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Original Article

Inhibition Effect of Ozone on Resistant Clinical Isolates

Özgür Yanılmaz, Burak Aksu

Department of Microbiology Laboratory, Marmara University Pendik Training and Research Hospital, İstanbul, Turkey

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Correspondence: Özgür Yanılmaz, Marmara Üniversitesi Pendik Eğitim ve Araştırma Hastanesi, Mikrobiyoloji Laboratuvarı, İstanbul, Turkey
E-mail: dryanilmaz@gmail.com

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ABSTRACT

Objective: Nowadays, the treatment of infections caused by hospital-acquired resistant bacteria has become very difficult. It is known that hospital infections can be limited and kept to a minimum with the use of appropriate sterilization–disinfection methods. In our study, we aimed to investigate the inhibition effect of ozone—which is low cost, has a nontoxic effect on humans, and does not leave chemical residues and wastes—on resistant microorganisms that cause nosocomial infections.

Methods: In this study, 80 strains of bacteria with various resistance patterns and isolated as a causative agent of nosocomial infection were included. Ten strains of each bacterium—MRSA, VRE, MDR *Pseudomonas aeruginosa*, ESBL (+) *Klebsiella pneumoniae*, carbapenemase (+) *K. pneumoniae*, colistin-resistant *K. pneumoniae*, colistin-resistant *Acinetobacter baumannii* complex, and colistin sensitive *A. baumannii* complex—were used. One liter of sterile distilled water (DW) was saturated with ozone for 1 h. A quantity of 0.1 mL of bacterial suspension was added onto 9.9 mL ozonated DW (final bacterial concentration 10^6 cfu/mL). From the suspensions kept at room temperature, samples were inoculated as a count plate on sheep blood agar with a 10 µL calibrated loop at 10 and 30 min. After 24 h of incubation, the number of growing colonies was calculated by evaluating the Petri dishes.

Results: The bacterial inhibition rates of ozonated water at 10 and 30 min exposure times were detected as 97.29–100% for Gram-positive nosocomial-resistant pathogens and 94.76–99.99% for Gram-negative nosocomial-resistant pathogens, respectively.

Conclusion: It has been determined that ozonated water can provide a very high antibacterial effect *in vitro* at a very low cost. In other studies, the antiviral activity of ozone, including SARS-CoV-2, has also been shown. The data we obtained suggest that ozone can be used in various disinfection–sterilization processes in hospitals. We believe that a cost-effective solution can be produced by supporting such studies with clinical research.

INTRODUCTION

Nowadays, the treatment of infections caused by hospital-acquired resistant bacteria has become very difficult. Bacteria have rapidly developed resistance against many antibacterial agents available for use. Both the low number of new antibacterial agents and the efforts to reduce costs have led to the search for new methods that can prevent nosocomial infections. It is known that nosocomial infections can be limited and kept to a minimum with the use of appropriate sterilization–disinfection methods. Many effective sterilants–disinfectants that are effective on microorganisms are used for this purpose.

Many published studies reported that ozone (O_3) gas is a highly oxidizing molecule with significant microbicidal potential, which can be used as an effective, practical, fast, and cheap disinfection method. Ozone is a compound made of three oxygen atoms. In nature, ozone occurs when sunlight, ultraviolet, or electrical charges affect oxygen. Ozone is a bioactive oxidizing disinfectant that breaks down into O_2 and O_1, a reactive molecule that disrupts the bacterial cell wall and the function of proteins and carbohydrates.[1,2] Ozone has strong bactericidal and sporicidal effects but is not harmful to the environment. It is a gas that dissolves quickly in water. It has short stability and very strong oxidation and disinfection ability in parallel. The disinfectant effect of ozone is approximately 2000 times higher compared with chlorine, and it loses its effect by converting to oxygen in a period usually measured in minutes and does not leave any residue.[3–5] Ozone has been proven to destroy microorganisms through progressive oxidation of vital cellular components including proteins and peptidoglycans in the cell wall, enzymes and nucleic acids in the cytoplasm, and unsaturated lipids in cell membranes.[6] In detail, ozone can react with polysaccharides and break glycosidic bonds; it can oxidize sulfhydryl groups, amino acids of proteins and peptides, and polyunsaturated fatty acids to acid peroxides.[7,8] These changes cause the cell envelope to deteriorate or break, causing cell contents to leak out and eventually cell lysis.[9] Membrane degradation causes ozone to penetrate easily into the cell, react with DNA and RNA (especially thymine, guanine, and uracil), and damage nucleic acids.[10] In addition, ozone decomposition in water creates free radicals such as hydroperoxyl (HO_2), hydroxyl (OH), and superoxide (O_{2-}).[8,10]

Ozone application to contaminants selected for surface disinfection has been found to have significant disinfectant effects in a linear relationship with the amount of ozone (2–150 mg) and the exposure time to this dose.[11]

