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SAFETY OF UTERINE LAVAGE WITH OZONATED SALINE IN MARES

(Segurança da lavagem uterina com solução salina ozonizada em éguas)

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ABSTRACT - This study aimed to assess the safety of uterine lavage using ozonated saline in mares. Each liter of saline (NaCl 0.9%) was ozonated at 50 of ozone per mL for 7 min using a medical ozone generator. Then, uterine lavage was performed during estrus or 4h post-breeding and each liter of saline remained inside the uterus for 10 min before being recovered. In experiment 1, two cycles of six mares were used in a crossover design. Lavage was performed with ozonated (Treated) or non-ozonated (Control) saline 4h after the infusion of dead sperm, and cytology samples and biopsies were collected 6 and 24h later. Experiment 2, endometrial biopsies were collected before lavage (T0) and at 15 (T15), 30 (T30), and 60 (T60) days post-treatment with ozonated (n=11) or non-ozonated (n=3) saline. In experiment 3 (n=10), the pregnancy outcome was evaluated subsequent to lavage with ozonated saline 4h after insemination. In Experiment 1, there were no difference at 6h post-breeding in PMNs count on endometrial cytology between treatment and control groups (P>0.05). However, PMNs tended (P=0.09) to be superior in the experimental treatment compared to the control at 24h post-breeding. In addition, the number of PMNs tended to be lower in the control cycle at 6 h (P=0.06) and 24 h (P=0.08) in endometrial biopsies. In Experiment 2, the endometrial architecture, the numbers of lymphocytes and PMN were not affected by the treatment (P>0.05). In Experiment 3, nine out of 10 mares were confirmed to be pregnant after treatment, and there was not pregnancy loss up to 60 days. In conclusion, uterine lavage with ozonante saline solution may cause a mild endometrial inflammation, however, doesn't harm the endometrium or impact pregnancy outcomes in healthy mares.

Key-words: endometritis; equine; fertility; ozone.

RESUMO - Este estudo tem como objetivo avaliar a segurança da utilização da lavagem uterina utilizando solução salina ozonizada em éguas. Cada litro de solução salina (0,9%) foi ozonizado com 50 de ozônio por mL durante 7 minutos, utilizando um gerador de ozônio medicinal. As lavagens uterinas foram realizadas durante o estro ou 4h após a cobertura, sendo cada litro de solução salina mantido no útero durante 10 minutos antes de ser recuperado. No experimento 1, dois ciclos de seis éguas foram utilizados em um sistema de crossover. As lavagens foram realizadas com solução salina ozonizada (Tratamento) e sem ozônio (Controle) 4h após a infusão de espermatozoides mortos. Amostras para citologia e biópsia foram coletadas 6 e 24h após. No experimento 2, as biópsias foram coletadas antes da lavagem (T0) e 15 (T15), 30 (T30) e 60 (T60) dias após o tratamento com solução salina ozonizada (n=11) e não ozonizada (n=3). No experimento 3 (n=10), as taxas de prenhez foram avaliadas após a utilização de lavagens uterinas com solução salina ozonizada 4h após a inseminação. No experimento 1, não ocorreu diferença no número de PMNs na citologia endometrial 6h após a cobertura entre os grupos de animais tratados e controle (P>0,05). Entretanto, o número de PMNs

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tendeu a ser inferior nos ciclos controles 6h (*P*=0,06) e 24h (*P*=0,08) nas biópsias endometriais. No experimento 2, a arquitetura endometrial, o número de linfócitos e PMNs, não foram afetados pelo tratamento (P>0,05). No experimento 3, nove de 10 éguas tiveram a prenhez confirmada após o tratamento, e não ocorreram perdas gestacionais até os 60 dias. Assim, as lavagens uterinas utilizando solução salina ozonizada podem causar uma leve inflamação endometrial, contudo, não prejudica o endométrio ou impacta nos índices gestacionais de éguas saudáveis.

Palavras-chave - endometrite; equino; fertilidade; ozônio.

