Journal of Equine Veterinary Science 112 (2022) 103900

Contents lists available at ScienceDirect

Journal of Equine Veterinary Science

journal homepage: www.j-evs.com

Original Research

Effectiveness of Ozone Therapy in The Treatment of Endometritis in Mares

Ana Caroline Araújo Ávila[a], Natália Castro Diniz[a], Rafael Torres Serpa[a], Maria Manoela Barata de Castro Chaves[a], Marco Antônio de Oliveira Viu[b], Rodrigo Arruda de Oliveira, DVM, Ms, PhD[a,*]

[a] Faculty of Veterinary Medicine, Laboratory of Animal Reproduction, University of Brasília (FAV/UnB), Brasília, DF
[b] Faculty of Veterinary Medicine, UFG, Jataí, GO

ARTICLE INFO

Article history:
Received 30 December 2021
Received in revised form 13 February 2022
Accepted 14 February 2022
Available online 18 February 2022

Keywords:
Bacteria
Cytology
Horse
Ozone
Uterine inflammation

ABSTRACT

Endometritis is defined as inflammation of the endometrium that may be acute or chronic, infectious, or non-infectious. Endometritis is an important cause of subfertility in mares. Considering the antimicrobial characteristics, immune-stimulating ability, and low cost of ozone (O_3) therapy, we aimed to evaluate the effectiveness of intrauterine O_3 therapy as an alternative treatment for endometritis in mares. Twenty-five mares with a known reproductive history of uterine infection and inflammation were allocated into three groups: Group 1 (control; n = 7), uterine lavage with Ringer's lactate solution; Group 2 (O_3-gas; n = 9), uterine lavage with Ringer's lactate solution followed by uterine insufflation with O_2-O_3 gas mixture containing 40 µg O_3 mL^{-1} for 10 minute; and Group 3 (O_3-oil; n = 9), uterine infusion of ozonized sunflower oil. Uterine inflammation was evaluated through a uterine cytological examination (cytobrush) and uterine culture (swab) for microbiological content before and after all treatments. In assessments of uterine cytology, the average number of neutrophils/field changed from 9.14 ± 3.02 to 7.71 ± 3.59 in the control group, from 10.67 ± 3.84 to 2.89 ± 3.59 in the O_3-gas group, and from 6.44 ± 2.79 to 6.55 ± 7.18 in the O_3-oil group post-treatment. The pre- and post-treatment findings in the mares treated with ozonized gas were significantly different ($P < .05$), unlike the findings for the mares in the control and O_3-oil groups. All mares (25/25) showed a positive uterine culture before treatment. After treatment, the percentage of mares showing positive culture results was 57%, 11%, and 22% in the control, O_3-gas, and O_3-oil groups, respectively. Our results showed the effectiveness of two groups (O_2-O_3 gas mixture and ozonized sunflower oil) for the treatment of uterine infections in mares. Thus, direct intrauterine O_3 gas infusion has been shown to be effective in treating endometritis in mares, reducing both inflammation and uterine infection.

1. Introduction

Brazil has the fourth-largest herd of horses in the world, which is responsible for more than 600,000 animals and 3.2 million direct and indirect jobs and moving BRL 16.15 billion [1]. In addition, Brazil is the country with the maximum utilization of the equine embryo transfer technique [2]. Many of these animals are donor and recipient mares that can develop uterine pathologies, such as endometritis.

Among reproductive system pathologies, which have been identified as the third most common set of clinical conditions after colic and respiratory system diseases [3], endometritis is the biggest cause of infertility in mares [4]. Endometritis is an inflammatory and/or infectious process, and persistent endometritis can make the uterine environment unfavorable to the conceptus, thereby reducing the mare's reproductive potential [5].

Endometritis has been treated with uterine lavage, ecbolic and anti-inflammatory drugs, and antimicrobials when necessary [6]. However, the lack of response to traditional therapy and the increasing incidence of antimicrobial-resistant pathogens have led

Conflict of Interest statement: None of the authors of this manuscript has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of this article.

