Theriogenology 219 (2024) 132–137

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Original Research Article

In vitro effects of different ozone preparations on microorganisms responsible for endometritis in the mare

Gian Guido Donato [a], Patrizia Nebbia [a], Maria Cristina Stella [a], Daniela Scalas [a], Denis Necchi [b], Alessia Bertero [a,*], Giovanna Romano [c], Lara Bergamini [d], Mariagrazia Lucia Poletto [a], Andrea Peano [a], Patrizia Robino [a,1], Tiziana Nervo [a,1]

[a] *Department of Veterinary Sciences, University of Turin, Largo Paolo Braccini 2, 10095, Grugliasco, Italy*
[b] *Keros Embryo Transfer Center, Westrozebekestraat 23A, 8980, Passendale, Belgium*
[c] *Centro Equino Arcadia, Frazione Mottura 106, 10068, Villafranca Piemonte, Italy*
[d] *Allevamento Le Fontanette, Via Fontanette 12, 10067, Vigone, Italy*

ARTICLE INFO

Keywords:
Ozone
Endometritis
Mare
Infertility
In vitro
Antimicrobial activity

ABSTRACT

Infectious endometritis is considered one of the major causes of infertility and it can affect up to 60% of barren mares. It is characterized by the presence of one or more microorganisms in the reproductive tract and it is treated with the administration of antibiotics, ecbolic agents and uterine lavages. Ozone, thanks to its antimicrobial properties that are based on its high oxidative potential, could represent an effective alternative treatment for endometritis. The aim of this study was to test *in vitro* the bactericidal and fungicidal properties of different ozone formulations, either as gas (experiment 1) or dissolved in two liquid matrices (experiment 2), specifically distilled water or oil (Neozone 4000, Cosmoproject, Parma, Italy), onto 6 different species of microorganisms isolated from mares with clinical endometritis, namely *Escherichia coli, Staphylococcus aureus, Streptococcus equi* subsp. *Zooepidemicus, Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Candida albicans*. In the first experiment, 3 clinical antibiotic-resistant strains per each species were exposed to different conditions: to O_2O_3 gas mixtures (15 and 40 μg/ml for 1, 3 and 5 min), to 100 % O_2 or left untreated. The results showed a reduction of the microbial count of over 99,9% for every pathogen, time and concentration of O_2O_3 gas mixtures tested. Furthermore, gaseous ozone showed both a time-dependant effect (5 vs 3 vs 1 min of exposure) and a concentration-dependant effect (40 vs 15 μg/ml) at 1 and 3 min, while after 5 min no differences were observed. In the second experiment, minimum inhibitory concentration (MIC), and minimum bactericidal/fungicidal concentration (MBC, MFC) of ozonated distilled water and ozonated oil were evaluated. Ozonated oil showed a bactericidal/fungicidal activity against all the strains tested (MIC range 12.5–25 *% v/v*, MBC/MFC range 12.5–50 *% v/v*) while ozonated distilled water didn't show an observable antimicrobial effect, discouraging its use as an antimicrobial agent for the treatment of endometritis.

The results of this *in vitro* study indicate that both gaseous ozone and ozonated oil exerted remarkable antimicrobial activities and are promising alternative treatments for infectious endometritis, even when caused by antibiotic-resistant bacteria, and encourage further experiments in an effort to scale down or even prevent the use of antibiotics in equine reproduction.

1. Introduction

Infectious endometritis is considered one of the major causes of infertility and it can affect up to 60% of barren mares [1]. It is characterized by the presence of one or more microorganisms in the reproductive tract that, in a large study, were isolated from 31% of the over 8000 uterine samples examined [2,3]. The aetiological agents frequently isolated in equine endometritis are *Streptococcus equi* subsp. *Zooepidemicus*, *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus and Klebsiella pneumoniae* [4]. Furthermore, yeasts, more

* Corresponding author.
E-mail address: alessia.bertero@unito.it (A. Bertero).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.theriogenology.2024.02.011
Received 13 October 2023; Received in revised form 8 February 2024; Accepted 9 February 2024
Available online 15 February 2024
0093-691X/© 2024 The Author(s). Published by Elsevier Inc. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

frequently *Candida* spp., and fungi can cause endometritis as well, and even if these infections account only for 1–5% of mare's endometritis, they can be extremely difficult to solve [5]. The canonical treatment for infectious endometritis involves the administration of antibiotics and/or alternative non-antibiotic therapies, associated with uterine lavages and ecbolic agents [2]. Since traditional therapies are not always effective [6] and considering the increasing incidence of antimicrobial resistance (AMR), the development of alternative therapies is a compelling field of research in the treatment of mare endometritis. According to the World Health Organisation (WHO), antibiotic resistance is indeed one of the greatest global public health threats facing humanity [7] and increasing evidence demonstrates that AMR is widespread in bacteria isolated from equine reproductive tracts [3,8].

Ozone is a highly unstable gaseous molecule made of three atoms of oxygen with a cyclic structure that naturally forms from oxygen (O_2) by the action of ultraviolet light and electrical discharges in the atmosphere [9]. Its antimicrobial properties are based on the high oxidative potential of this gas, which is able to attack the constituents of cell membranes, cell envelopes, cytoplasm, spore coats, and virus capsids, producing the destruction of bacterial cell walls and cytoplasmic membranes [10]. This causes an increase in permeability ultimately leading to the entry of ozone into the cells [10]. Moreover, ozone therapy is also able to damage nucleic acid and stimulates the innate immune system to contrast the microorganisms [10,11]. In this scenario, ozone, thanks to its antimicrobial properties [11,12], could represent an important resource for reducing or bypassing antibiotic requirement.

