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In Vitro Evaluation of Ozone Activity on Recent Clinically Isolated Bacterial Strains

P. Tordiglione[1*], F. S. M. Araimo Morselli[1], I. Scarpa[1], G. Puggioni[2], C. Mancini[2], G. Rosa[1], A. Giordano[2]

[1]Department of Anaesthesiology and Critical Care Medicine, Sapienza University of Rome, Rome, Italy
[2]Department of Public Health and Microbiology, Sapienza University of Rome, Rome, Italy
Email: [*]p.tordiglione@policlinicoumberto1.it

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ABSTRACT

This study aims to evaluate the cozone bactericidal activity in different suspension media (saline, broth and whole blood) at different exposure times. Methicillin-resistant *Staphylococcus aureus*, *Enterococcus faecalis*, ESBL- positive *Escherichia coli*, MDR *Pseudomonas aeruginosa* were suspended in different media. We used a bacterial concentration of 0.2 MF for all experiments, as this concentration is consistent with the results of septic shock blood experiments. We performed ozone insufflations in a "sealed environment". The total number of insuffla- tions for each experiment ranged from one to four. The gas concentration was maintained at 80 mcg/ml. We con- firmed the bactericidal activity of ozone on saline for all the bacterial strains. Experiments in broth revealed no changes in the bacterial growth. Ozone is primarily bactericidal against *E. coli* and bacteriostatic on *P. aerugi- nosa*, *S. aureus* and *E. faecalis* on whole blood. This study confirms the bactericidal efficacy of topical ozone ap- plications and supports the need for further evaluations of the therapeutic potential of major ozone autohemo- therapy. The results in *E. coli* promote further investigations of ozone activity on other Enterobacteriaceae and its potential use in the treatment of urinary infections. In general, these results suggest that ozone-therapy might be an alternative therapy to overcome antibiotic resistance.

KEYWORDS

Ozone; Bactericidal Activity; *in Vitro*; Media; Therapeutic Treatment

1. Introduction

The microbicidal activity of ozone has been demonstrated since the late 1800. The first municipal water purification plant dates back to 1906. On June 26, 2001, the US Food and Drug Administration (FDA) formally approved the use of ozone (gaseous phase and ozonized water) as an antimicrobial agent for the treatment, storage and preservation of food products [1].

Ozone is the most powerful oxidizing agent, showing ten times the effectiveness of chlorine, and it's currently used to potabilize water [2-4], disinfect swimming pool water [5] and decontaminate bioclean rooms [6].

Ozone bactericidal activity seems to be primarily resulting from direct oxidative damage and the effects of ozone have been tested on different bacterial strains, including *E. coli*, Salmonella sp., *S. aureus* and *Bacillus subtilis* [7-18].

Using electron microscopy analysis, Thanomsub *et al.* showed the destruction of the bacterial membrane with consequent cell lysis [19]. The treatment of *Bacillus subtilis* spores with various oxidizing agents (including ozone) damages the inner membrane, making the spores more sensitive to subsequent thermal and osmotic stresses, with the increased rapid penetration of methylamine within the core [20]. The literature also confirms the synergic action of ozone with antibiotic therapy *in vivo* [21-26].

The *in vitro* ozone bactericidal activity is compromised when applied to blood and blood derivatives [27].

[*]Corresponding author.

The aim of this study is to evaluate the ozone bactericidal activity on different bacterial strains and media under different circumstances.

2. Materials and Methods

2.1. Microorganisms and Media

These experiments were divided into three phases according to the suspension media used: saline, nutrient broth (BHI) and whole blood.

We focused our study on the four multi-resistant bacterial strains frequently found in nosocomial sepsis [28-33]. The following bacterial strains were isolated from clinical samples obtained from patients at the Neurosurgery Intensive Care Unit, suspended in glycerol and frozen at −80˚C:

- Methicillin-resistant *Staph. aureus*;
- *Ent. faecalis*;
- ESBL-positive *E. coli*;
- MDR *Ps. aeruginosa*.

The preparation of the bacterial strains was performed using the following steps:

1) Thawing of the bacterial strain for testing;

2) Seeding culture medium Trypticase Soy Agar (TSA);

3) Incubation at 37˚C for 18 - 24 hours;

4) Suspension of the bacterial strains in saline and dilution until reaching 0.2 MF (McFarland);

5) Using a drop (0.02 ml) of the bacterial suspension;

6) Inoculating into 2.5 ml of the selected medium.