Animal Welfare/Ethical statement: All procedures were approved by the Ethics Committee in Animal Experimentation (CEUA) of the Institute of Biological Sciences at the University of Brasília, under protocol no. 23106.068932/2018-12

* Corresponding author at: Rodrigo Arruda de Oliveira, Faculty of Veterinary Medicine, Laboratory of Animal Reproduction, University of Brasília (FAV/UnB), Brasília, DF.

E-mail address: rodrigoarruda@unb.br (R.A. de Oliveira).

https://doi.org/10.1016/j.jevs.2022.103900
0737-0806/

to the development of alternative therapies to treat mares suffering from chronic endometritis [7], including acupuncture [8,9], platelet-rich plasma [10], and the promising ozone (O_3) therapy [11,12].

Intrauterine treatment with O_3 therapy has been shown to be effective in reducing uterine inflammation in cows [13] as well as in local promotion of endometrial angiogenesis without inducing tissue damage in mares [11]. Moreover, O_3 therapy has an antimicrobial effect [14,15]. O_3 gas activates erythrocyte metabolism and immune cells [16] and shows the ability to react with many compounds, such as phospholipids, lipoproteins, bacterial cell envelopes, and viral capsids [17]. The anti-inflammatory and bactericidal effects of ozonated oils have been demonstrated both *in vitro* and *in vivo* [15,18,19,20,21]. Therefore, this study aimed to evaluate the effectiveness of treatment using two O_3 presentations (O_2-O_3 gas mixture and ozonized sunflower oil) in comparison with conventional control treatment for endometritis.

2. Material and Methods

2.1. Animals

All procedures were approved by the Ethics Committee in Animal Experimentation of the Institute of Biological Sciences at the University of Brasília, under protocol no. 23106.068932/2018-12. Twenty-five light-horse breed mares aged 5–15 years (8.4 ± 4.4) and weighing 400–500 kg were used for this study. The mares were kept under natural light in an outdoor paddock in a private Stud Farm located in Brasília, Federal District, Brazil, under pasture, mineral supplementation, and water *ad libitum*.

The mares were selected based on their reproductive history. Females that presented with subfertility conditions, such as difficulty in pregnancy, low percentage of embryonic recovery, and presence of uterine fluid (purulent or nonpurulent), were sent for uterine cytological examinations and endometrial culture. Only mares that showed positive results in microbiological cultures were included in the experiment.

2.2. Experimental Design

All mares were evaluated over 3 consecutive cycles. Uterine culture and cytological examinations were performed in the first and third cycles in all 25 mares to evaluate the findings of pre- and post-treatment. In the second cycle, the mares were randomly allocated into 3 experimental groups: control (G1), O_3-gas (G2) and O_3-oil (G3). In G1 (n = 7), mares were treated with Ringer lactate uterine lavage (until recovery of clear flushing) and 20 IU of oxytocin (intramuscular [IM]). In G2 (n = 9), mares were treated with Ringer lactate uterine lavage (until recovery of clear flushing), and the uterus was insufflated with O_2-O_3 gas mixture at a concentration of 40 μg O_3 mL^{-1} for 10 minute. The gas infusion was performed through an insemination pipette for mares (PROVAR, São Paulo, Brazil) and the tubing of a drip set accoupled to an O_3 generator (Ozone & Life, São José dos Campos, Brazil). This procedure was repeated for 2 consecutive days and was followed by IM administration of 20 IU of oxytocin on the second day. In G3 (n = 9), mares were treated with Ringer lactate uterine lavage (until recovery of clear flushing), followed by intrauterine infusion (uterine body and bifurcation) of 50 mL of commercial ozonized sunflower oil (peroxide index 600 mEq kg^{-1}, Ozone & Life, São José dos Campos, Brazil). The oil infusion was performed through an insemination pipette for mares, and the uterus was massaged by rectal palpation. After 24 hour of infusion, uterine lavage was performed with a Ringer lactate solution until completely clean flushing was obtained, which was followed by IM administration of 20 IU of oxytocin. In all groups, treatment was started when grade 2 edema in the uterus was verified according to a 0–5 scale [22], with an ovarian follicle diameter of 35–40 mm, without corpus luteum and relaxed cervix.