In veterinary and human reproductive medicine, ozone therapy gained popularity in the last years because of its efficacy in the treatment of reproductive pathologies that could affect female fertility [13]. The term "Ozone therapy" includes a wide range of treatments and pharmaceutical forms with different mechanisms of action such as gas, foam, pearls, ozonated water solutions and oil [13–18]. The heterogeneity in the protocols and formulations, however, limits standardization and possibly accounts for the different and sometimes conflicting findings reported in the literature [19].

The aim of this study was to evaluate the *in vitro* antimicrobial activities of different ozone formulations, either as gas, ozonated water or ozonated oil against six different microorganisms involved in equine endometritis, namely *Escherichia coli, Staphylococcus aureus, Streptococcus equi* subsp. *Zooepidemicus, Pseudomonas aeruginosa, Klebsiella pneumoniae and Candida albicans*, to provide insights for clinical use in equine endometritis.

2. Materials and methods

All experiments have been approved by the Ethics and Animal Welfare Committee of the Department of Veterinary Sciences of the University of Turin (Italy) (0002180, 2021).

2.1. Sample collection

Mares admitted for infertility to the Veterinary Teaching Hospital (OVU) of the University of Turin (Italy) during the breeding seasons 2021, 2022 and 2023 underwent a complete examination of the reproductive tract, including transrectal palpation, ultrasound examination (MyLabOne, Esaote, Genova, Italy), microbiological and cytological evaluation [20]. Those that exhibited one or more clinical signs of endometritis (intrauterine fluid accumulation, short inter-oestrous intervals, excessive edema [1]) and the evidence of inflammatory uterine cytology were recruited for this study and double guarded uterine swab samples (Minitube GmbH, Tiefenbach, Germany) were collected to research bacteria and yeasts.

2.2. Isolation of bacterial and yeast strains

Swab samples were streaked on Columbia agar with 5% sheep blood (CA-SB) and on Sabouraud dextrose agar (SDA) (Thermo Fisher Diagnostics, Altrincham, UK). CA-SB plates were incubated for 24/48 h at 37 °C with or without 5% CO_2 atmosphere, whereas SDA plates were incubated for 48 h at 35 °C. Isolates were identified by matrix assisted laser desorption ionization-time of flight/mass spectrometry (MALDI-TOF/MS) (Bruker Daltonik GmbH, Bremen, Germany).

Three antibiotic-resistant strains belonging to each of the following species, *E. coli, S. aureus, S. equi* subsp. *Zooepidemicus, P. aeruginosa, K. pneumoniae and C. albicans* were selected for this study. Each strain was isolated from a different mare (n = 18) and was selected if there was evidence of resistance to one or more antibiotics. To determine this parameter, the bacteria were tested *in vitro* for their antimicrobial susceptibility to the following panel of antimicrobials (Oxoid Ltd., Basingstoke, UK): amikacin (30 μg), amoxicillin (10 μg), ampicillin (10 μg), amoxicillin/clavulanic acid (30 μg), ceftiofur (30 μg), clindamycin (2 μg), gentamicin (10 μg), penicillin (1U), tetracycline (30 μg), trimethoprim-sulfamethoxazole (25 μg), enrofloxacin, (5 μg) and marbofloxacin (5 μg; Fatro S.p.A., Ozzano Emilia, IT). The disk diffusion method was performed and interpreted for each species according to Clinical and Laboratory Standards Institute (CLSI) M27-A3 (2018) and to EUCAST guidelines. The results of the antimicrobial susceptibility tests are reported in the supplementary materials (S1).

2.3. Experiment 1: antimicrobial activity of the gaseous ozone

The action of gaseous ozone was tested on three strains for each microbial species. A starting inoculum of bacterial cells was prepared by inoculating three or four colonies grown on CA-SB plates in sterile saline solution and adjusting the bacterial suspension to a 0.5 McFarland turbidity standard, corresponding to ≈1 to 2 × 10^8 colony forming units/ml (CFU/ml) [12]. One hundred microlitres of this suspension was inoculated onto either Triptic soy agar plates (TSA, Thermo Fisher Diagnostics, Altrincham, UK) (for *E. coli, P. aeruginosa, S. aureus* and *K. pneumoniae)* or CA-SB plates (for *S. equi* subsp. *zooepidemicus)*. For *C. albicans* the starting yeast inoculum of 0.5 McFarland corresponding to ≈5 × 10^6 cells/ml was diluted in sterile saline to yield a final suspension of 0.5–2.5 × 10^3 CFU/ml [21]. Again, 100 μL of this suspension was inoculated onto SDA plates. The inoculum size was checked by plating serial dilutions on TSA for bacteria and on SDA for yeasts and determining the colony counts in triplicate after incubation at 37 °C for 24/48 h.

Three clinically relevant strains per each species were exposed to the following different treatments and all the examinations were performed in duplicate.

- Group 1: samples were exposed to a continuous flow of gaseous ozone at a concentration of 15 and 40 μg/ml for 1, 3 and 5 min.
- Group 2: samples were exposed to a continuous flow of 100% O_2 for 3 min (negative control)
- Group 3: samples weren't exposed to neither ozone nor oxygen (negative control)

Samples were exposed to the O_2O_3 gas mixtures (Group 1) and O_2 (Group 2) inside a glass hermetic box with a polypropylene lid. This box was coupled to an ozone generator (VetBo3x, Eco3 S.r.l., Brandizzo, Italy) (Group 1) through a silicon tube, while another silicon tube was used to let the gas out [11] (Fig. 1). For the Group 2, the glass hermetic box was coupled to an oxygen cylinder.