2.2. Ozone Generation

A Medica-srl machine, model E80, was used to generate medical ozone (Ozonline International, Medica S.r.l. Via Sante Vincenzi, 48 - 40138 Bologna
Italy) from oxygen and electricity. The machine converts medical oxygen into a mixture of O_3 (0.05%) and O_2 (99.95%) through an electrochemical process.

The Medica-srl machine is equipped with a photometer, calibrated according to the classic iodometric titration of ozone, and a voltage system which regulates the concentration within a range from 5 to 80 µg/ml.

In all the experiments, we used an ozone concentration of 80 µg/ml, corresponding to the maximum concentration within the therapeutic range recommended *in vivo* [34-38].

The volume of the O_2/O_3 gas mixture used for each insufflation was 6 ml, corresponding to 480 µg of ozone.

2.3. Treatment of the Bacterial Strains with Ozone

2.3.1. Suspension Medium: Saline

The four bacterial strains were suspended in saline and diluted until reaching 0.2 MF in a final volume of 2.5 ml for each suspension. The obtained bacterial suspensions were sown onto TSA culture medium (Bio-Mérieux Italia) as (T_0). We standardized the inoculum using a calibrated loop of 10 µl and seeding in four quadrants.

We placed a 21-gauge needle (Troge/Hamburg), premounted with a three-way cock (Axel S.r.l. connector), on the tubes containing the bacterial suspensions (BD 7-ml Vacutainer Red tube, Belliver Industrial Estate, Plymouth), thereby avoiding any leakage of the gas during the insufflation ("insufflation in closed tube").

Because of excessive pressure, the insufflation could open the tubes. To avoid this problem, we aspirated the air from the tubes using a 60-ml syringe (Luer Lock Omnifix/B. Braun) for a total of 360 ml, creating a vacuum.

We proceeded with the insufflation of 6 ml of O_2/O_3 mixture.

This procedure was applied in all experiments.

The tubes were subsequently shaken in a monodirectional oscillator for 40 minutes.

The experiment was terminated with a second seeding (T_1).

2.3.2. Suspension Medium: Brain Heart Infusion (BHI)

The bacterial strains were suspended in saline, diluted until 0.2 MF and suspended in 2.5 ml of BHI (Bio-Mérieux Italia).

The bacterial suspensions were sown onto TSA culture medium (Bio-Mérieux Italia) as (T_0).

We proceeded with the insufflation of 6 ml of O_2/O_3 mixture.

The tubes were subsequently shaken in a monodirectional oscillator for 40 minutes.

The experiment was terminated with a second seeding (T_1).

2.3.3. Suspension Medium: Whole Blood

We performed the same procedure on medium containing the fresh whole blood of healthy donors. The blood was collected using a 21-gauge butterfly needle (Pic Indolor, Mirage, Artsana S.p.a. Grandate, CO, Italy) and BD Vacutainer Light Blue tubes containing citrate (Belliver Industrial Estate, Plymouth).

We performed seven different experiments:

1) We inoculated the bacterial suspensions in 2.5 ml of whole blood; the obtained bacterial suspensions were subsequently sown onto TSA culture media (Bio-Mérieux Italia) as (T_0).

The experiment continued, according to the following steps:

- Insufflation of the O_2/O_3 mixture;
- Mechanical agitation for 5 minutes;
- Second seeding (T_1);

- Mechanical agitation for 20 minutes;
- Third seeding (T_2);
- Mechanical agitation for 20 minutes; and
- Fourth seeding (T_3).

Ozone: 480 µg × 1 = 480 µg.

Total time of mechanical agitation: 45'.

2) We inoculated the bacterial suspensions in 2.5 ml of whole blood; the obtained bacterial suspensions were subsequently sown onto TSA culture media (Bio-Mérieux Italia) as (T_0).

The experiment continued according to the following steps:

- Insufflation of the O_2/O_3 mixture;
- Mechanical agitation for 5 minutes;
- Second seeding (T_1);
- Second insufflation of the O_2/O_3 mixture;
- Mechanical agitation for 40 minutes; and
- Third seeding (T_2).

Ozone: 480 µg × 2 = 960 µg.

Total time of mechanical agitation: 45'.