INTRODUCTION

Ozone is a naturally occurring molecule consisting of three oxygen atoms, which has been investigated and used for decades due to its immunomodulation and antimicrobial properties (JORDAN; CARLSON, 1913; SENESE et al., 1998; SAGAI; BOCCI, 2011). Ozone is an unstable short-lived oxygen molecule that decomposes to pure oxygen and produces oxygen-free radicals (STÜBINGER; SADER; FILIPPI, 2006). These oxidizing agents react with proteins and lipids of the cells and bacterial plasma membrane, causing ultrastructural changes and cell death (MUSTAFA, 1990; THANOMSUB et al., 2002). Interestingly, neutrophils naturally generate ozone as part of their bacterial destruction mechanism (WENTWORTH, 2002).

Ozone has been widely used in the treatment of water, in the food industry (RESTAINO et al., 1995; JYOTI; PANDIT, 2004), and in human and veterinary medicine as a complementary low-cost treatment. This therapy has been used to treat wounds (GORDILLO et al., 2008; ARAUJO et al., 2017), orthopedic issues (COELHO et al., 2015; SEYAM et al., 2018; VENDRUSCOLO et al., 2018), urogenital disorders (ZOBEL et al., 2012; BAYRAK et al., 2014), gastrointestinal conditions (ALVES et al., 2004), infectious diseases (AKEYT; WALTON, 1985; CARPENDALE TF; FREEBERG., 1991), intravenous therapy (HADDAD et al., 2009), and reproductive diseases (ENGINLER et al., 2015). Intrauterine application of ozone has been carried out in goats (DJURICIC; VALPOTIC; SAMARDZIJA, 2015) and cows (DJURICIC et al., 2012a, 2012b; ZOBEL; TKALČIĆ, 2012; ĐURIĮIŠ et al., 2013; ZOBEL, 2013; DURIČIĆ; LIPAR; SAMARDŽIJA, 2014; ZOBEL et al., 2014) and improved the fertility rate of female cows treated for retained fetal membranes (DJURICIC et al., 2012a; ZOBEL; TKALČIĆ, 2012; ĐURIĮIŠ et al., 2013; DJURICIC; VALPOTIC; SAMARDZIJA, 2015) or during the normal puerperal period (DJURICIC et al., 2012b; ZOBEL et al., 2014), as well as those affected by metritis and/or endometritis (ZOBEL, 2013; DURIČIĆ; LIPAR; SAMARDŽIJA, 2014). In mares, few cases of successful treatment of endometritis with uterine ozone insufflation have been reported, besides presenting superficial technical descriptions (KEMPCHEN et al., 2013; CAMPOS et al., 2018).

Endometritis is the main cause of fertility disorders in horses (TROEDSSON, 1999). Treating the condition is still challenging due to its multifactorial causes and individual variations in clinical signs. Treatment therapies often consist of a combination of antibiotics, uterine lavage with saline solution either associated antimicrobials or alone, intrauterine infusions, anti-inflammatories and ecbolic medication (SCOGGIN, 2016). However, due to the bacterial resistance that has been reported in equine reproduction disorders, the development of new, alternative therapies to antibiotic treatment is required (CANISSO; SEGABINAZZI; FEDORKA, 2020), and a possible agent of interest in treating resistant microorganisms is ozone. However, this therapy has been empirically used in the field by many equine practitioners. Therefore, to the best of the present authors' knowledge, the effects of ozone on the endometrial architecture, post-breeding endometrial inflammatory response, and fertility of mares are still unknown. The main hypothesis of the present study was that intrauterine ozonated saline is a safe therapy in mares. This study aimed to evaluate the safety of uterine lavage with ozonated saline (NaCl 0.9%) in mares by measuring the endometrial cell variables, degree of inflammation, and fertility after treatment.