2.3. Uterine Cytology and Endometrial Culture

Uterine culture was carried out during estrus using a sterile disposable collector for mares (PROVAR, São Paulo, Brazil), with a double-protection swab system to avoid contamination. The tip of the collector was protected between the thumb and palm of the gloved hand and was introduced throughout the vagina until it reached the cervix. At the cervix, the sanitary protection was torn, and the swab was exposed, coming into contact with the uterine mucosa. Circulatory movements were performed with the swab, which remained in contact with the uterine mucosa for 40 s. Then, the swab was placed back inside the collector's protection so that it could be removed from the reproductive tract without contact with the cervical, vaginal, and vulvar environments [23]. Subsequently, the swab was uncoupled from the collector and attached to a tube containing Stuart medium suitable for transport. The samples were kept at 5°C until microbiologic analyses. Swabs were plated on blood agar and incubated for 48 hours at 36.7°C. The plates were observed at 24 and 48 hours for bacterial growth. Bacterial growth was classified as: no growth; mixed flora (2 or more organisms); or single isolate (1 bacterial isolate only). Where bacterial growth was detected, Gram-positive or Gram-negative organisms were identified using the Gram staining technique [24].

Uterine cytological examination was performed using a double-protection system with a cytobrush [25,26]. After assembly, the smears were prepared by rolling the cytobrush on a slide. The material was then stained with a simplified method of staining cytology (panoptic). Cell counting was performed according to the method described by Cocchia et al [23]..

Both culture and uterine cytology were performed in estrus when the mares had grade ≥2 uterine edema and ovarian follicle diameter of 35–40 mm. The mean time between first and third cycles of cytology and cultures was 52 days.

2.4. Statistical Analysis

The data were subjected to descriptive statistical analysis, followed by the Kolmogorov–Smirnov normal distribution test using GraphPad Prism 9. The t-test was used for 2-parameter comparisons, followed by the non-parametric Mann–Whitney U and Wilcoxon tests. When comparing more than 2 parametric groups, the analysis of variance (ANOVA) summary and the Kruskal–Wallis test were used for non-parametric variables that did not show a normal probability distribution.

3. Results

The uterine cytological findings showed a significant difference after treatment ($P < .001$) only for mares treated with O_2-O_3 gas mixture. Thus, O_3-gas (G2) showed better control of the inflammatory response than the control (G1) and O_3-oil groups (G3) (Table 1).

Independently of the experimental group, all mares showed positive cultures before the treatment. The most common pretreatment agents were *Escherichia coli* (48%, 12/25), followed by Aspergillus spp. (24%, 7/25), Streptococcus spp. (12%, 3/25), *Staphylococcus aureus* (12%, 3/25), and Candida spp. (4%, 1/25). One of the mares showed, concomitantly, infection of *Escherichia coli* and *Staphylococcus aureus*. After the treatments, the most common agent was still *Escherichia coli* (72%, 5/25), followed by *Enterobacter* (14%, 1/25) and Candida spp. (14%,1/25) (Fig. 1).

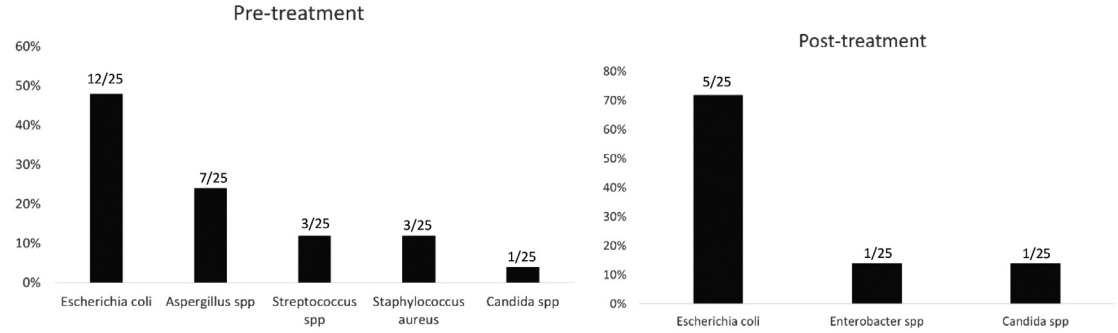

Fig. 1. Uterine microbial isolates (%) from equine uteri (n = 25) before and after intrauterine treatment with Ringer'e lactate solution, O_2-O_3 gas mixture or ozonized sunflower oil.