3) We inoculated the bacterial suspensions in 2.5 ml of whole blood, and the obtained bacterial suspensions were subsequently sown onto TSA culture media (Bio-Mérieux Italia) as (T_0).

The experiment continued according to the following steps:

- Insufflation of the O_2/O_3 mixture;
- Mechanical agitation for 5 minutes;
- Second seeding (T_1);
- Second insufflation of the O_2/O_3 mixture;
- Mechanical agitation for 20 minutes;
- Third insufflation of the O_2/O_3 mixture;
- Mechanical agitation for 20 minutes; and
- Third seeding (T_2).

Ozone: 480 µg × 3 = 1440 µg.

Total time of mechanical agitation: 45'.

4) We inoculated the bacterial suspensions in 2.5 ml of whole blood, and the obtained bacterial suspensions were subsequently sown onto TSA culture media (Bio-Mérieux Italia) as (T_0).

The experiment continued according to the following steps:

- Insufflation of the O_2/O_3 mixture;
- Mechanical agitation for 5 minutes;
- Second seeding (T_1);
- Second insufflation of the O_2/O_3 mixture;
- Mechanical agitation for 20 minutes;
- Third seeding (T_2);
- Mechanical agitation for 20 minutes;
- Fourth seeding (T_3);
- Mechanical agitation for 20 minutes; and
- Fifth seeding (T_4).

Ozone: 480 µg × 1 = 480 µg.

Total time of mechanical agitation: 65'.

5) We inoculated the bacterial suspensions in 2.5 ml of whole blood, and the obtained bacterial suspensions were subsequently sown onto TSA culture media (Bio-Mérieux Italia) as (T_0).

The experiment continued according to the following steps:

- Insufflation of the O_2/O_3 mixture;
- Mechanical agitation for 5 minutes;
- Second seeding (T_1);
- Mechanical agitation for per 20 minutes;
- Third seeding (T_2);
- Second insufflation of the O_2/O_3 minutes;
- Mechanical agitation for 20 minutes;
- Fourth seeding (T_3);
- Third insufflation of the O_2/O_3 mixture;
- Mechanical agitation for 20 minutes;
- Fourth insufflation of the O_2/O_3 mixture;
- Mechanical agitation for 20 minutes; and
- Fifth seeding (T_4).

Ozone: 480 µg × 4 = 1920 µg.

Total time of mechanical agitation: 85'.

6) We inoculated the bacterial suspensions in 2.5 ml of whole blood, and the obtained bacterial suspensions were subsequently sown onto TSA culture media (Bio-Mérieux Italia) as (T_0).

The experiment continued according to the following steps:

- Insufflation of the O_2/O_3 mixture;
- Mechanical agitation for 5 minutes;
- Second seeding (T_1);
- Mechanical agitation for 40 minutes;
- Third seeding (T_2);
- Second insufflation of the O_2/O_3 mixture, consistent with the resumption of the bacterial growth;
- Mechanical agitation for 20 minutes; and
- Fourth seeding (T_3).

Ozone: 480 µg × 2 = 960 µg.

Total time of mechanical agitation: 65'.

7) We inoculated the bacterial suspensions in 2.5 ml of whole blood, and the obtained bacterial suspensions were subsequently sown onto TSA culture media (Bio-Mérieux Italia) as (T_0).

The experiment continued according to the following steps:

- Insufflation of the O_2/O_3 mixture;
- Mechanical agitation for 5 minutes;
- Second seeding (T_1);
- Second insufflation of the O_2/O_3 mixture;
- Mechanical agitation for 5 minutes;
- Third insufflation of the O_2/O_3 mixture;
- Mechanical agitation for 5 minutes;
- Fourth insufflation of the O_2/O_3 mixture;
- Mechanical agitation for 5 minutes;

- Third seeding (T_2);
- Mechanical agitation for 20 minutes;
- Fourth seeding (T_3);
- Mechanical agitation for 40 minutes; and
- Fifth seeding (T4).

Ozone: 480 µg × 4 = 1920 µg.

Total time of mechanical agitation: 80'.

Before and after each experiment on whole blood, we performed a complete blood count.

All experiments, regardless of the suspension medium, were consistent with a control bacterial growth curve obtained without ozone.

2.4. Statistical Analysis

The results were expressed as the means ± SD of three independent measurements for each experiment. The statistical evaluations were performed using the statistical software SPSS ver. 10. Significance was defined as a *P* value < 0.05.

