M1	34.00^{Aa} (11.26)	27.00^{Aa}(7.80)
M2	18.10^{Ab}(8.81)	11.44^{Ab}(4.88)
M3	15.11^{Ab}(6.35)	9.00^{Ab}(5.27)
Left uterine horns (%)		
Moments	RL Group	FITO Group
M1	23.20^{Aa}(7.83)	24.56^{Aa}(14.98)
M2	17.20^{Aa}(8.30)	11.88^{Ab}(5.25)
M3	15.50^{Aa}(5.50)	11.50^{Ab}(5.68)
Right uterine horns (%)		
Moments	RL Group	FITO Group
M1	28.90^{Aa}(10,69)	25.78^{Aa}(10,29)
M2	15.50^{Ab}(5,66)	14.33^{Ab}(5.07)
M3	15.89^{Ab}(6,86)	10.00^{Ab}(5.20)

** Treatment averages with different upper-case letters in the lines and lower-case letters in the columns differ significantly by the Tukey Test (P>0.05).

Table 8. Results of culture, endometrial cytology, and B mode ultrasound of mixed-breed and Mangalarga Marchador mares carried out at M1 (24h after intrauterine inoculation with *E. coli* and before starting the uterine infusion with phytotherapy and Ringer's lactate solution), M2 (48h after uterine infusion with phytotherapy and Ringer's lactate solution) and M3 (72h after uterine infusion with phytotherapy and Ringer's lactate solution) in the RL and FITO groups

RL Group (n=10)				
Moments	Nº of animals	Culture (bacterial growth)	Cytology (≥ 2 PMNs per field 400x)	US (presence of intrauterine liquid)
M1	7	+	+	+
	1	+	-	-
	2	+	+	-
M2	5	+	+	+
	5	-	+	-
M3	5	+	+	+
	3	-	-	-
	2	-	+	-
Fito group (n=10)				
M1	9	+	+	+
	1	+	+	-
M2	8	+	+	+
	2	-	-	-
M3	7	+	+	+
	2	-	+	-
	1	+	+	-

Experiment 3: According to the results obtained in the culture and antibiogram exams carried out seven days after the end of the experiment 2, 13 mares (65%, 13/20) were subjected to antibiotic therapy through intrauterine infusion using 100ml of Gentamicin (Gentrin® Infusão Uterina, Ourofino Saúde Animal, São Paulo, Brazil) for three days, regardless of the group they were in.

One week after this treatment, they were subjected to another Doppler ultrasound exam. The comparison of the percentage of uterine vascular perfusion between moments M0, subjection to the treatment with Fitoclean® (FITO), and subjection to the treatment with Gentamicin (GENTA) is found in Table 9.

After the treatment with intrauterine infusion of antibiotics, the observed vascularization presented a reduction in body and uterine horns when compared to the FITO moment. However, it was not equal to the vascularization presented at M0. However, there was no bacterial growth in the samples obtained after treatment with intrauterine infusion with antibiotics.

Table 9. Comparison of the average endometrial vascular perfusion of mixed-breed and Mangalarga Marchador mares before inoculation with *E. coli* (M0), subjection to treatment with Fitoclean® (FITO - 48h after intrauterine inoculation with *E. coli*), and after treatment with Gentamicin (GENTA)

Moments	Uterus (%)*	Left horn (%)*	Right horn (%)*
M0 (n=20)	13.74 (12.00)	13.21 (10.03)	10.28 (8.62)
FITO (n=13)	15.11 (6.35)	15.50 (5.50)	15.89 (6.86)
GENTA (n = 13)	14.62 (8.42)	15.00 (6.20)	12.40 (10.21)

*Average (Standard Deviation) (%)

DISCUSSION

A reported case indicated an increase in uterine vascularization due to endometritis in seven mares (Pereira *et al.,* 2014). However, the color Doppler ultrasound exam was carried out only after the infection was already established; moreover, the vascularization values were not described, which makes comparison impossible. This was the only research found in the literature which applies the use of color Doppler in mares with endometritis. Studies related to the evaluation of uterine inflammatory reaction using color Doppler are also scarce. In beef cattle, the inflammatory uterine reaction caused by artificial insemination was evaluated but no alteration was observed in the vascularization between the control group and the inseminated group (Oliveira *et al.,* 2014a). The same research group did not observe a difference in vascularization when the artificial inseminations were carried out using a lower quality semen (with a greater percentage of cellular damage) evaluated by fluorescent probes (Oliveira *et al.*, 2014b).