MATERIAL AND METHODS

Animals

All experimental protocols conducted in the present study were revised and approved by the Animal Care and Use Committee of "Júlio de Mesquita Filho" São Paulo State University (UNESP), Botucatu/SP, under protocol #0149/2019. The study was carried out from November 2017 to April 2018. A total of 30 crossbred mares belonging to the Department of Animal Reproduction and Veterinary Radiology at UNESP were enrolled in this study, all aged six to 13 years, multiparous, and weighing 350 to 450 kg. Mares were not repeated among experiments. Complete breeding soundness examinations of all mares revealed no fluid in the uterine lumen, negative aerobic bacterial endometrial cultures, and normal cytology (<5% of polymorphonuclear cells, PMNs) (CARD, 2005). The mares were maintained on pasture, receiving an additional 10 kg of silage per day, and water and mineral salt *ad libitum*.

Endometrial exfoliative cytology

Samples were obtained using disposable cytological collectors for mares (Provar Commercial LTDA, Sao Paulo, Brazil) (ALVARENGA; IWANA DE MATOS, 1990). After

collection, the cytobrush was immediately and carefully rubbed on slides, which were then air-dried and Dip Quick stained (Instant Prov; NewProv, Brazil). The cytological smears were evaluated using an optical microscope under a 100x oil immersion objective. Two hundred randomly cells were counted in each smear and the percentage of PMNs was recorded.

Endometrial Biopsies

Endometrial biopsies were performed before and during experiments for histological evaluation, using stainless-steel alligator type biopsy forceps (Botuphama, Botucatu, Brazil). The samples were conditioned in 10% buffered formalin before paraffin embedding. The slides were prepared with 5-micrometer slices of tissue stained with hematoxylin & eosin, and were evaluated under an optical microscope (Primo Star, Zeiss, Germany) at 1000x magnification. The degree of inflammation and any evidence of endometrial degeneration were subjectively reported, as outlined in Kenney and Doig (KENNEY; DOIG, 1986), and the number of PMNs and limphocytes were counted in 10 fields without artifacts or damaged epithelial tissue.

Preparation of ozonated saline and uterine lavage

The ozonated solution was prepared immediately before uterine lavage using an ozone generator (O&L1.5 RM, Ozonio Life®, São José dos Campos, SP, Brazil). In a brief, the ozone generator was connected to a cylinder of pure oxygen and calibrated to release 50 ug of ozone/mL per minute. The saline solution was linked to the ozone generator using an 18G needle, and another 18G needle was placed on the top of the saline bottle to relieve the pressure and to allow the influx of exceeded ozone not retained in the saline solution. One liter of saline solution (NaCl 0.9%, JP Farma, Ribeirao Preto, SP, Brazil) at 5° C was ozonized for 7 minutes immediately before uterine lavage.

To minimize contamination, the perineum was washed with mild soap, rinsed, and dried with paper towels before all intrauterine procedures. In addition, sterilized materials were used for all intrauterine procedures. For uterine lavage, a bullet tip silicone catheter 28FR 75cc (PETS-inc, Canton, USA) was placed into the uterus through the cervix, in order to fill the uterus with ozonated saline solution. Two lavages of one liter each were accomplished for all experiments, the uterus was massaged transrectally to distribute equally the ozonated saline solution and then, each liter was maintained in the uterus for 10 min, after which it was recovered via the catheter.

Experiment 1 – Effect of intrauterine ozone therapy on post-breeding inflammation

In Experiment 1, six mares were selected and assigned to treatment or control cycles in this cross-over designed study. The reproductive tract and follicle growth were examined daily by transrectal ultrasonography (Sono Scape A5V, Domed, SP, Brazil). Ovulation was induced by a single dose of 250 ug histrelin acetate after a ≥ 35 mm follicle and uterine edema were observed. Intrauterine infusion with 1 billion dead sperm extended in 20 mL PBS was performed 24 h after ovulation was induced. Uterine lavage was performed 4 h after intrauterine infusion with sperm, and endometrial cytology samples and biopsies were collected 6 and 24 h after sperm infusion. In the treatment group, uterine lavage was accomplished using two liters of ozonated saline as described above, while non-ozonated saline (NaCl 0.9%) was used in the control cycle. After the estrous cycle began in one of the groups, all mares experienced a washout cycle to minimize potential carry-over effects and were assigned to the opposite group for the following estrus. Before each cycle, it was necessary to confirm negative aerobic bacterial cultures and negative cytology from the uterus as a prerequisite for starting the experimental treatment. Mares presenting positive bacterial cultures were treated before new cycle enrollment and were allowed an additional washout cycle.