3. Results

3.1. Suspension Medium: Saline

After contact with the O_2/O_3 mixture, we observed a total reduction of the bacterial load and the absence of growth for all four bacterial strains under examination (**Figure 1**).

3.2. Suspension Medium: Whole Blood

We performed seven experiments, adding ozone to the bacterial suspensions to verify the total dosage and intervals between insufflations.

Experiment I - Ozone: 480 µg × 1; total time of mechanical agitation: 45'.

Staph. aureus, *Ps. aeruginosa*, *Ent. faecalis*: reduction of bacterial growth at T_1 (5') and bacterial resumption at T_3 (45'), except for *Staph. aureus* which showed bacteriostatic activity.

E. coli: reduction of bacterial growth at T_1 (5') and total absence of colonies at T_2 and T_3. Bactericidal activity (**Figure 2**).

Experiment II - Ozone: 480 µg × 2; total time of mechanical agitation: 45'.

Staph. aureus, *Ps. aeruginosa*, *Ent. faecalis*: Bacteriostatic activity.

E. coli: reduction of the bacterial growth at T_1 (5') and total absence of colonies at T_2. Bactericidal activity (**Figure 3**).

Experiment III - Ozone: 480 µg × 3; total time of mechanical agitation: 45'.

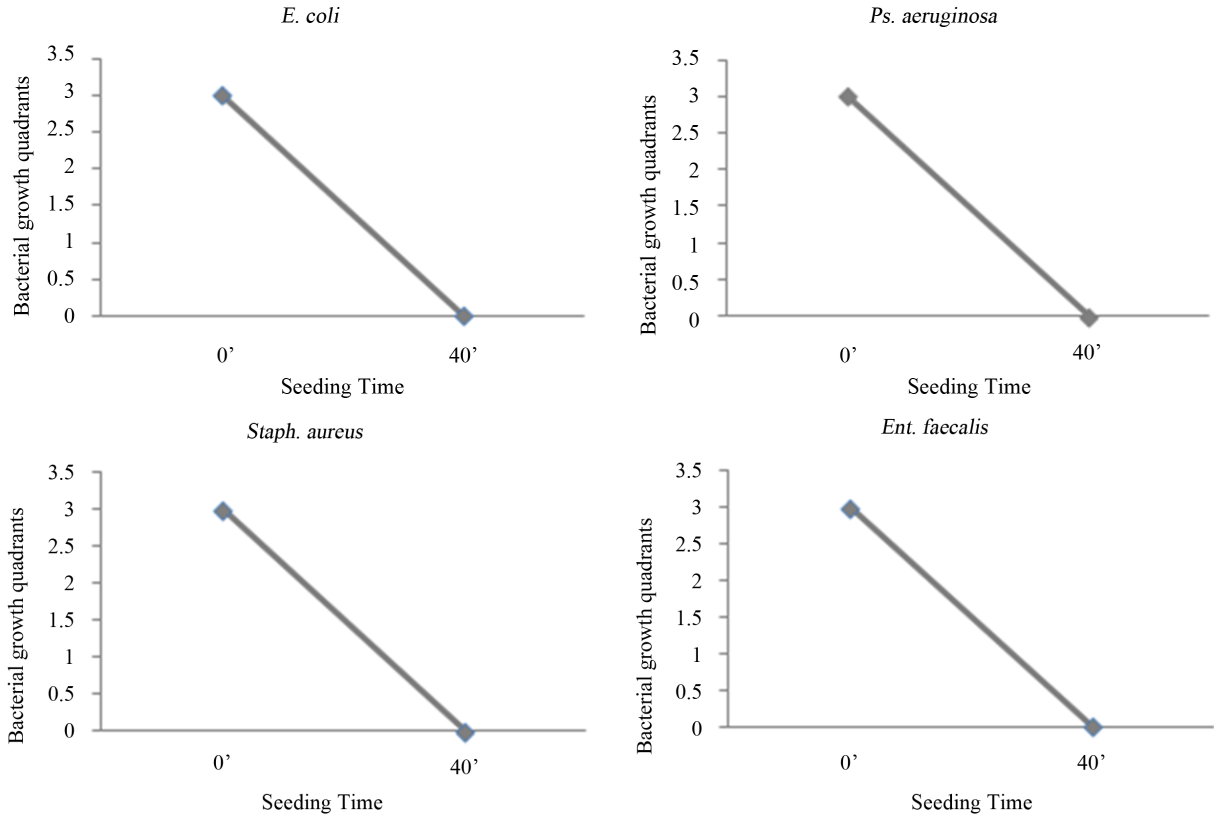

Figure 1. Experience on saline: seeding at T_0, with O_3 treatment, mechanical agitation for 40 minutes, and subsequent seeding at T_1. T_0 (0'); O_3 (0'); T_1 (40'). Ozone: 480 µg × 1. Total time of mechanical agitation: 40'.