A bacterial growth inferior to this study (66.6%, 8/12) was observed 24h after the intrauterine inoculation of 10^6 UFC/10ml of *E. coli* (Christoffersen *et al.,* 2012a). The lower concentration of *E. coli* used for inoculation probably influenced this result. In this study, in 75% (15/20) of mares it was possible to make a confirmation between bacterial growth, cytology (≥ two PMN cells per field in a 400X zoom field), and the presence of intrauterine liquid detected through B mode ultrasound. These cytology results are inferior to that obtained by (Christoffersen *et al.,* 2012b), which reported neutrophilia in all mares 24h after the intrauterine inoculation. In one of the mares, the confirmation of endometritis was based only on the culture and US results while in four mares the confirmation was based on results from culture and cytology (Table 3).

Almost all the animals presented positive uterine culture and endometrial cytology. Only one mare (5%) presented a negative result in the uterine cytology exam. According to (Ferris, 2016), this may happen when bacteria are present in the deep region and not on the surface of the endometrium. In another four mares (20%), although the culture and cytology exams identified infection, the presence of intrauterine liquid was not identified during B mode ultrasound, characterizing a subclinical endometriosis. Manifestation of endometritis varies greatly from one case to another, and detection can be difficult due to subtle or absent clinical signs during a phase in the estrus cycle. In other words, some mares may not manifest infection, which is referred to as subclinical endometritis (Leblanc and Causey, 2009). However, through the pronounced increase of vascular endometrial perfusion in M1 when compared to moment M0 (free of endometritis) (Table 2) it was possible to identify uterine inflammation, even though there was no production of intrauterine liquid.

Despite this, in 80% (16/20) of mares, intrauterine liquid was identified during B mode ultrasound 24h after inoculation (Reghini *et al.*, 2016) and we also identified a high response (92%, 12/13) of intrauterine retention 24h after artificial insemination in mares which presented chronic endometrial degeneration. This percentage is higher when compared to our study; however, (Reghini *et al.*, 2016) used animals which were known to have a severe degenerative process, which may have favored the identification of a higher percentage. In this work, the result diverged between cytology and microbiology in only one of the mares, which presented negative cytology. This result is superior compared to Oliveira *et al.* (2010), who obtained a positive cytology in only 25% (4/16) of the mares which had a positive microbiological exam.

There are situations in which the endometrial cytology may be normal (with no inflammatory cells) and the infection still be present (Dascanio and Ferris, 2014). One of the mares in this experiment presented normal cytology (see Table 2) but showed a bacterial growth in the culture exam. When evaluating the vascular perfusion of the same mare at moment M1, the color Doppler ultrasound revealed lower vascularization in both uterine horns when compared to the uterine body. Dascanio and Ferris (2014) believe that the negative result in the cytology exam may be related to a localized infection, which may also have been responsible for the discrepancy between vascularization in different regions. This means that the infection was located in the uterine body, promoting large vascularization and bacterial growth in this region.

In M1, 50% (10/20) of growth was *E. coli*. This result corroborates the findings of (Camozzato *et al.*, 2014), who reported 59.2% of growth for the same time. They were also close to the results of Leblanc and Causey (2009), who obtained 42.8% (3/7), and Christoffersen *et al.* (2012b), who obtained a growth of 46.6% (7/15 cycles), both in mares susceptible to endometritis. The authors affirm that the low number of mares which presented bacterial growth post inoculation may have been due to the concentration of *E. coli* used (10^6 UFC), inferior to that used in this experiment (10ml – 1>5 x 10^9 UFC/ml). Similar to the present experiment, besides the growth of *E. coli*, Christoffersen *et al.* (2012a) also describe the development of other bacteria: *Streptococcus zooepidemicus* in four of seven mares, and *Panthoea agglomerans* in other mares. The authors affirmed that the bacteria may establish infections in a latent/inactive manner, becoming active through the infusion of a substance which induces inflammation (*E. coli*).