Sperm processing consisted of semen collected from two warmblood stallions in an artificial vagina (Botucatu model, Botuphama, Botucatu, Brazil). After collection, semen was immediately filtered using a nylon filter. Then, the pooled semen was centrifuged at 600 × *g* for 10 min and all seminal plasma was removed. The sperm pellet was resuspended in PBS at a concentration of 500 million cells ml. The sample was then separated into 2 ml aliquots, subjected to two freeze-thaw cycles to kill sperm cells, and then stored at -20 °C until use.

Experiment 2 – Effect of intrauterine ozone on endometrial architecture

Mares (*n*=14) with the endometrium histologically classified as types I or IIA (KENNEY; DOIG, 1986) were enrolled in this experiment. Mares had uterine lavage with ozonated saline (*n*=11) or non-ozonated saline (Control, *n*=3) performed during estrus and had endometrial biopsies collected one week before treatment (T0) and at 15 (T15), 30 (T30) and 60 (T60) days after treatment, to evaluate the effect of intrauterine ozonated saline on the endometrial architecture. Estrus was confirmed when mares presented a dominant follicle (≥ 35 mm) and endometrial edema diagnosed by transrectal ultrasonography (Sono Scape A5V, Domed, SP, Brazil). Mares were treated once when in

estrus, and the treatment consisted of uterine lavage performed with 2 liters of ozonated saline (*n*=11) prepared immediately before, or non-ozonated saline (*n*=3). Each liter of solution remained inside the uterus for 10 min before being recovered. Immediately after uterine lavage, ovulation was induced with 250 ug of intramuscular histrelin acetate (Strelin®, Botupharma, Brazil). Over the following two days, mares were examined by transrectal ultrasonography to detect ovulation. The diestrus phase was confirmed when a corpus luteum in one ovary was observed (MCCUE; SCOGGIN; LINDHOLM, 2011) and all biopsies were collected when mares were in diestrus.

Experiment 3 – fertility trial

Ten crossbreed mares (5 to 12 years), with known fertility histories that have been used in previous studies of our group for at least three breeding seasons were enrolled in Experiment 3. All mares were under the same conditions and management as described in Experiments 1 and 2. Daily transrectal palpation and ultrasonography were used to monitor follicle growth. Estrus detection and ovulation induction were performed as described in Experiment 2. At 24 h after the induction of ovulation, all mares were artificially inseminated with fresh semen (1 billion total sperm) from a 4-year-old warmblood stallion with confirmed fertility to minimize variability. Semen was diluted at 50×10^6 sperm/mL in a skimmed milk-based extender (BotuSemen®, Botupharma, Brazil). Uterine lavage with two liters of ozonated saline was performed 4 hours after insemination. During the following day, mares were examined by transrectal ultrasonography to detect ovulation and intrauterine fluid accumulation. Mares were treated with oxytocin (20 IU, im, Ocitocina Forte, UCB, Ribeirão Preto, SP, Brazil) if necessary. Pregnancy diagnosis was carried out at 14, 30, and 45 days after ovulation.

Statistical analysis

Statistical analysis was performed using GraphPad Prism (Istat 8.0 software, GraphPad Software Inc. USA). To evaluate the Gaussian distribution, PMNs, and lymphocytes in endometrial cytology samples and biopsies were evaluated using the Shapiro-Wilk normality test. ANOVA-RM and posthoc Tukey's tests were used to compare continuous parametric data, whereas nonparametric data were tested using the Kruskal-Wallis test, followed by Dunn's test. Differences in endometrial architecture were evaluated as nonparametric data. Significance was set at $P \leq 0.05$ for all tests and P values between 0.05 and 0.1 were considered a statistical trend. Fertility results were descriptively presented below.