Ozone has applications in gas and liquid forms for disinfection purposes. Both water-based and gaseous applications of ozone are used in the food industry for disinfection and sanitation of water systems. In addition, studies are being made to degrade biofilms created by pathogenic organisms in periodontitis.[1,12] As ozone is a disinfectant in the form of gas, it is effective on both airborne and microbial loads on surfaces. Gaseous O_3 is easily soluble in water and highly oxidative. Its oxidative capacity is higher than that of hydrogen peroxide and peracetic acid. This feature, together with its solubility, makes it an excellent candidate for use as a sterilizer.[13]

It has been determined that many bacteria, which are fatal in terms of public health, show sensitivity to ozone application on plastic, cotton, fabric, and cardboard surfaces.[14] Ozone is used and recommended in many applications for surface disinfection and sanitation of foodstuffs in the food industry.[15] Ozone should be used where it is produced because it has a short half-life.[16]

In our study, we aimed to investigate the antimicrobial effect of ozone gas—which is low cost, has a nontoxic effect on humans, and does not leave chemical residues and waste—on resistant microorganisms that cause nosocomial infection.

MATERIALS AND METHODS

In our study, the Ozone Generator L-450 device (Jiangmen Headita Machinery, China), which produces 450 mg ozone per hour, was used. Eighty strains of nosocomial infections with various resistance patterns, isolated in Marmara University Pendik Training and Research Hospital and stored in a deep freezer, were included in the study. Ten strains of each bacterium—MRSA, VRE, MDR *P. aeruginosa*, ESBL (+) *K. pneumoniae*, carbapenemase (+) *K. pneumoniae*, colistin-resistant *K. pneumoniae*, colistin-resistant *A. baumannii* complex, and colistin sensitive *A. baumannii* complex—were used.

All bacteria were made ready for the study by passage from stocks two times on sheep blood agar medium (bioMérieux, France). Then, 0.5 McFarland turbidity (1×10^8 cfu/mL) suspensions of all bacteria were prepared from the second subcultures made. One liter of sterile distilled water (DW) was saturated with ozone for 1 h. Then, 0.1 mL of bacterial suspension was added to 9.9 mL ozonated DW (final concentration 10^6 kob/mL). From the suspensions kept at room temperature, inoculation was made on sheep blood agar medium as a count plate with a calibrated 10 µL loop at 10th and 30th minutes. In addition, the same DW without being saturated with ozone was inoculated as 10 µL sample on sheep blood agar for negative control. All bacterial suspensions were diluted 1/100 and 10 µL samples of each inoculated on sheep blood agar for positive control of growth. The number of growing colonies was calculated by evaluating all Petri dishes after 24 h of incubation at 37°C.

Statistical analyses were performed using Mann–Whitney U test to evaluate the effects of ozone exposure on Gram-positive and Gram-negative bacteria. Statistical analyses were performed using SPSS version 20.0 (SPSS, Inc., Chicago, IL, USA). A value of $p \le 0.05$ was considered significant.

RESULTS

The inhibition rate detected in Gram-positive bacteria was higher than the rate in Gram-negative bacteria at both the 10th and 30th minutes (for 10th minute, $p<0.046$; for 30th minute, $p<0.015$). While the inhibition rate for Gram-positive bacteria at the 10th minute was 98.52%, it was determined that ozone inhibited all Gram-positive bacteria at the 30th minute. The inhibition rates for Gram-negative bacteria were determined as 95.64% and 99.98% at the 10th and 30th minutes, respectively. When we evaluated the bacteria groups separately, among all the bacteria studied, the most prominent inhibition was identified as MRSA. Post-incubation bacterial inhibition was 99.76% at 10th minute and 100% at 30th minute for MRSA. Inhibition rates for VRE were 97.29% and 100% at the 10th and 30th minutes, respectively. When Gram-negative bacteria groups were examined, it was seen that inhibition rates were similar. For *P. aeruginosa*, the inhibition rate at the 10th minute was 97.82%, while the inhibition rate at the 30th minute was 99.99%. Inhibition rates for *K. pneumoniae* at the 10th and 30th minutes, respectively, were 94.96% and 99.98% in ESBL (+) patients, 94.76% and 99.91% in patients with carbapenemase (+), and 94.89% and 99.99% in patients with colistin resistance. Inhibition rates for *A. baumannii complex* at the 10th and 30th minutes were 96.40% and 99.99% in colistin-resistant isolates, 94.99% and 99.99% in colistin sensitive isolates, respectively. It was observed that the inhibition rates for all bacteria were 96.36% at the 10th minute and 99.98% at the 30th minute (Table 1).