All samples were subsequently incubated at 37 °C for 24 h or 48 h (*C. albicans)* and the microbial count (CFU/ml) was performed. The CFU/ml observed after the exposure to O_2O_3 gas mixtures (Group 1) were compared with the count observed in the Group 2 and 3 to evaluate the effect of gaseous ozone. Furthermore, differences between the effect of the various times and concentrations of O_2O_3 gas mixtures were assessed.

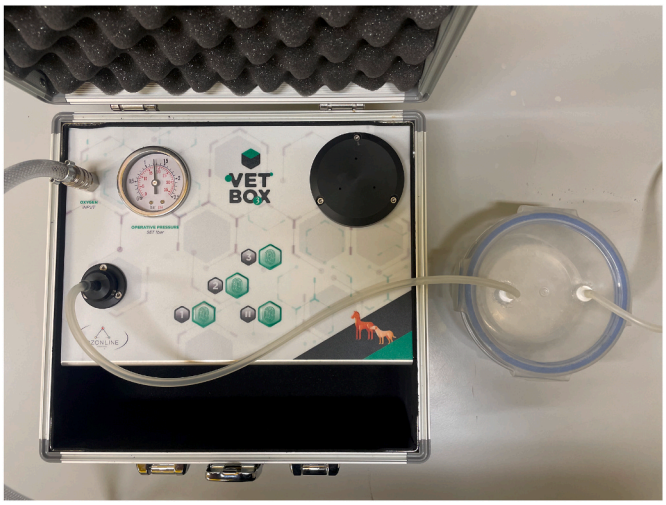

Fig. 1. Ozone generator (Vet Bo3x, Eco3 S.r.l, Brandizzo, Italy) coupled to a glass hermetic box containing a tested sample.

2.4. Experiment 2: antimicrobial activity of the ozonated water and oil (minimum inhibitory concentrations and minimum bactericidal/fungicidal tests)

The action of ozone dissolved in liquid matrices was tested on three strains for each microbial species and all the examinations were performed in duplicate. The ozonated water was obtained by continuously bubbling 100 ml of sterile distilled water for 10 min using O_2O_3 gas mixture (concentration 40 μg/ml). After preparation, ozonated water was immediately used. The ozonated oil was a commercial ozonated sunflower oil, Neozone 4000 (Cosmoproject, Parma, Italy) with a peroxide index of 4000 $mEqO_2$/Kg. Non-ozonated distilled water and sunflower oil were also tested in this experiment as control groups.

Minimum inhibitory concentrations (MICs) and minimum bactericidal/fungicidal concentrations (MBCs/MFCs) were determined by broth dilution method according to the CLSI. The inoculum was 10^5 CFU/ml for bacteria and 10^3 CFU/ml for *C. albicans*. Doubling concentrations of ozonated oil, ozonated water, sunflower oil and water controls in the Mueller Hinton broth medium (Thermo Fisher Diagnostics, Altrincham, UK) for bacteria and RPMI 1640 broth medium (Sigma, Milan, Italy) for yeasts were used (range 50% v/v – 6.25% v/v).

MICs were visually read as the lowest concentration at which no bacterial/fungal growth was observed, determined as the absence of turbidity in the tubes after incubation [22].

MBCs/MFCs were read as the lowest concentration resulting in no growth on subculture and corresponding in the death of 99.9% or more of the initial inoculum [22].

2.5. Statistical analysis

In Experiment 1 the data are expressed as mean number of CFU/ml ± standard error of the mean (SEM). The results of Experiment 2 are expressed as modal values and ranges of MIC and MBC/MFC (% v/v).

Data were analysed using GraphPad Prism, 10.1.1 for macOS, (GraphPad Software, La Jolla, USA). Data were tested for normality using Shapiro-Wilk test. Friedman test and Dunn's as post hoc were performed to assess differences in CFU/ml between the different groups and the different conditions of Experiment 1. Fisher's exact test applying Bonferroni corrections for multiple comparisons was performed to assess differences in MIC values between the groups of Experiment 2.

3. Results

3.1. Experiment 1

For every microorganism, a statistically significant decrease in the number of CFU/ml was observed in the treated group (Group 1) compared to the control groups (Groups 2 and 3) ($p < 0.001$) (Table 1). For all the bacterial species, whether Gram + or Gram -, a reduction of over 99.9% of the bacterial load was observed at every time and concentration tested. Furthermore, regarding *C. albicans,* exposure to O_2O_3 gas mixtures determined a reduction between 93.5% and 98.3% in the number of CFU/ml, depending on the experimental condition evaluated (respectively 15 μg/ml for 3 min and 40 μg/ml for 3 min).