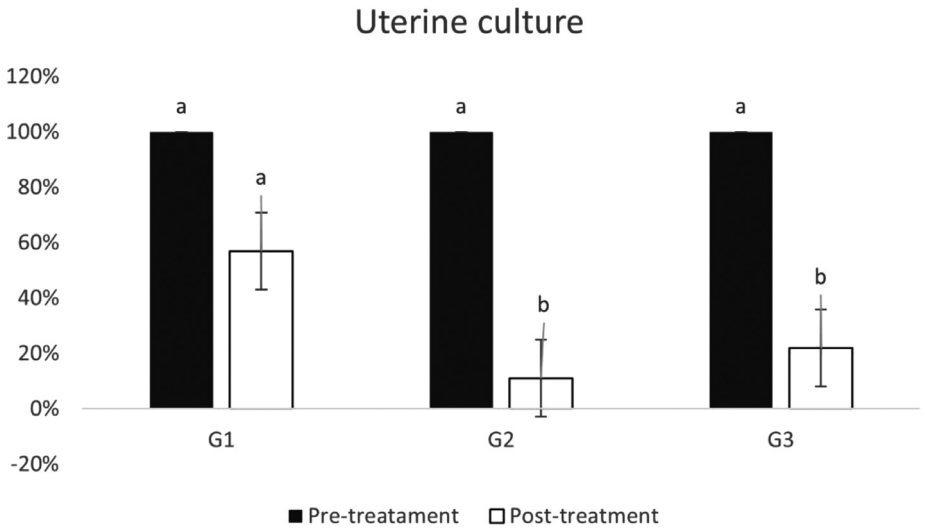

Fig. 2. Incidence (%) of positive cultures from equine uteri before and after intrauterine treatment with Ringer's lactate solution (G1; n = 7), O_2-O_3 gas mixture (G2; n = 9) and Ozonized sunflower oil (G3; n = 9). [a,b]Different letters in the same group indicate a statistical difference ($P < .05$).

Table 1
Uterine cytology (Mean ± SD for polymorphonuclear neutrophil) before and after intrauterine treatment with Ringer's lactate solution (G1), O_2-O_3 gas mixture (G2) or ozonized sunflower oil (G3). Both culture and uterine cytology were performed in estrus when the mares had grade ≥2 uterine edema and ovarian follicle diameter of 35–40 mm.

Groups	Pre-Treatment	Post-Treatment
G1	9.14 ± 3.02^{a1}	7.71 ± 3.59^{a1}
G2	10.67 ± 3.84^{a1}	2.89 ± 3.59^{b2}
G3	6.44 ± 2.79^{a1}	6.55 ± 7.18^{a1}

[a,b]Different letters in the same row indicate a statistical difference ($P < .05$).
[1,2]Different numbers in the same column indicate a statistical difference ($P < .05$).
G1, control (n = 7); G2, direct infusion of intrauterine O_2-O_3 gas mixture (n = 9); and G3, intrauterine infusion of ozonized sunflower oil (n = 9). Pre-treatment (cycle 1), treatment (cycle 2), Pos-treatment (cycle 3).

The rate of identification of microbiological agents in the uterine culture before treatment did not differ among the groups ($P > .05$). However, after treatment, the percentage of mares showing positive cultures was significantly lower ($P < 0.5$) in the O_3-gas and O_3-oil groups than that of the control group (11%, 1/9; 22%, 2/9; and 57%, 4/7, respectively) (Fig. 2).

4. Discussion

The present study describes, for the first time, 2 presentations of O_3 (O_2-O_3 gas mixture and ozonized sunflower oil) as an alternative treatment for endometritis in mares. Under the current study conditions, O_3-gas was found to be safe and more effective for controlling uterine infection and inflammation in endometritis. Because of its wide-ranging mechanism of action, O_3 therapy is considered an integrative treatment showing various effects in animals and humans [27], including antioxidant [17], antimicrobial [14,15,28], and anti-inflammatory activities [13,29].