RESULTS

In Experiment 1, there was no significant difference ($P>0.05$) in the number of PMNs in cytology samples at 6 h post-breeding in either treated or control cycles. However, PMN counts tended ($P=0.09$) to be higher in mares treated with intrauterine ozonated saline compared to in those in the control cycle at 24 h post-breeding. In both cycles, the number of PMNs decreased over time ($P<0.05$) (Table 1). Similarly, in endometrial biopsies, the number of PMNs tended to be lower at 6 h ($P=0.06$) and 24 h ($P=0.08$) in the control cycle compared to the treated cycle. Lymphocyte concentrations in endometrial biopsies did not differ ($P>0.05$) between cycles at any time point (Table 1).

Table 1 - Percentage of polymorphonuclear cells (PMNs) in endometrial cytology samples, and leucocyte number (X in ten high power fields) in the endometrial biopsies of mares treated with uterine lavage using non-ozonized saline solution (Control) or ozonized saline solution (Treated) at 6 h and 24 h post-breeding.

	Cytology (%)		**Biopsy**			
	PMNs		**PMNs**		**Lymphocytes**	
	6 h	**24 h**	**6 h**	**24 h**	**6 h**	**24 h**
Control	67.1±11.3	9.3±4.0*	21.2±7.6*	14.8±7.2*	27.3±8.7	26.6±7.5
Treated	71.1±10.6	22.1±6.1*	29.0±4.5*	20.8±3.0*	26.5±9.3	28.6±5.1

Columns with (*) denotes a statistical trend ($P<0.1>0.05$). PMNs, polymorphonuclear neutrophils.

In Experiment 2, the endometrial architecture of mares was not influenced ($P>0.05$) by uterine lavage with ozonated or non-ozonated saline. In addition, the uterine therapy did not affect ($P>0.05$) the number of lymphocytes or PMN cells in endometrial biopsies. In Experiment 3, nine mares (90%) became pregnant after artificial insemination and uterine lavage using ozonated saline solution 4 h post-breeding. In addition, no embryonic losses up to 45 days were diagnosed.

DISCUSSION

In this study, the effect of uterine lavage with ozonated saline were investigated *in vivo* in mares. To the best of the present authors' knowledge, the effects of ozone on the post-breeding endometrial inflammatory response, endometrial architecture, and fertility of mares have not been reported yet. Ozone therapy can be administered in many ways,

including as an ozonated gas, solution, oil, spray, pearls, or foam (MASSLENIKOV; KONTORCHIKOVA; GRIBKOVA, 2008; TRAVAGLI; ZANARDI; BOCCI, 2009; DJURICIC et al., 2012b; ZOBEL et al., 2014). The complete removal of air and ozone after uterine insufflation may be difficult, leading to residual air that can cause further uterine inflammation (BRADECAMP, 2011; FERRER et al., 2012). Therefore, in the current study intrauterine treatment was carried out with ozonated solution. Uterine lavage is regularly used to manage mares with delayed uterine *clearance* and endometritis, and consists of the mechanical removal of microorganisms, residual sperm, debris, and fluid accumulated in the uterine lumen (TROEDSSON; SCOTT; LIU, 1995; BLANCHARD et al., 2003). Therefore, ozonated solution has had the added benefit of mechanically cleaning the uterus by removing contaminants. Lavage can also enhance the contact with and action of ozone on the endometrial surface. The aqueous medium also ensures that the ozonated molecules from ozone react with tissue and local cells, immediately transforming into stable hydroperoxides, which are the active ozone biological messenger (MAURO et al., 2019).