Despite the very high antimicrobial activity of gaseous ozone in all tests, this experiment stresses also a time-dependent effect. Indeed, considering the microbial counts of all the microorganisms for each different time, a significantly lower number of CFU/ml was observed at both concentrations after 3 min of exposure (15 μg/ml, average microbial count of $2.1 \times 10^2 \pm 3.1 \times 10$ CFU/ml; 40 μg/ml, average microbial count of $9.8 \times 10 \pm 1.8 \times 10$ CFU/ml) compared to 1 min (15 μg/ml, average microbial count of $7.9 \times 10^2 \pm 1.6 \times 10^2$ CFU/ml, $p < 0.01$; 40 μg/ml, average microbial count of $2.8 \times 10^2 \pm 3.9 \times 10$ CFU/ml, $p < 0.01$). The same result was observed when comparing 5 min vs 3 min at the concentration of 15 μg/ml ($8 \times 10 \pm 1.8 \times 10$ CFU/ml, $p = 0.004$), while no difference was observed at 40 μg/ml ($5.7 \times 10 \pm 1.3 \times 10$ CFU/ml, $p > 0.1$). Furthermore, the antimicrobial effect of ozone resulted also concentration-dependant at 1 and 3 min of exposure since at a higher concentration the number of CFU/ml was significantly lower (15 vs 40 μg/ml at 1 and 3 min, respectively $p < 0.05$ and 0.02), while no differences were observed between the two ozone concentrations after 5 min of exposure ($p > 0.1$).

3.2. Experiment 2

The absence of turbidity was only observed in the tubes containing ozonated oil, which showed an inhibition of bacterial growth that was significantly different from that of other liquid matrices (ozonated water, non-ozonated oil and non-ozonated water, all p values < 0.001), with MIC values comprised between 12.5 and 25% (*v/v*). Furthermore, ozonated oil showed bactericidal activity (MBC/MFC) at a concentration between 12.5 and 50% (*v/v*) depending on the pathogen. Ozonated oil was effective against all the tested microorganisms. On the other hand, ozonated water, non-ozonated water and non-ozonated oil did not show any inhibitory effect on bacterial growth even at a concentration of 50% (*v/v*), therefore MBC/MFC were not evaluated. The MIC and MBC/MFC values of ozonated and non-ozonated oil and water are reported in Table 2.

4. Discussion

To the best of the author's knowledge, this study described for the first time the *in vitro* antimicrobial effect of different ozone preparations on clinical isolates responsible for equine endometritis.

In this experiment ozonated distilled water didn't have any observable bactericidal effect. These findings are in contrast to literature describing the potent bactericidal effect of ozonated water solutions [23,24]. On the other side, in the equine reproduction field, two recent studies have stated that ozonated saline and Ringer do not have a significant antimicrobial or antibiofilm activity against microorganisms responsible for endometritis [25,26]. These apparent controversial data could be explained by the action of different variables that may ultimately affect the final concentration of ozone in a liquid matrix, which is rarely measured and reported and that mostly depends on the protocol used to ozonize the water [27]. The lack of a precise measurement of the concentration of ozone dissolved in water constitutes indeed a limitation of the present study. Thus, it can be hypothesized that the protocol we

Table 1
Mean of CFU/ml ± SEM of each species after exposure to 0_20_3 gas mixture (Group 1), 100% O_2 (Group 2) or left untreated (Group 3). Three strains for each microbial species were tested and two independent replicates per each strain were performed.

Microroganism	15 μg/ml 1′	15 μg/ml 3′	15 μg/ml 5′	40 μg/ml 1′	40 μg/ml 3′	40 μg/ml 5′	100 % O2	Control untreated
	CFU/ml							
E. coli (n = 3)	$1.8 \times 10^2 \pm 4.3 \times 10$	$1.4 \times 10^2 \pm 6.7 \times 10$	$2.5 \times 10 \pm 7.6$	$1.6 \times 10^2 \pm 5.1 \times 10$	$5.7 \times 10 \pm 3 \times 10$	$3 \times 10 \pm 1.3 \times 10$	1–2 x 10^8	1–2 x 10^8
S. aureus (n = 3)	$2.3 \times 10^3 \pm 5.4 \times 10^2$	$3.8 \times 10^2 \pm 9 \times 10$	$1.7 \times 10^2 \pm 7.1 \times 10$	$4.4 \times 10^2 \pm 6.3 \times 10$	$1.8 \times 10^2 \pm 5.2 \times 10$	$9.2 \times 10 \pm 5.1 \times 10$	1–2 x 10^8	1–2 x 10^8
S. zooepidemicus (n = 3)	$7.8 \times 10^2 \pm 2.9 \times 10^2$	$1.6 \times 10^2 \pm 7.2 \times 10$	$3.7 \times 10 \pm 1.7 \times 10$	$1.5 \times 10^2 \pm 7.5 \times 10$	$1.3 \times 10^2 \pm 6.4 \times 10$	$6.8 \times 10 \pm 3.9 \times 10$	1–2 x 10^8	1–2 x 10^8
P. aeruginosa (n = 3)	$6.8 \times 10^2 \pm 9.6 \times 10$	$2.7 \times 10^2 \pm 7.9 \times 10$	$1.5 \times 10^2 \pm 5.2 \times 10$	$5.8 \times 10^2 \pm 8.7 \times 10$	$7.8 \times 10 \pm 2 \times 10$	$6.3 \times 10 \pm 3 \times 10$	1–2 x 10^8	1–2 x 10^8
K. pneumoniae (n = 3)	$6.8 \times 10^2 \pm 9.7 \times 10$	$2.1 \times 10^2 \pm 6.6 \times 10$	$6.2 \times 10 \pm 1.9 \times 10$	$2.6 \times 10^2 \pm 7.2 \times 10$	$1.1 \times 10^2 \pm 4.2 \times 10$	$5.8 \times 10 \pm 3.1 \times 10$	1–2 x 10^8	1–2 x 10^8
C albicans (n = 3)	$8.7 \times 10 \pm 5.4 \times 10$	$9 \times 10 \pm 5.7 \times 10$	$3.1 \times 10 \pm 2.4 \times 10$	$7.2 \times 10 \pm 4.5 \times 10$	$2.3 \times 10 \pm 1.7 \times 10$	$3.0 \times 10 \pm 2.4 \times 10$	$1.4 \times 10^3 \pm 5 \times 10$	$1.3 \times 10^3 \pm 6.1 \times 10$
Significance*	*a*	*b*	*c*	*b*	*c*	*c*	*d*	*d*

*The presence of different letters in the same row indicates a statistically significant difference ($p < 0.05$).