Our study showed a decreased inflammatory response in equine uterus exposed to O_2-O_3 gas mixture (G2), with the reduction in the number of polymorphonuclear neutrophil (PMNs) per field indicating the efficacy of the treatment for inflammation control. A similar result was found in a study involving puerperal dairy cows [13], reporting a reduction in uterine inflammation that could be verified by uterine cytological assessment 72 hours after treatment with 50 mL of ozonized distilled sterile infusion (50 μg/mL) in the uterus in the early postpartum period. Better reproductive efficiency has also been observed in dairy cows after postpartum uterine ozonized foam infusion [30]. To our knowledge, there are no studies using these presentations of O_3 (O_2-O_3 gas mixture and ozonized sunflower oil) as an alternative treatment for endometritis in mares, which have a low cost and practical approach for the treatment of subfertility in domestic animals.

The direct infusion of O_3-gas (G2) better controlled the inflammatory process, probably by removing the cause of the inflam-

mation, through the anti-inflammatory effect of O_3 by the inhibition of proinflammatory cytokines and phospholipase A2 and by the stimulating effects of immunosuppressive cytokines, such as interleukin-10 and tumor necrosis factor-β1 (anti-inflammatory and tissue repair) [31,32]. In contrast, ozonated sunflower oil (G3) acted as a more aggressive agent because of the low pH and difficult removal from the uterine environment. Furthermore, its presence and maintenance in the uterus for a long period might prolong inflammation response.

No difference was observed in the O_3-oil treatment (G3) concerning the inflammatory response. After the infusion of ozonized oil, the mares showed some discomfort, raising their tail similar to the urination posture for a few minutes; however, they soon returned to normal. Uterine flushings obtained 24 hours after ozonized oil treatment included lumps and mucus in mares with cervical tortuosity. Due to its highly reactive nature, O_3 is not carried by the oil; the O_3 reacts with the components of the oil, generating subcomponents, such as peroxides, and changing the pH [19,20]. These substances and their physicochemical changes will react with the uterus. The control group also showed no difference.

The difference between pre- and post-treatment rates of positive cultures in mares treated with O_2-O_3 gas mixture (G2) and ozonized sunflower oil (G3) was significant ($P < .05$) in comparison with the control group. When O_3 reacts with unsaturated fatty acids present in vegetable oils, it can form a series of peroxide species that are responsible for the broad biological and germicide activities of ozonized vegetable oils [20]. The acidity value normally increases during ozonation of sunflower oil due to the formation of carboxylic acids by the decomposition of hydroperoxides formed during ozonation [19]. These acids are in equilibrium with the peroxidic compounds, fundamentally responsible for the germicidal action. In addition, these carboxylic acids contribute to the biological action by modifying the intra- and extracellular pH and, with this, exchange processes occur in the membranes of microorganisms [19,20]. The antimicrobial effect of ozonated sunflower oil has also been reported [15], who succeeded in inactivating *Pythium insidiosum* isolated from horses by treatment with ozonated sunflower oil, which inhibited the *in vitro* growth of the oomycete. Exposure to O_3 may induce the degradation of membrane elements [28,33], oxidation of enzymatic sulfhydryl groups [34,35], and fragmentation of the microbial genetic material [36]. As a result of this nonspecific mechanism of action, O_3 can inactivate fungi [21,35] and bacteria [15,34,36].

The most common agents found in this study were *Escherichia coli*, Aspergillus sp., and Streptococcus sp., which is in agreement with the literature that states that infectious endometritis can occur in the presence of bacteria and/or fungi [4,37-40]. Therefore, O_3 has been shown to be effective in controlling infections against *Candida albicans* yeast [41], and it was demonstrated the structural changes caused by Gram-negative (*Escherichia coli*) and Gram-positive (*Staphylococcus aureus*) bacteria by exposure to O_3 and microorganisms that were also isolated in uterine cultures before treatment [42]. It is important that the peroxide index of the ozonized oil is >500 mEq kg^{-1} to have activity against microorganisms [15,21].

The combination of conventional treatment with O_3 therapy has been shown to be more effective in controlling uterine infection, which can be explained by the removal of the uterine fluid by flushing before uterine infusion with ozonized gas or oil. It was demonstrated that the activity of O_3 against *Escherichia coli* is influenced by the composition of the organic substrates, reinforcing the need for adequate removal of organic matter before sanitization [34].