Although ozone is relatively unstable in water solution, this molecule has a half-life that varies from 20 to 30 min in distilled water at 20° C (WICKRAMANAYAKE, 1991; KHADRE; YOUSEF; KIM, 2001). The decomposition of ozone is affected by the temperature and pH of the medium, low pH values and temperatures increasing its half-life, which can result in more efficient antimicrobial activity (GALDEANO et al., 2018). At higher temperatures, ozone becomes more unstable and has reduced water solubility (GALDEANO et al., 2018). In the present study, lactate ringer was stored at 5° C and then ozonated immediatelly before treatment, therefore the authors assume that ozonated saline as used in the present study maintains ozone action. Of interest, ozonated water solutions have been reported to efficiently disinfect surfaces, equipments and food (KIM et al., 2000; CÉSAR et al., 2012), reducing microbial count (e.g., *E. coli*; *Staplyloccocus aureus* and *Pseudomonas spp)* and total coliforms (CARDOSO et al., 2003; BIALOSZEWSKI et al., 2011).

A slight increase in the endometrial inflammation was observed in mares after intrauterine ozone therapy. Despite this event, histopathological analysis of endometrial biopsies over time (15, 30, and 60 days) confirmed that this therapy is safe to be used in mares. It is known that intrauterine infusion with some chemicals, such as enrofloxacin (RODRIGUEZ et al., 2012) and 1% povidone-iodine solution (OLSEN et al., 1992), can cause severe tissue damage and changes to the endometrial architecture, with

consequent irreversible reproductive effects. Therefore, the histological analysis of the endometrium provides important information related to the safety of this therapy.

Ozonated saline exerts superficial influence on the surface of the endometrium, which did not affect the inner layers of the stratum compactum or stratum spongiosum (SCROLLAVEZZA et al., 1997). In addition, although high concentration of ozone (50 ug/mL) was released in the saline, ozonated saline did not harm the endometrium. According to Martínez-Sánchez (2020) the ozone concentration in the saline fluids corresponds to 10% of the ozone concentration at the generator outlet, which theoretically would makes our solution at final concentration of 5 µg of ozone per mL. Of interest, other athors have demonstrated the antimicrobial activity against *E. coli*, *Staplyloccocus aureus*, *Pseudomonas spp and Candida albicans* of ozonated water at lower concentration (0.01 – 3.6 ug/mL) (CARDOSO et al., 2003; BIALOSZEWSKI et al., 2011; CÉSAR et al., 2012). However, the antimicrobial activity of ozone into the uterus of mares remains to be determined.

Uterine lavage with ozonated saline solution at 4 h after infusion with dead sperm tended to increase PMN counts in cytology samples at 24 h post-treatment, as well as in biopsies at 6 and 24 h post-sperm infusion in the present study. Exacerbated uterine inflammation can be harmful to the arrival of an embryo and to the establishment of pregnancy (ZENT; TROEDSSON; XUE, 1998; BUCCA et al., 2008). However, post-breeding inflammation is a natural physiological phenomenon in mammals that is important in eliminating seminal plasma, excess sperm, microorganisms, and debris from the uterus to promote a health environment for embryo survive (TROEDSSON; LIU; CRABO, 1998). Of interest, intrauterine infusion with leukocytes has been reported to quickly eliminate bacteria from the uterus of mares (NEVES et al., 2007), and the recruitment of new PMN cells is beneficial in controlling uterine infections (BLANCHARD et al., 2003). Therefore, the recruitment of PMNs caused by intrauterine ozone therapy added to its antimicrobial properts might be an alternative to treat infectious endometritis. Interestingly, ozone increased the phagocytic competence of PMNs in one *in vitro* study (DUCUSIN et al., 2003), and improved cellular immunity when used in cows in the form of ozonated autohemoadministration (OHTSUKA et al., 2006). Intrauterine ozone insufflation was reported to successfully treat infectious endometritis refractory to antibiotics in mares (KEMPCHEN et al., 2013). However, these previous reports lack in details and did not investigate the histological effects of ozone in the endometrium, necessary to validate the effectiveness and safety of the treatment. Besides the study in normal animals, mares

susceptible to persistent breeding-induced endometritis are the main purpose for further studies, as exacerbated post-breeding inflammation is the main persisting issue in these mares (CANISSO; SEGABINAZZI; FEDORKA, 2020).