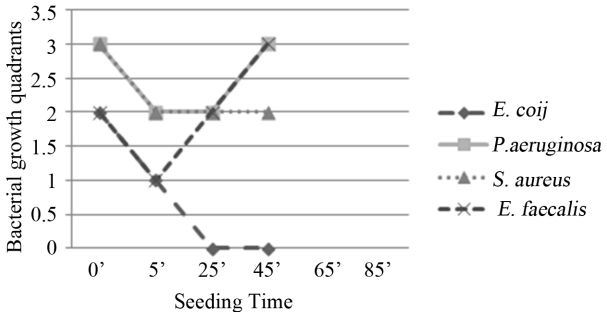

Figure 2. Experiment I on whole blood: seeding T_0, O_3, mechanical agitation for 5 minutes, seeding T_1, mechanical agitation for 20 minutes, seeding T_2, mechanical agitation for 20 minutes, seeding T_3. T_0 (0’); O_3 (0’); T_1 (5’); T_2 (25’); T_3 (45’). Ozone: 480 µg × 1. Total time of mechanical agitation: 45’.

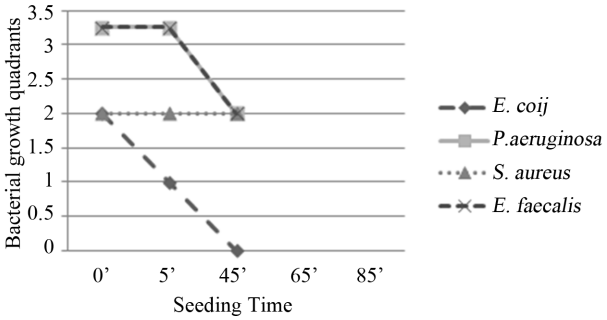

Figure 3. Experiment II on whole blood: seeding T_0, O_3, mechanical agitation for 5 minutes, seeding T_1, O_3, mechanical agitation for 40 minutes, seeding T_2. T_0 (0’); O_3 (0’); T_1 → O_3 (5’); T_2 (45’). Ozone: 480 µg × 2. Total time of mechanical agitation: 45’.

Staph. aureus and *Ent. faecalis* remained in the two quadrants in each seeding, while *Ps. aeruginosa* showed a growth reduction at T_1 (5’) and also remained in one quadrant at T_2 (45’). Bacteriostatic activity.

E. coli: reduction of bacterial growth at T_1 (5’) and total absence of colonies at T_2 (45’). Bactericidal activity (**Figure 4**).

Experiment IV - Ozone: 480 µg × 1; total time of mechanical agitation: 65’.

Staph. aureus and *Ps. aeruginosa*: a reduction of bacterial growth at T_2 (25’) which extended to T_3 (45’). Bacteriostatic activity.

Ent. faecalis: although a higher susceptibility to ozone than the first two bacteria was observed, these bacteria showed an evident resumption of growth at T_4 (65’).

E. coli: progressive reduction of the bacterial growth from T_0 to T_3 (45’). Bactericidal activity (**Figure 5**).

Experiment V - Ozone: 480 µg × 4; total time of mechanical agitation: 85’.

Staph. aureus and *Ent. faecalis*: reduction of bacterial growth for both bacteria, respectively at T_2 (25’) and T_1 (5’) with evident resumption at T_4 (85’).

P. aeruginosa and *E. coli*: showed a progressive reduction in bacterial growth from T_0 to T_3 (45’) which did not increase from T_3 (45’) to T_4 (85’). Bacteriostatic activity (**Figure 6**).

Experiment VI - Ozone: 480 µg × 2; total time of mechanical agitation: 65’.