Since the initial effect of O_3 therapy involves the induction of controlled oxidative stress by triggering an antioxidant response [43], it was investigated the feasibility of its use in healthy mares to propose its further use in animals with endometritis, with the aim of verifying whether O_3 therapy improved oxidative stress during pathological conditions [12]. Therefore, our results also demonstrate the effectiveness of O_3 therapy for mares with endometritis.

5. Conclusion

Under the experimental conditions described in this study, direct infusion of O_3-gas in association with uterine lavage proved to be effective in controlling uterine infection and inflammation. Due to its antimicrobial activity, O_3 may be useful in preventing the spread of antibiotic-resistant bacteria and antibiotic resistance genes.

Financial disclosure

CAPES (Coordenacão de Aperfeiçoamento de Pessoal de nível Superior), Grant Scholarship to Ana Caroline Araújo Ávila.

References

[1] FEI (Fédération Équestre Internationale). Equestrian Nation: Brazil. 2021. https://www.fei.org/stories/lifestyle/my-equestrian-life/equestrian-nation-brazil.
[2] Viana JHM. 2019 Statistics of embryo production and transfer in domestic farm animals. Embryo Technology Newsletter 2020;38:1–15. https://www.iets.org/Portals/0/Documents/Public/Committees/DRC/IETS_Data_Retrieval_Report_2019.pdf.
[3] Troedsson MHT. Uterine clearance and resistance to persistent endometritis in the mare. Theriogenology 1999;52:461–71. doi:10.1016/S0093-691X(99)00143-0.
[4] Canisso IF, Segabinazzi LGTM, Fedorka CE. Persistent Breeding-Induced Endometritis in mares - a multifaceted challenge: from clinical aspects to immunopathogenesis and pathobiology. Int J Mol Sci 2020;21:1432. doi:10.3390/ijms21041432.
[5] Watson ED. Post-breeding endometritis in the mare. Anim Reprod Sci 2000;61:221–32. doi:10.1016/s0378-4320(00)00110-x.
[6] Canisso IF, Stewart J, Da Silva MAC. Endometritis: Managing persistent post-breeding endometritis. Vet Clin North Am 2016;32:465–80. doi:10.1016/j.cveq.2016.08.004.
[7] Scoggin CF. Endometritis: Nontraditional therapies. Vet Clin North Am 2016;32:499–511. doi:10.1016/j.cveq.2016.08.002.
[8] Schofiel WA. Use of acupuncture in equine reproduction. Theriogenology 2008;70:430–4. doi:10.1016/j.theriogenology.2008.05.001.
[9] Swift LA, Christensen BW, Samocha MB, Le Jeune SS, Millares-Ramirez EM, Dujovne GA. Randomized comparative trial of acupuncture and exercise versus uterine ecbolics in the treatment of persistent postbreeding endometritis in mares. J Equine Vet Sci 2020;86:102821. doi:10.1016/j.jevs.2019.102821.
[10] Segabinazzi LGTM, Canisso IF, Podico G, Cunha LL, Novello G, Rosser MF, Loux SC, Lima FS, Alvarenga MA. Intrauterine blood plasma platelet-therapy mitigates persistent breeding-induced endometritis, reduces uterine infections, and improves embryo recovery in mares. Antibiotics 2021;10:490. doi:10.3390/antibiotics10050490.
[11] Ferreira JC, Amaral RS, Cardoso LM, Marchió SP, Rodrigues MA, Alves CEF, Meira C. Endometrial angiogenesis induced by uterine insufflation with an oxygen–ozone gas mixture in mares. Reprod Domest Anim 2021;56:1176–83. doi:10.1111/rda.13958.
[12] Almeida BFM, Amatti LZ, Souza GG, Garcia LV, Montechiesi DF, Ignacio FS, Oliveira PL, Costa LR, Floriano BP, Bosculo MRM, Joaquim JGF, Rubio CP. Effect of uterine ozone therapy and anticoagulant sampling on oxidative stress parameters in mares. Res Vet Sci 2021;136:503–11. doi:10.1016/j.rvsc.2021.04.002.
[13] Escandón BM, Espinoza JS, Perea FP, Quito F, Ochoa R, López GE, Galarza DA, Garzón JP. Intrauterine therapy with ozone reduces subclinical endometritis and improves reproductive performance in postpartum dairy cows managed in pasture-based systems. Trop Anim Health Prod 2020;52:2523–8. doi:10.1007/s11250-020-02298-3.
[14] Đuričić D, Valpotić H, Žura Žaja I, Samardžija M. Comparison of intrauterine antibiotics versus ozone medical use in sheep with retained placenta and following obstetric assistance. Reprod Domest Anim 2016;51:538–40. doi:10.1111/rda.12715.
[15] Ferreira JC, Pires RH, Costa GB, Carrijo BN, Guiotto FG, Rodrigues VS. The in vitro effect of ozone therapy against equine Pythium insidiosum. J Equine Vet Sci 2021;98:103305. doi:10.1016/j.jevs.2020.103305.
[16] Silva RA, Garotti JEG, Silva RS, Navarini A, Pacheco A Jr. Analysis of the bactericidal effect of ozone pneumoperitoneum. Acta Cir Bras 2009;24:124–7. doi:10.1590/S0102-86502009000200009.
[17] Smith NL, Wilson AL, Gandhi J, Vatsia S, Khan SA. Ozone therapy: an overview of pharmacodynamics, current research, and clinical utility. Med Gas Res 2017;7:212–19. doi:10.4103/2045-9912.215752.