Infectious endometritis is the most common cause of equine subfertility (RIDDLE; LEBLANC; STROMBERG, 2007). In particular, bacterial uterine infections are responsible for 25 to 60% of mares failing to become pregnant (COLLINS, 1964; BAIN, 1966). A Brazilian report described successful treatment with intrauterine ozone insufflation in mares suffering from bacterial and fungal endometritis (CAMPOS et al., 2018). Interestingly, ozonated saline was reported to be 100% effective *in vitro* against gram-positive bacteria, but slightly less effective (96.2%) against gram-negative bacteria (GIULIANI et al., 2018). Furthermore, *in vitro* application of ozonated saline proved to be effective against methicillin-resistant *Staphylococcus aureus*, but was only partially effective against *Pseudomonas aeruginosa,* which maintained bacterial viability of 60% (BURGASSI et al., 2009), both common agents of endometritis in the mare (CANISSO; STEWART; COUTINHO DA SILVA, 2016; CANISSO; SEGABINAZZI; FEDORKA, 2020)

The increasing incidence of antibiotic-resistant bacteria could makes ozone a suitable alternative treatment for infectious endometritis in mares. Even though antimicrobial activity was not tested in the present study, ozone is known to be effective against bacteria, fungi, and viruses (OUF et al., 2016; GIULIANI et al., 2018) and does not induce bacterial resistance (GIULIANI et al., 2018). Ozone acts in a similar manner to hydrogen peroxide (H_2O_2), which proved to be effective against cultivated bacteria *in vitro* from mares with endometritis (FERRIS et al., 2016). Despite ozone is considered a good method of destroying biofilms *in vitro* (BIALOSZEWSKI et al., 2011), one study reported that ozonated solutions were not able to *in vitro* destroy the biofilms formed by gram-negative bacteria isolated from the uterus of mares (LONCAR et al., 2017). Loncar et al. (2017) hypothesized that ozone molecules could have been degraded before contact with bacteria in the aforementioned study, based on the assumption that ozone rapidly dissociates back into oxygen atoms (STÜBINGER; SADER; FILIPPI, 2006). Although this process is responsible for generating the oxygen-free radicals that have bactericidal and viricidal properties (BURLESON; MURRAY; POLLARD, 1975; STÜBINGER; SADER; FILIPPI, 2006). It may explain the lack of efficacy in the aforementioned study (LONCAR et al., 2017). Therefore, further investigations are needed to evaluate the efficacy of ozone treatment in mares with infectious endometritis.

In the present study, 10 mares were artificially inseminated and had their uterus flushed with ozonated saline solution 4 h after breeding in order to confirm the safety of

intrauterine ozone therapy for the ongoing pregnancy. Nine mares (90%) became pregnant after treatment and no embryonic losses up to 45 days of pregnancy were diagnosed. These results confirm our hypothesis that intrauterine ozonated saline is a safe treatment for post-breeding mares and does not affect subsequent pregnancy outcomes. Interestingly, intrauterine ozone therapy has been reported to improve fertility and to reduce days open in cows during the normal post-partum period (DJURICIC et al., 2012b; ZOBEL et al., 2014). Furthermore, this therapy has been shown to be effective in cows with retained fetal membranes, resulting in reproductive performance similar to that of animals with a normal peripartum period (DJURICIC et al., 2012a; ĐURIĮIŠ et al., 2013). In addition, the efficacy of intrauterine ozonated solution was proven in cows suffering from metritis and endometritis as it increased fertility rate after treatment (DURIČIĆ; LIPAR; SAMARDŽIJA, 2014). In goats with retained fetal membranes, intrauterine application of ozone induced similar results to those of antibiotic therapy, supporting the efficacy of this alternative treatment (DJURICIC; VALPOTIC; SAMARDZIJA, 2015).

CONCLUSION

In conclusion, an ozonated solution prepared as described in this study is safe for intrauterine treatment in mares. Future studies administering intrauterine ozone to treat uterine diseases should be undertaken in mares to understand the benefits of this treatment, as well as the effects of intrauterine ozone therapy in mares susceptible to endometritis.

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