Staph. aureus, *Ps. aeruginosa* and *Ent. faecalis*: further reduction of bacterial growth for all bacteria, respectively at T_2 (45’), T_1 (5’) and T_3 (65’), and progressive reduction of the bacterial growth in subsequent seedings. Bactericidal activity.

E. coli: evident reduction of bacterial growth at T_1 (5’) and total absence of colonies at T_2 (45’) and T_3 (65’). Bactericidal activity (**Figure 7**).

Experiment VII - Ozone: 480 µg × 4; total time of mechanical agitation: 80’.

The results for *Staph. aureus* and *Ps. aeruginosa* were similar: after an evident reduction at T_2, the bacterial growth resumed.

Ent. faecalis: bacterial growth was observed in three quadrants at T_0 and in two quadrants at T_1 (5’); the growth returned in three quadrants at T_2 (20’) and remained constant until the last seeding (T_4: 80’). Bacteriostatic activity.

E. coli: the evident reduction of bacterial growth at T_1 (5’) and total absence of colonies in subsequent seedings. Bactericidal activity (**Figure 8**).

The blood counts showed no significant changes.

The curves of the control bacterial growth showed a progressive increase in the bacterial growth.

4. Discussion

The study was divided into three phases, according to the suspension medium used for the bacterial cultures (saline, BHI and whole blood), to understand the interaction between the ozone, bacterium and the suspension medium. Indeed, the milieu in which microbes are present determines the effectiveness and outcome of the ozone treatment [39].

As shown in previous studies [7,11,16], we confirmed the ozone bactericidal activity on all bacterial strains when suspended in saline. Interestingly enough, ozone loses bactericidal activity with BHI suspension medium.

The results are interesting and discordant when the suspension medium is whole blood. Burgassi *et al.* observed that fresh plasma (as a biological substance with antioxidant systems), compromises ozone bactericidal activity.

Moreover, these authors suggested the incompatibility of using whole blood because it coagulates in the presence of a bacterial suspension [27].

To overcome this technical problem, we used tubes containing citrate, the same anticoagulant present in the bags for major ozone autohemotherapy (AHT-O_3).

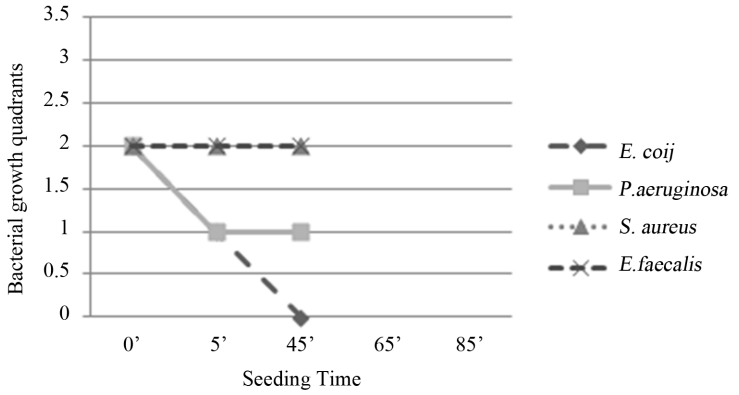

Figure 4. Experiment III on whole blood: seeding T_0, O_3, mechanical agitation for 5 minutes, seeding T_1, O_3, mechanical agitation for 20 minutes, O_3, mechanical agitation for 20 minutes, seeding T_2. T_0 (0’); O_3 (0’); T_1 → O_3 (5’); O_3 (25’); T_2 (45’). Ozone: 480 µg × 3. Total time of mechanical agitation: 45’.