[18] Anzolin AP, Silveira-Kaross NL, Bertol CD. Ozonated oil in wound healing. What has already been proven? Med Gas Res 2020;10:54–9. doi:10.4103/2045-9912.279985.
[19] Moureu S, Violleau F, Haimoud-Lekhal DA, Calmon A. Ozonation of sunflower oils: Impact of experimental conditions on the composition and the antibacterial activity of ozonized oils. Chem Phys Lipids 2015;186:79–85. doi:10.1016/j.chemphyslip.2015.01.004.
[20] Ugazio E, Tullio V, Binello A, Tagliapietra S, Dosio F. Ozonated oils as antimicrobial systems in topical aplications. Their characterization, current apllications, and advances in improved delivery techniques. Molecules 2020;25:334. doi:10.3390/molecules25020334.
[21] Carrijo BN, Pires RH, Costa GB, Guiotto FG, Rodrigues VS, Ferreira JC. Ozone gas and ozonized sunflower oil as alternative therapies against *Pythium insidiosum* isolated from dogs. Ozone Sci Eng 2021. doi:10.1080/01919512.2021.1937511.
[22] Samper JC. A review of a practitioner's perspective on endometrial edema. Pferdeheilkunde 2010;26:14–18. doi:10.21836/PEM20100103.
[23] Cocchia N, Paciello O, Auletta L, Uccello V, Silvestro L, Mallardo K, Paraggio G, Pasolini MP. Comparison of the cytobrush, cottonswab, and low-volume uterine flush techniques to evaluate endometrial cytology for diagnosing endometritis in chronically infertile mares. Theriogenology 2012;77:89–98. doi:10.1016/j.theriogenology.2011.07.020.
[24] Schöniger S, Sieme H, Mönnig F, Richter J, Martinsson G, Verspohl J, Schoon HA. Endometrial health and disease in the mare—comparison of histopathological findings and microbiological results of two different culture methods: uterine swab and endometrial tissue. Pferdeheilkunde 2014;30:26–36.
[25] Walter J, Neuberg KP, Failing K, Wehrend A. Cytological diagnosis of endometritis in the mare: Investigations of sampling techniques and relation to bacteriological results. Anim Reprod Sci 2012;132:178–86. doi:10.1016/j.anireprosci.2012.05.012.
[26] Bohn AA, Ferris RA, McCue PM. Comparison of equine endometrial cytology samples collected with uterine swab, uterine brush, and low-volume lavage from healthy mares. Vet Clin Pathol 2014;43:594–600. doi:10.1111/vcp.12194.
[27] Bocci VA. Scientific and medical aspects of ozone therapy. State of Art. Arch Med Res 2006;37:425–35. doi:10.1016/j.arcmed.2005.08.006.
[28] Cho M, Kim J, Kim JH, Yoon J, Kim JH. Mechanisms of *Escherichia coli* inactivation by several disinfectants. Water Research 2010;44:3410–18. doi:10.1016/j.watres.2010.03.017.
[29] Tartari APS, Moreira FF, Pereira MCS, Carraro E, Cidral-Filho FJ, Salgado AI, Kerppers II. Anti-inflammatory effect of ozone therapy in an experimental model of rheumatoid arthritis. Inflammation 2020;43:985–93. doi:10.1007/s10753-020-01184-2.
[30] Zobel R, Martinec R, Ivanović D, Rošić N, Stančić Z, Žerjavić I, Flajsig B, Plavec H, Smolec O. Intrauterine ozone administration for improving fertility rate in Simmental cattle. Vet Arh 2014;84:1–8.