Table 2
MIC and MCB/MFC values (% v/v), presented as modal values and ranges of ozonated oil, ozonated water and controls (non-ozonated oil and water) on antibiotic-resistant bacteria and C. albicans. Three strains per species and two independent replicates per strain were performed.

Microorganism		Ozonated oil		Ozonated water		Non-ozonated oil		Non-ozonated water	
		MIC (% *v/v*)	MBC/MFC (% *v/v*)	MIC (% *v/v*)	MBC/MFC (% *v/v*)	MIC (% *v/v*)	MBC/MFC (% *v/v*)	MIC (% *v/v*)	MBC/MFC (% *v/v*)
E. coli (n = 3)	modal value	12.5	25	>50%	nt*	>50%	nt	>50%	nt
	range	12.5/25	25/50	−/−		−/−		−/−	
S. aureus (n = 3)	modal value	12.5	12.5	>50%	nt	>50%	nt	>50%	nt
	range	−/−	12.5/25	−/−		−/−		−/−	
S. zooepidemics (n = 3)	modal value	12.5	25	>50%	nt	>50%	nt	>50%	nt
	range	12.5/25	12.5/25	−/−		−/−		−/−	
P. aeruginosa (n = 3)	modal value	25	25	>50%	nt	>50%	nt	>50%	nt
	range	12.5/25	−/−	−/−		−/−		−/−	
K. pneumoniae (n = 3)	modal value	25	25	>50%	nt	>50%	nt	>50%	nt
	range	−/−	−/−	−/−		−/−		−/−	
C. albicans (n = 3)	modal value	12.5	12.5	>50%	nt	>50%	nt	>50%	nt
	range	−/−	−/−	−/−		−/−		−/−	

Nt* = non tested.

used to ozonize the water (40 μg/ml for 10 min), was not sufficient to reach or maintain the critical ozone concentration needed to kill the microorganisms. Again, this underpins the need of standardized protocols for ozone administration that should have a satisfactory bactericidal effect and can be performed in field conditions.

On the other hand, ozonated sunflower oil showed antimicrobial activity against all the species tested, while the control non-ozonated sunflower oil didn't show any effect. Ozonation of oils is a procedure carried out to improve the short half-life of gaseous ozone or water-based solutions. Briefly, ozone reacts with carbon-carbon double bonds of unsaturated fatty acids present in triglycerides of vegetable oils. This reaction produces several oxygenated products, such as ozonides, hydroperoxides, aldehydes, peroxides, diperoxides and polyperoxides, that are responsible for the antibacterial and fungicidal activity [28,29]. To give an objective meaning to the word "ozonated", however, it is necessary to describe the quantity of peroxides in the solution [10]. The peroxide value is a number that expresses in milliequivalents of active oxygen the quantity of peroxides contained in 1000 g of the substance and is generally considered to be correlated to its antimicrobial activities [10,28]. Diaz et al. observed that at higher peroxide indexes corresponded a higher antimicrobial activity [29], while Ferreira and coauthors [30] noted that only ozonated oil with a peroxide value higher than 500 mEq kg−1 was able to inactivate the growth of Phytium insidiosum *in vitro*. The oil we used in the experiment is Neozone 4000 (Cosmoproject, Parma, Italy). It is a commercial standardized product, with a measured peroxide index (over 4000 mEq kg^{-1}) that maintains satisfactory stability at different storage conditions [28]. This ozonated oil is the base of different ozone preparations, including Riger Spray®, that was widely used with good results in mares, cows, sheep and goats to treat endometritis, metritis, and placental retention [13,14]. Therefore, it can be speculated that the beneficial effects on fertility reported in the previous studies could have been due to the bactericidal effect of the ozonated oil, among other properties of ozone therapy. The use of ozonated oil to treat endometritis in the mare was recently reported *in vivo* by Ávila et al. [15], who observed that the treatment did not cause an increase in uterine inflammation or adverse effects more serious than mild and transient discomfort, but it was effective in reducing the rate of positive uterine culture. The data we obtained using ozonated oil in a larger number of species of uterine pathogens support its use as a therapeutic agent. Moreover, the evidence that it acts on bacteria with a high resistance pattern to antibiotics enforces its use as an alternative to antibiotics therapy in equine reproduction. In conclusion, ozonated oil showed a potent bactericidal effect and represents an effective ozone formulation to treat mares with endometritis under field conditions, bypassing the use of gaseous ozone which needs an ozone generator and an oxygen cylinder that can be expensive and not easily available or utilizable in the field.

Also gaseous ozone proved to be a strong bactericidal agent, since all the concentrations and times of exposure achieved a reduction in the number of CFU/ml of over 99.9% on all the bacteria tested, whether Gram + or Gram -. Furthermore, regarding *C. albicans,* a reduction

between 93.5% and 98.3% in the number of CFU/ml depending on the experimental condition was observed.

