Potent Activity of a High Concentration of Chemical Ozone against Antibiotic-Resistant Bacteria

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INTRODUCTION

Health care-associated infections (HAIs) are a significant public health problem worldwide, favoring multidrug-resistant (MDR) microorganisms. The SARS-CoV-2 infection was negatively associated with the increase in antimicrobial resistance, and the ESKAPE group had the most significant impact on HAIs. The inefficiency of antimicrobials against these pathogens is due to several resistance mechanisms, as a result, these pathogens can survive in the hospital environment for extended periods and be transported from one individual to another, thus spreading in the community and hospital. Considering the increasing prevalence of MDR microorganisms in hospitals, which has become a severe threat to public health, the need for safe and validated technologies capable of ensuring the disinfection of air environments, room surfaces, and sanitary materials has become evident against the current pandemic or future events. In this sense, the study of alternative methods and/or agents for disinfection and sanitization should receive special attention, and ozone (O3) can be a valid option with different objectives.

OBJECTIVE

The aim of the study was investigated the bactericidal effect of a high concentration of O3 gas on some reference and ESKAPE bacteria (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.).

MATERIALS E METHODS

- **Bacterial Strains:** Standard strains (*Staphylococcus aureus* (ATCC 6538), *Salmonella enterica* subsp. enterica serovar *choleraesuis* (ATCC 10708), *Escherichia coli* (ATCC 25922), and *Pseudomonas aeruginosa* (ATCC 15442)) were obtained from the American Type Culture Collection (ATCC). Representative MDR strains of the ESKAPE group were also used, with four clinical strains isolated from HAIs, which were: methicillin-resistant *S. aureus* (MRSA), carbapenemase-producing *K. pneumoniae* (KPC+), *A. baumanni*i PDR carrying the bla_{OXA-23} gene and representing one of the genotypes disseminated in Brazil (ST15/CC15), and an environmental strain of *P. aeruginosa* (XDR) from hospital effluent.
- **Ozone Generating and Monitoring:** The ozone generating equipment (SANITECH O3-80-Sanitization, Astech Serv. and Fabrication Ltd.a., Petrópolis, Brazil) is adjustable from 10 to 80 ppm, and the capacity to treat the room air up to 1000 m3 (not habitable) was used. The environmental concentration of O3 emitted was monitored and measured using two portable electrochemical ozone detection modules (model ZE14-O3).
- **Ozone Treatment:** The ozone generated was infused into two hermetically sealed containers, with a volume of approximately 1 m3 each. The plates inoculated with the different microorganisms were placed on each container's shelves. After closing the lid of each container, we started the exposure to ozone using only one SANITECH O3-80-Sanitization ozone generator, producing ozone at a concentration of 80 ppm (maximum). ATCC strains were exposed to ozone for 1, 10, 20, 30, and 40 min.
- **Cell Viability:** The cell viability was measured on a selected bacterial suspension of 10^5 CFU mL−1 after 40 min exposure to O3 based on previous results (cell count—CFU mL−1). Resazurin as metabolic indicators.
- **Scanning Electron Microscopy (SEM):** SEM visualizes morphological changes in the bacteria species. examined in a Jeol JSM 6390 (Tokyo, Japan) scanning electron microscope.

RESULTS / DISCUSSION

- **Monitoring of Ozone Concentration:** Monitoring the O3 concentration inside each container showed that the average ozone emission from the equipment (1 to 40 min) ranged from 21.1 ppm to 71.7 ppm, with the average of all measurements being 43.9 ppm. The mean ozone concentration in the 40 min time chosen for testing with the MDR strains was 30.8 ppm. The ambient temperature ranged from 22.5 ◦C to 24.3 ◦C, with an average of 23.4 ◦C. Regarding the relative humidity of the air, it went from 71.4% RH to 75.5% RH, with an average of 74.2% RH.
- **Ozone treatment:** The culture exposure at different times (1 to 40 min) with a high level of gaseous O3 was able to inhibit the in vitro growth of all bacterial strains tested with a statistically significant reduction in colony count compared to the control group (not treated with ozone).
- **Cell Viability:** Ozone treatment significantly reduced bacterial growth in *S. aureus* (MRSA), leading to an inhibition of about 99.6%, followed by *P. aeruginosa* XDR (29.2%) (Figure 1). No difference was found in bacterial viability after ozone treatment in strains of S. aureus (ATCC 6538), P. aeruginosa (ATCC 15442), S. enterica (ATCC 10708), E. coli (ATCC 25922), *A. bau*mannii (PDR), and *K. pneumoniae* (KPC+).