In this study, other bacteria also developed in lower frequency during cultivation, probably due to an ascending contamination during previous gynecological manipulation, since there was growth of a large part of these bacteria in the first culture exam (M0). There is the possibility of environmental contamination during the collection of uterine material, because these bacteria are usually found in the feces and the environment and can be introduced into the uterus during the collection of material (Oliveira *et al.*, 2010). Additionally, some gynecological procedures (artificial insemination, embryo transfer, biopsy or uterine swab, uterine infusion) may carry bacteria to the uterine environment.

The result of the mixed bacterial growth observed in this study (3/20, 15%) is close to a study reported using mares with fertility issues, where 17.1% (49/287) of animals with positive bacterial culture presented more than one bacterium in uterine samples (*E. coli* and *Streptococcus dysgalactiae subsp. equisimilis* were the most common growths) (Frontoso *et al.*, 2008). In our study, the mixed growth was probably due to contamination that occurred before the experiment began, which may have led to a chronic case of endometritis in these mares. The presence of two bacteria in the uterine environment was not reflected in any alteration in uterine vascularization in these mares, which presented perfusion similar to the others for the same period.

In the present study, in 80% (16/20) of mares the presence of intrauterine fluid was identified through B mode ultrasound. Predisposing factors to the accumulation of intrauterine fluid are reduced myometrial contraction, poor lymphatic drainage, excessively distended uterus, and cervical incompetency (Barbacini *et al.*, 2003), which happens more frequently in older mares and is associated with reduced fertility (Canisso *et al.*, 2016). Seventy-five percent (15/20) of the mares in this study were above 10 years of age,

which 13 (13/15, 86.6%) accumulated intrauterine liquid. Advanced age has long been associated with persistent endometritis (Ricketts and Alonso, 1991; Carnevale and Ginther, 1992; Troedsson, 1999; Barbacini *et al.*, 2003). There is great disparity in the capacity to solve uterine inflammation among young mares (fertile mares) and old mares (sub fertile mares) after intrauterine inoculation with *Streptococcus zooepidemicus* (Hughes and Loy, 1969). Older or multiparous animals, or those with a low body score, present reproductive tract conditions which favor susceptibility to persistent endometritis (Woodward and Troedsson, 2014).

The treatments applied in the RL and FITO groups were not efficient to eliminate the uterine pathogen in the studied mares, according to the findings shown in Table 8. The treatment using phytotherapy solutions has been used empirically to try to resolve the cases of endometritis and consequently increment the reproductive indexes of animals. However, in this study it was demonstrated that its effectiveness was similar to that obtained with uterine flush using Ringer's lactate solution, a therapy whose benefits are already described in the literature. The uterine flush is a useful procedure in managing endometritis in susceptible mares, which objective is to aid with uterine clearance to eliminate inflammatory cells (Scoggin, 2016). Uterine flush can eliminate biofilm (Livini *et al.*, 2013). *Escherichia coli* isolated from the uterus have been associated with the formation of biofilm, a protection strategy developed by the bacteria against antibiotics (Ferris, 2016).

Despite the difference in vascular perfusion observed in groups 1 and 2 at different moments, the uterine cleaning was not observed in the results of the culture, cytology, and B mode ultrasound exams. Nor was any reduction observed in the bacterial concentration of *E. coli* utilizing an ozonized saline solution (Loncar *et al.*, 2017). However, in the same study, dimethylsulfoxide significantly reduced the number of colonies forming units for *E. coli* compared to experimental group 1 in 80% (8/10) of isolates.

CONCLUSIONS

From the results obtained in this study, we conclude that it is possible to identify acute endometritis through color Doppler ultrasound. Moreover, the results demonstrated that treatments using Ringer's lactate solution and phytotherapy were not efficient in eliminating the uterine pathogen in the studied mares, and that both presented a similar efficacy on endometritis. Finally, the vascular perfusion identified did not present a correlation with the uterine laboratory exams performed.

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