These results are in agreement with previously published data: Fontes et al. [12] observed that the growth of different bacterial isolates was completely inhibited after 5 min of exposure to an ozone-oxygen mixture at a concentration of 20 μg/ml. Lillo et al. [11] performed a similar experiment on clinical isolates of bacteria responsible for bovine metritis and stated that already after 1 min of exposure to ozone at a concentration of 20 μg/ml the growth was minimal or totally inhibited. In the present study, a higher concentration of bacteria was inoculated on the plate (10^7 CFU vs 10^3 CFU). Furthermore, we performed a dose-dependent analysis reaching 15 μg/ml of O_2O_3, in the attempt to identify a lower effective antibacterial dose. A minimal effective dose of gaseous ozone for topical application has not been clearly determined yet and ozone, due to its mechanism of action based on its oxidating properties, if delivered in high doses could have a detrimental effect, overwhelming the antioxidant system of the cells and organism and resulting in tissue damage [9,11,13,31]. Lowering the ozone supply, either reducing the time of administration or its concentration, has the purpose of identifying a standard therapy able to reduce these risks. It was recently proved, moreover, that the intrauterine administration of gaseous ozone in the mare at a concentration of 39 μg/ml and a total dose of 125 μg/kg causes a systemic oxidative stress evidenced by decreased total antioxidant capacity and increased total oxidant capacity [32]. However, it is important to perform more studies to understand whether this effect is beneficial or not, in order to adjust the doses [32].

According to the results of the present study, 15 and 40 μg/ml achieved a similar inhibition of bacterial growth after 5 min of exposure. This finding is particularly interesting, especially considering the results of two recently published *in vivo* studies. In the first one, Ávila et al. [15] performed 10 min of continuous intrauterine insufflation of ozone at a concentration of 40 μg/ml, repeated for two consecutive days, and 8 out of 9 mares showed a negative bacteriological culture after the treatment. In the second study, Kohne et al. [16], reported that 5 out of 9 mares showed a negative culture after the insufflation of 240 ml of ozone at a concentration of 80 μg/ml repeated twice at a 48-h interval. These results prove that a higher concentration is not always more effective, but probably the volume of O_2O_3 mixture insufflated plays an important role. Two-hundred forty ml is, indeed, a volume generally not sufficient to fill the entire uterine cavity and probably ozone could not reach all the surface of the uterus and does not have enough contact time with endometrium to carry out its antimicrobial action, especially due to the gaseous nature of the molecule and its short half-life. Thus, it can be hypothesized that focalized infection could be missed [4]. Therefore, the insufflation of lower concentrations of ozone in the mixture (up to 15 μg/ml, which proved to be effective in this study) but using higher volumes/time of exposure could be a successful strategy to achieve optimal antimicrobial activity while minimizing the total dose of ozone administered to the mare and therefore potential local cytotoxicity and systemic impact [33].

Another aspect to consider is that in mares with clinical infectious endometritis, sometimes not only one microorganism is involved but mixed infections do occur and may be the cause of therapeutic failure [34]. In this work, however, ozone, due to its mechanism of action that is based on the oxidation of biological components of bacteria [12], proved to be effective against all the pathogens tested, whether Gram +, Gram – or yeasts. Ozone therapy could therefore bypass this problem and be effective even when the infection is caused by multiple pathogens.

Finally, in the present study, ozone showed its bactericidal effect against antibiotic-resistant bacteria. The phenomenon of antibiotic resistance is widespread also in the equine reproduction field [3,8]. However, the use of antibiotics is very common in the mare and in a recent descriptive study, Mouncey et al. [35] described that post-covering antibiotics are still routinely administered, even in the absence of clinical signs of endometritis, bacterial culture and antimicrobial sensitivity test, a dangerous habit that may lead to an increase of antibiotic-resistant bacterial strains. In this scenario, since the development of novel antibacterial agents is insufficient to counter this growing threat, alternative bactericide treatments are required [36] and ozone could play an important role in the future in the treatment of numerous infective pathologies, including equine endometritis. It was recently described in human medicine the strategy of using micro doses of ozone in association with antibiotics to improve the performance of both therapies, especially against resistant strains of Gram-negative bacteria [31]. Furthermore, in a case report, Kempchen et al. showed that intrauterine gaseous insufflation successfully resolved endometritis caused by methicillin-resistant *Staphylococcus aureus* in two mares [37]. In our study, we confirmed and expanded this finding since ozone proved to be effective *in vitro* against all the antibiotic-resistant strains tested. These results, obtained on clinical isolates responsible for equine endometritis, create a solid scientific rationale for its use as antimicrobial agent *in vivo* that could contribute to prevent or at least to scale down the use of antibiotics.

5. Conclusions

In the present study ozonated distilled water didn't show a significant antibacterial effect, discouraging its use as an antimicrobial agent for the treatment of endometritis.

On the other hand, both gaseous ozone and ozonated oil showed antimicrobial activity against antibiotic-resistant bacterial and yeast strains. Furthermore, even lower than previously reported concentrations of gaseous ozone showed efficacy *in vitro*. Thus, ozone therapy proved to be a promising treatment for infectious endometritis and the results of this study create a solid scientific rationale for its use as an antimicrobial agent in an effort to scale down or prevent the use of antibiotics in equine reproduction.