[31] Bocci VA, Luzzi E, Corradeschi F, Paulesu L, Rossi R, Cardaioli E, Di Simplicio P. Studies on the biological effects of ozone: 4. Cytokine production and glutathione levels in human erythrocytes. J Biol Regul Homeost 1993;7:133–8.
[32] Janic B, Umstead TM, Phelps DS, Floros J. An *in vitro* cell model system for the study of the effects of ozone and other gaseous agents on phagocytic cells. J Immunol Methods 2003;272:125–34. doi:10.1016/s0022-1759(02)00440-4.
[33] Couto EP, Alencar ER, Gonçalves VSP, Santos AJP, Ribeiro JL, Ferreira MA. Effect of ozonation on the *Staphylococcus aureus* inoculated in milk. Semina 2016;37:1911–18. doi:10.5433/1679-0359.2016v37n4p1911.
[34] Souza SMO, Alencar ER, Ribeiro JR, Ferreira MA. Inactivation of *Escherichia coli* O157:H7 by ozone in different substrates. Braz J Microbiol 2019;50:247–53. doi:10.1007/s42770-018-0025-2.
[35] Souza Ferreira WF, Alencar ER, Blum LEB, Ferreira MA, Mendonça MA, Racanicci AMC, Urruchi WMI. Ozonation of Brazil nuts in aqueous media at different pH levels: ozone decomposition, *Aspergillus flavus* inactivation, and effects on nut color and crude oil lipid profile. Ozone Sci Eng 2020;43:351–62. doi:10.1080/01919512.2020.1799189.
[36] De Rezende AJ, Alencar ER, Ferreira MA, Ferreira WFS. Control of *Listeria monocytogenes* in refrigerated ozonated water. Ozone Sci Eng 2021. doi:10.1080/01919512.2021.1912586.
[37] Nielsen JM. Endometritis in the mare: a diagnostic study comparing cultures from swab and biopsy. Theriogenology 2005;64:510–18. doi:10.1016/j.theriogenology.2005.05.034.
[38] LeBlanc MM, Magsig J, Stromberg AJ. Use of a low-volume uterine flush for diagnosing endometritis in chronically infertile mares. Theriogenology 2007;68:403–12. doi:10.1016/j.theriogenology.2007.04.038.
[39] LeBlanc MM. Advances in the diagnosis and treatment of chronic infectious and postmating-induced endometritis in the mare. Reprod Domest Anim 2010;45:21–7. doi:10.1111/j.1439-0531.2010.01634.x.
[40] Junior JAAN, Filho EFO, Melo AGP, Correia MTS, Carneiro GF. Caracterização fúngica e formação de biofilme em éguas com endometrite. Rev Bras Reprod Anim 2021;45:33–8. doi:10.21451/1809-3000.RBRA2021.004.
[41] Zargaran M, Fatahinia M, Mahmoudabadi AZ. The efficacy of gaseous ozone against different forms of *Candida albicans*. Curr Med Mycol 2017;3:26–32. doi:10.18869/acadpub.cmm.3.2.26.
[42] Thanomsub B, Anupunpisit V, Chanphetch S, Watcharachaipong T, Poonkhum R, Srisukonth C. Effects of ozone treatment on cell growth and ultrastructural changes in bacteria. J Gen Appl Microbiol 2002;48:193–9. doi:10.2323/jgam.48.193.
[43] Sagai M, Bocci V. Mechanisms of action involved in ozone therapy: is healing induced via a mild oxidative stress? Med Gas Res 2011;1:29. doi:10.1186/2045- 9912-1-29.