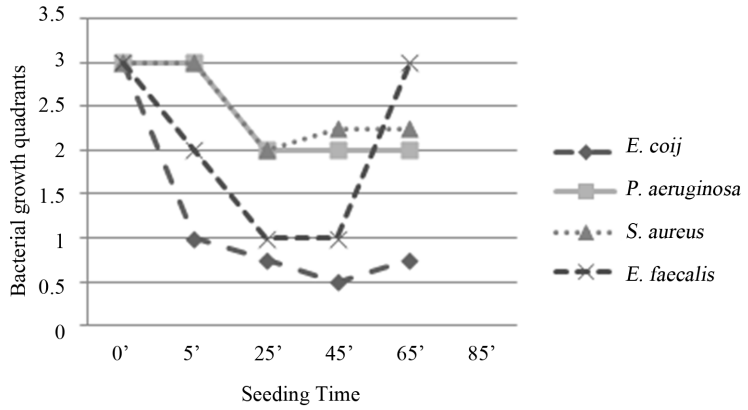

Figure 5. Experiment IV on whole blood: seeding T_0, O_3, mechanical agitation for 5 minutes, seeding T_1, mechanical agitation for 20 minutes, seeding T_2, mechanical agitation for 20 minutes, seeding T_3, mechanical agitation for 20 minutes, seeding T_4. T_0 (0’); O_3 (0’); T_1 (5’); T_2 (25’); T_3 (45’); T_4 (65’). Ozone: 480 µg × 1. Total time of mechanical agitation: 65’.

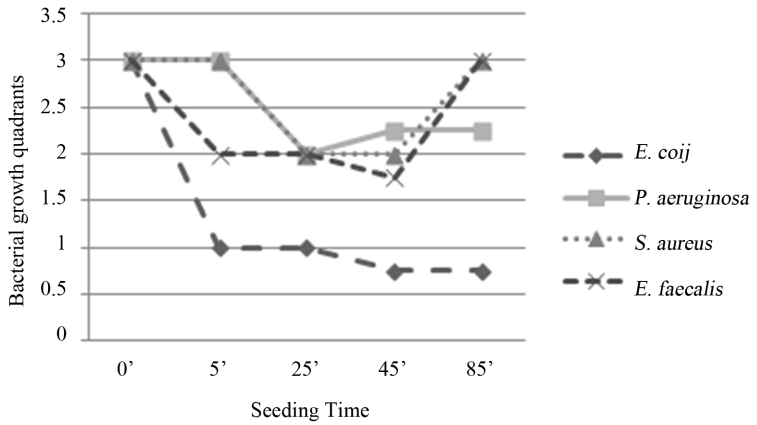

Figure 6. Experiment V on whole blood: seeding T_0, O_3, mechanical agitation for 5 minutes, seeding T_1, mechanical agitation for 20 minutes, seeding T_2, O_3, mechanical agitation for 20 minutes, seeding T_3, O_3, mechanical agitation for 20 minutes, O_3, mechanical agitation for 20 minutes, T_4. T_0 (0’); O_3 (0’); T_1 (5’); T_2 → O_3 (25’); T_3 → O_3 (45’); O_3 (65’); T_4 (85’). Ozone: 480 µg × 4. Total time of mechanical agitation: 85’.

Compared to the results reported in the literature, our study on whole blood shows that the single insufflation with an O_2/O_3 mixture is sufficient to achieve a bactericidal effect on *E. coli*. We also found that a second insufflation was essential for improving the results; specifically, the most encouraging results were obtained in ex-

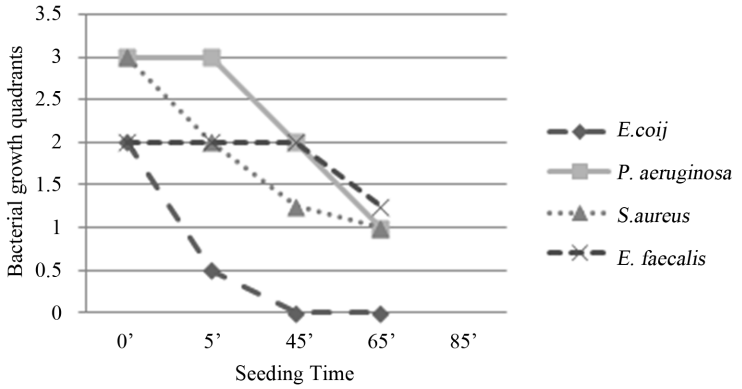

Figure 7. Experiment VI on whole blood: seeding T_0, O_3, mechanical agitation for 5 minutes, seeding T_1, mechanical agitation for 40 minutes, seeding T_2, O_3, mechanical agitation 20 minutes, seeding T_3. T_0 (0’); O_3 (0’); T_1 (5’); T_2 → O_3 (45’); T_3 (65’). Ozone: 480 µg × 2. Total time of mechanical agitation: 65’.