Declaration of interest

None.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CRediT authorship contribution statement

Gian Guido Donato: Writing – original draft, Investigation, Conceptualization. **Patrizia Nebbia:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Maria Cristina Stella:** Writing – original draft, Investigation, Conceptualization. **Daniela Scalas:** Writing – original draft, Investigation, Conceptualization. **Denis Necchi:** Writing – review & editing, Conceptualization. **Alessia Bertero:** Writing – review & editing, Formal analysis. **Giovanna Romano:** Investigation. **Lara Bergamini:** Investigation. **Mariagrazia Lucia Poletto:** Investigation. **Andrea Peano:** Methodology. **Patrizia Robino:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Tiziana Nervo:** Writing – review & editing, Supervision, Methodology, Conceptualization.

Acknowledgments

We thank Dr. Elisa Guastalli and Sara Albini for the collaboration and help with the project. We thank Paolo Valenti (Medical Swan ITALIA S.a.S) and Eco 3 S.r.l. For providing the ozone generator machine. We thank Cosmoproject S.p.a. For providing the ozonated oil.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2024.02.011.

References

[1] LeBlanc M, Causey R. Clinical and subclinical endometritis in the mare: both threats to fertility. Reprod Domest Anim 2009;44:10–22. https://doi.org/10.1111/j.1439-0531.2009.01485.x.

[2] Morris LH, McCue PM, Aurich C. Equine endometritis: a review of challenges and new approaches. Reproduction 2020;160:R95–110. https://doi.org/10.1530/REP.

[3] Davis HA, Stanton MB, Thungrat K, Boothe DM. Uterine bacterial isolates from mares and their resistance to antimicrobials: 8,296 cases (2003–2008). J Am Vet Med Assoc 2013;242:977–83. https://doi.org/10.2460/javma.242.7.977.

[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. https://doi.org/10.3390/ijms21041432.

[5] Scott CJ. A review of fungal endometritis in the mare. Equine Vet Educ 2020;32:444–8. https://doi.org/10.1111/eve.13010.

[6] Scoggin CF. Endometritis: nontraditional therapies. Vet Clin N Am Equine Pract 2016;32:499–511. https://doi.org/10.1016/j.cveq.2016.08.002.

[7] World Health Organization. Antimicrobial resistance 2021. Antimicrobial resistance. World Health Organization; 2021. https://www.who.int/news-room/fact-sheets/detail/antibiotic-resistance. [Accessed 5 October 2023].

[8] Ferrer MS, Palomares R. Aerobic uterine isolates and antimicrobial susceptibility in mares with post-partum metritis. Equine Vet J 2018;50:202–7. https://doi.org/10.1111/evj.12738.

[9] Bocci V, Borrelli E, Travagli V, Zanardi I. The ozone paradox: ozone is a strong oxidant as well as a medical drug. Med Res Rev 2009;29:646–82. https://doi.org/10.1002/med.20150.

[10] Ugazio E, Tullio V, Binello A, Tagliapietra S, Dosio F. Ozonated oils as antimicrobial systems in topical applications. Their characterization, current applications, and advances in improved delivery techniques. Molecules 2020;25. https://doi.org/10.3390/molecules25020334.

[11] Lillo E, Cordisco M, Trotta A, Greco G, Carbonari A, Rizzo A, et al. Evaluation of antibacterial oxygen/ozone mixture in vitro activity on bacteria isolated from cervico-vaginal mucus of cows with acute metritis. Theriogenology 2023;196:25–30. https://doi.org/10.1016/j.theriogenology.2022.10.031.

[12] Fontes B, Cattani Heimbecker AM, de Souza Brito G, Costa SF, van der Heijden IM, Levin AS, et al. Effect of low-dose gaseous ozone on pathogenic bacteria. BMC Infect Dis 2012;12. https://doi.org/10.1186/1471-2334-12-358.

[13] Sciorsci RL, Lillo E, Occhiogrosso L, Rizzo A. Ozone therapy in veterinary medicine: a review. Res Vet Sci 2020;130:240–6. https://doi.org/10.1016/j.rvsc.2020.03.026.

[14] Donato GG, Appino S, Bertero A, Poletto ML, Nebbia P, Robino P, et al. Safety and effects of a commercial ozone foam preparation on endometrial environment and fertility of mares. J Equine Vet Sci 2023:121. https://doi.org/10.1016/j.jevs.2023.104222.

[15] Ávila ACA, Diniz NC, Serpa RT, Chaves MMBC, Viu MAO, de Oliveira RA. Effectiveness of Ozone therapy in the treatment of endometritis in mares. J Equine Vet Sci 2022;112. https://doi.org/10.1016/j.jevs.2022.103900.

[16] Kohne M, Hofbauer L, Bottcher D, Tonissen A, Hegger A, Gorgens A, et al. Comparison of systemic trimethoprim-sulfadimethoxine treatment and intrauterine ozone application as possible therapies for bacterial endometritis in equine practice. Front Vet Sci 2023:10. https://doi.org/10.3389/fvets.2023.1102149.

[17] Camargo Ferreira J, Silva Amaral R, Mara Cardoso L, Panelli Marchió S, Aldrovani Rodrigues M, Fonseca Alves CE, et al. Endometrial angiogenesis induced by uterine insufflation with an oxygen–ozone gas mixture in mares. Reprod Domest Anim 2021;56:1176–83. https://doi.org/10.1111/rda.13958.

[18] Escandón BM, Espinoza JS, Perea FP, Quito F, Ochoa R, López GE, et al. 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. https://doi.org/10.1007/s11250-020-02298-3.