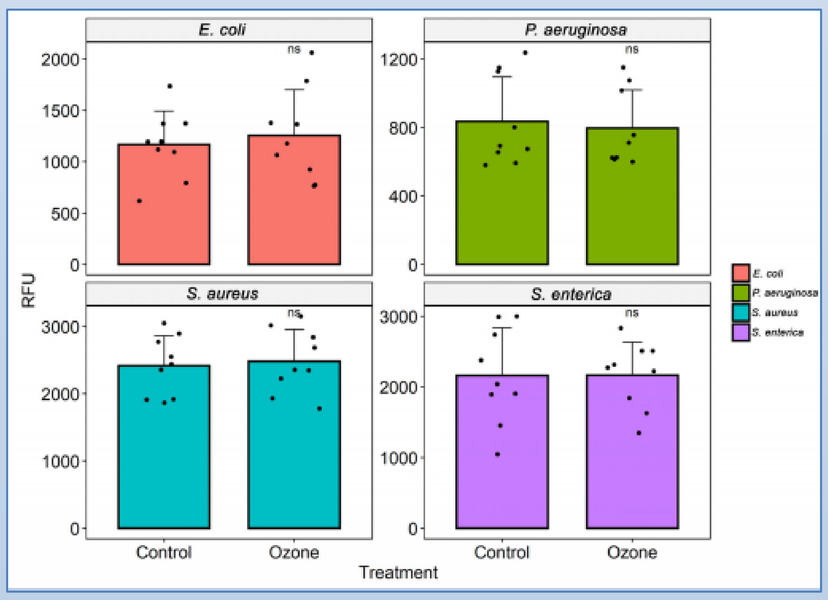

Figure 1. Analysis of cell viability after ozone treatment in different bacterial strains (S. aureus (ATCC 6538), S. enterica (ATCC 10708), E. coli (ATCC 25922), and P. aeruginosa (ATCC 15442)). The measurement of fluorescence intensity (relative fluorescence units, RFU) after the conversion of resazurin to resofurin by viable bacteria was performed in the control group (no treatment) and bacterial suspensions (105 CFU/mL) after exposure to ozone for 40 min. Results represent values from 3 randomly chosen colonies in the control group (no treatment) and after treatment with ozone. The black dots represent the values of fluorescence emission after addition of resazurin.

- **Scanning Electron Microscopy (SEM):** Scanning electron microscopy was performed to confirm membrane damage to bacterial species. Morphological analysis showed that *S. aureus* (MRSA) and *P. aeruginosa* (XDR) present membrane alterations after O3 treatment. All bacterial controls showed smooth and homogeneous surfaces. The therapy produced some cell wall protrusions (Figure 2).

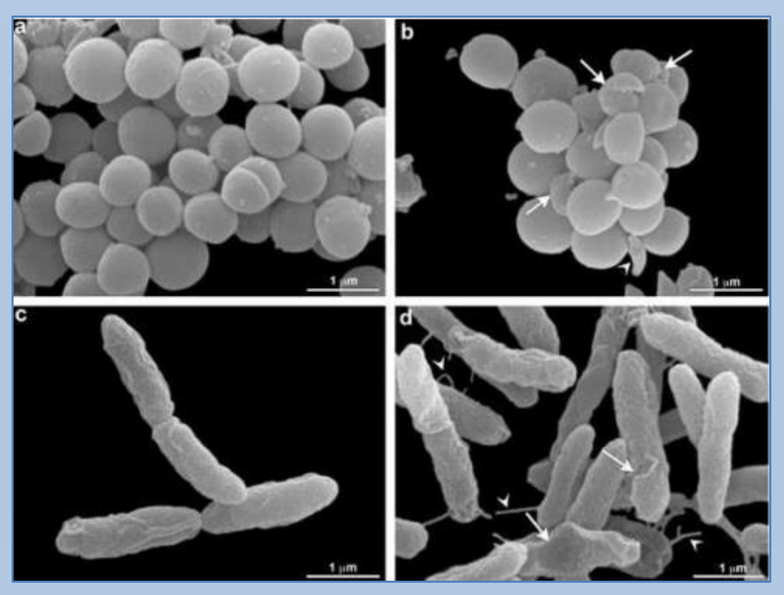

Figure 2. Morphological analysis of O3 treatment by electron microscopy. *S. a*ureus (MRSA) (a,b) and *P. aeruginosa* (XDR) (c,d) are seen without (a,c) and under O3 treatment (b,d). An alteration in *S. aureus* (MRSA) shape is seen (arrowhead) in b. Damage in bacteria is observed after treatment (arrows) (b). Note that *control cells are rounded and present in a homogeneous surface (a). Damaged cells are observed after treatment in* P. aeruginosa (XDR) (arrows) (d). Some cell wall protrusions are observed in treated cells (arrowhead) (d). These aspects were not verified in control cells (c).

CONCLUSION

Our results evidenced the antimicrobial potential of gaseous ozone in bacteria that are currently a significant problem worldwide. In the future, this resource may be a part of the protocol for the disinfection of hospital environments and surfaces, ensuring the control of microbial development.

Apoio: CAPES/CDTS/INCQS/Fiocruz.