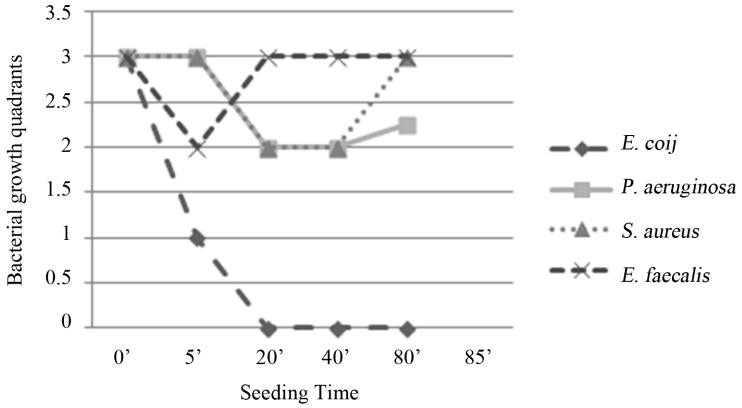

Figure 8. Experiment VII on whole blood: seeding T_0, O_3, mechanical agitation for 5 minutes, seeding T_1, O_3, mechanical agitation for 5 minutes, O_3, mechanical agitation for 5 minutes, O_3, mechanical agitation for 5 minutes, seeding T_2, mechanical agitation for 20 minutes, seeding T_3, mechanical agitation for 40 minutes, seeding T_4. T_0 (0’); O_3 (0’); T_1 (5’); O_3 (10’); O_3 (15’); O_3 (20’); T_3 (40’); T_4 (80’). Ozone: 480 µg × 4. Total time of mechanical agitation: 80’.

periment VI, where we performed a second insufflation at 45 minutes after the first. We observed that the bacterial growth typically resumed after approximately 40 minutes from the first exposure to the ozone oxidative insult, suggesting that the best results were obtained in experiment VI.

Although additional ozone insufflations prolonged bactericidal activity (*Staph. aureus*, *Ent. faecalis*, *Ps. aeruginosa*), the effects of these treatments were not significantly different from those obtained in the experiments with only two insufflations.

Based on the differential results obtained on whole blood between *E. coli* and *Ps. aeruginosa*, and the failure of the experiments on broth culture, suggested that the mechanism of ozone, contrary to the literature [19,20], could also have a metabolic basis. On the other hand, changes in the size and morphology of the colonies, observed in all the bacterial strains after exposure to ozone, confirm the oxidative mechanism.

The bactericidal activity on *E. coli*, contrasts with the increased effectiveness of ozone against Gram-positive bacteria [19]. The primary target of ozone on *E. coli* is the sulfhydryl group in the bacterial membrane [40]. Thus, we confirmed the correlation between ozone bactericidal activity and membrane permeability, which is specific for each microorganism [39].

The results of our study on whole blood represents an important confirmation of the ozone bactericidal activity in the topical treatment of wounds, and the daily persistence of topical ozone preparations kills even the most resistant bacteria [25,41].

The *in vitro* results were not consistent with the observations of AHT-O_3 *in vivo*, except for the portion of blood directly exposed to ozone. In fact, these results are consistent with those of Burgassi *et al.* [27], showing that the AHT enriched with ozone, even at highest ozone concentration (80 µg/ml), is not able to oxidize and destroy circulating bacteria in the blood.

However, the evaluation of the ozone bactericidal activity on whole blood performed in the present study in-

volves exclusively its direct action *in vitro*. While *in vivo*, AHT enriched with ozone induces a series of effects that might also be indirectly useful in the treatment of infections, such as: immune modulation [42-48], antioxidant systems activation [49-52] and improvement of the microcirculation [53-56].

To clarify the mechanism of ozone, it would be useful to examine its affects on other Enterobacteriaceae (suspension medium: whole blood), such as Serratia and Klebsiella, and Aspergillus. Indeed, Aspergillus produces large colonies whose variations in size or color might be an important index for metabolic alterations.

In conclusion, we confirmed the bactericidal efficacy of topical ozone preparations, according to the evidence of antibody-catalyzed ozone formation in bacterial killing [57], and suggest the need for further evaluations of the therapeutic potential of AHT-O_3.

5. Conclusion

Further *in vitro* investigation on whole blood might reveal the synergic action between traditional antibiotics and ozone treatment, and might provide the basis for developing successive studies *in vivo* in patients affected through multidrug resistant nosocomial infections.

Conflict of Interest

The authors declare that they have no conflict of interest, neither commercial nor financial, in any of the products described in this article.

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