[19] Orlandin JR, Machado LC, Ambrósio CE, Travagli V. Ozone and its derivatives in veterinary medicine: a careful appraisal. Vet Anim Sci 2021;13. https://doi.org/10.1016/j.vas.2021.100191.

[20] Nervo T, Nebbia P, Bertero A, Robino P, Stella MC, Rota A, et al. Chronic endometritis in subfertile mares with presence of chlamydial DNA. J Equine Vet Sci 2019;73:91–4. https://doi.org/10.1016/j.jevs.2018.12.003.

[21] Mandras N, Roana J, Scalas D, Del Re S, Cavallo L, Ghisetti V, et al. The inhibition of non-albicans candida species and uncommon yeast pathogens by selected essential oils and their major compounds. Molecules 2021;26. https://doi.org/10.3390/molecules26164937.

[22] Yousef MS, Abdelhamid HN, Hidalgo M, Fathy R, Gómez-Gascón L, Dorado J. Antimicrobial activity of silver-carbon nanoparticles on the bacterial flora of bull semen. Theriogenology 2021;161:219–27. https://doi.org/10.1016/j.theriogenology.2020.12.006.

[23] Song M, Zeng Q, Xiang Y, Gao L, Huang J, Huang J, et al. The antibacterial effect of topical ozone on the treatment of MRSA skin infection. Mol Med Rep 2018;17:2449–55. https://doi.org/10.3892/mmr.2017.8148.

[24] Marino M, Maifreni M, Baggio A, Innocente N. Inactivation of foodborne bacteria biofilms by aqueous and gaseous ozone. Front Microbiol 2018;9. https://doi.org/10.3389/fmicb.2018.02024.

[25] Loncar KD, Ferris RA, McCue PM, Borlee GI, Hennet ML, Borlee BR. In vitro biofilm disruption and bacterial killing using nonantibiotic compounds against Gram-negative equine uterine pathogens. J Equine Vet Sci 2017;53:94–9. https://doi.org/10.1016/j.jevs.2017.02.003.

[26] Mazzuchini MP, Segabinazzi LG, Canisso IF. Antimicrobial activity of alternative therapies for microorganisms causing chronic endometritis in mares. J Equine Vet Sci 2023;125:104730. https://doi.org/10.1016/J.JEVS.2023.104730.

[27] Bocci V. Preparation of ozonated water and oil for the topical therapy – ozone as a drinking water disinfectant: ozone disinfection to prevent nosocomial infections. Ozone 2010;24:9–16. https://doi.org/10.1007/978-90-481-9234-2_3.

[28] Cirlini M, Caligiani A, Palla G, de Ascentis A, Tortini P. Stability studies of ozonized sunflower oil and enriched cosmetics with a dedicated peroxide value determination. Ozone Sci Eng 2012;34:293–9. https://doi.org/10.1080/01919512.2012.692992.

[29] Díaz M, Gavín J, Gómez M, Curtielles V, Hernández F. Study of ozonated sunflower oil using 1H NMR and microbiological analysis. Ozone Sci Eng 2006;28:59–63. https://doi.org/10.1080/01919510500479239.

[30] Ferreira JC, Pires RH, Costa GB da, Carrijo BN, Guiotto FG, Rodrigues VS. The in vitro effect of ozone therapy against equine Pythium insidiosum. J Equine Vet Sci 2021;98. https://doi.org/10.1016/j.jevs.2020.103305.

[31] Roth A, Maruthamuthu MK, Nejati S, Krishnakumar A, Selvamani V, Sedaghat S, et al. Wearable adjunct ozone and antibiotic therapy system for treatment of Gram-negative dermal bacterial infection. Sci Rep 2022;12. https://doi.org/10.1038/s41598-022-17495-3.

[32] Almeida BF, Amatti LZ, de Souza GG, Garcia LV, Montechiesi DF, Ignácio FS, et al. Effect of uterine ozone therapy and anticoagulant sampling on oxidative stress parameters in mares. Res Vet Sci 2021;136:503–11. https://doi.org/10.1016/j.rvsc.2021.04.002.

[33] Lillo E, Pellegrini F, Rizzo A, Lanave G, Zizzadoro C, Cicirelli V, et al. In vitro activity of Ozone/Oxygen gaseous mixture against a Caprine Herpesvirus Type 1 strain isolated from a goat with vaginitis. Animals 2023;13:1920. https://doi.org/10.3390/ani13121920.

[34] LeBlanc M. The current status of antibiotic use in equine reproduction. Equine Vet Educ 2009:156–67. https://doi.org/10.2746/095777308X357621.

[35] Mouncey R, Arango-Sabogal JC, de Mestre A, Verheyen K. Descriptive study of medication usage and occurrence of disease and injury during gestation in thoroughbred broodmares. J Equine Vet Sci 2022;118:104104. https://doi.org/10.1016/j.jevs.2022.104104.

[36] Skorup P, Fransson A, Gustavsson J, Sjöholm J, Rundgren H, Özenci V, et al. Evaluation of an extracorporeal ozone-based bactericide system for the treatment of Escherichia coli sepsis. Intensive Care Med Exp 2022;10. https://doi.org/10.1186/s40635-022-00443-w.

[37] Kempchen S, Lenk S, Behrendt D, Mayer V, Gremmes S. Case report of the treatment of two mares by uterus insufflation with an ozone oxygen mix after positive test results for MRSA in endometrial swabs. Tierärztliche Umsch 2013;68:546–50.