DISCUSSION

Bacteria respond to antibacterial molecules developed by scientists as a result of long and exhausting studies with various resistance mechanisms established in a very short time. While inappropriate/insufficient use of antibiotics may be the biggest cause of this problem, exposure to antibacterial agents is also one of the factors that trigger the

Table 1. Antibacterial efficacy results obtained with ozonated water applied at different times

Bacteria	Growth inhibition (%)	
	10. minute (±Standard deviation)	30. minute (±Standard deviation)
Methicillin-resistant *Staphylococcus aureus*	99.76 (±0.38)	100 (±0.00)
Vancomycin-Resistant *Enterococci*	97.29 (±3.91)	100 (±0.00)
Multidrug-Resistant *P. aeruginosa*	97.82 (±3.22)	99.99 (±0.01)
Extended spectrum betalactamases (+) *K. pneumoniae*	94.96 (±4.96)	99.98 (±0.05)
Carbapenemase (+) *K. pneumoniae*	94.76 (±4.77)	99.91 (±0.19)
Colistin resistant *K. pneumoniae*	94.89 (±4.90)	99.99 (±0.01)
Colistin resistant *A. baumannii complex*	96.40 (±4.43)	99.99 (±0.01
Colistin susceptible *A. baumannii complex*	94.99 (±4.32)	99.99 (±0.02)
Gram (+) bacteria	98.52 (±3.04)	100 (±0.00)
Gram (-) bacteria	95.64 (±4.61)	99.98 (±0.09)
All bacteria	96.36 (±4.45)	99.98 (±0.08)

development of resistance. In particular, resistance development is much higher in hospital-acquired infections than in community-acquired infections. It is possible to prevent nosocomial infections with appropriate disinfection–sterilization methods. Therefore, the use of antibiotics due to nosocomial infection can be minimized; thus, cost and labor can be minimized by reducing the length of stay. Therefore, low-cost disinfection–sterilization applications that do not compromise efficiency gain great importance.

In their study, Tuncay et al.[17] investigated the effect of various disinfection methods on *Enterococcus faecalis*, which is one of the bacteria that cause endodontic treatment failure by forming biofilms in teeth. Although they determined the NaOCl method as the most effective method, they found disinfection with ozone as effective as photoactive disinfection. Therefore, they stated that it can be used as a washing liquid at the end of dental canal treatments. Ximenes et al.[18] studied the antibacterial effect of ozone on *Streptococcus mutans*, *Lactobacillus acidophilus*, and *E. faecalis*, which also cause dental caries, and showed that ozone at a concentration of 20 ppm caused 3-log reduction on all three bacteria. In our study, the effectiveness of ozone against VRE, which is a resistant nosocomial infection agent, was found to be 97.29% at the 10th minute and 100% at the 30th minute.

Lopes et al.[19] investigated the disinfectability of slotted cannulas with ozone in patients with mechanical ventilation with a tracheostomy. Gram-negative bacteria were used in this study because they were more isolated as an agent than other bacteria in this hospital. In the study, it was found that ozone application caused a 5-log reduction in bacterial load, and it was concluded that it could be used in the disinfection of semi-critical materials. In accordance with this study, we found ozone to be very effective in Gram-negative bacteria despite their resistant hospital origins. Ozone caused an approximate 4-log reduction on Gram-negative bacteria.

In their study, Martinelli et al.[1] investigated the effect of ozonated water on some microorganisms. After 20 min of ozonization, they found 98.9% decrease in *Staphylococcus aureus*, which is also in our study list, and this decrease remained at 57.4% in *P. aeruginosa*. Although our *S. aureus* results were consistent, *P. aeruginosa* inhibition was detected at much higher rates in our study. The more interesting result is that the reduction in *Escherichia coli* used in this study remained at 26.4%. As we included resistant strains isolated from the hospital in our study, we did not study *E. coli*, but the value found does not seem to be compatible with other studies in the literature. Venta et al.[20] determined that tomatoes, which were artificially inoculated with *E. coli*, successfully disinfected the bacteria when exposed to ozonated water at a dose of 0.5 and 1.0 mg/L for 15–30 min. Habibi-Najafi and Haddad-Khodaparast[21] found that the coliforms on the surface of cold-stored fresh date palm fruits were completely destroyed by applying ozone in gas form at a dose of 5 ppm for 60 min.

In their study on bacterial agents commonly detected in cystic fibrosis patients, Dana Towle et al.[22] found that all agents, including the mucoid *P. aeruginosa* species, could be completely killed by ozone, depending on the exposure time. Our data also seem to be compatible with this study.

In the study conducted by Breidablik et al.[23] on nursing students' hand disinfection, the effectiveness of water contaminated with *E. coli* ATCC 25922 and ozonated as an alternative to alcohol-based hand disinfectant was investigated. As a result of the study, it was determined that the disinfectant efficiency of ozonated water was higher. In addition, no skin reaction was found with ozonated water. This study cannot give information about other microorganisms. Our research supports this study because it includes many resistant bacteria.

In their study, Martins et al.[24] found that when SARS-CoV-2 is exposed to ozonated water for 1 min, a 2-log decrease in the number of viruses occurs. In addition, Alimohammadi and Naderi stated that ozone gas has an inactivation effect on SARS-CoV-2, which has sulfhydryl groups rich in cystine.[25] Thus, the disinfectant effect of ozonated water has been detected on viruses as well as bacteria and fungi.

The share of health expenditures in state budgets is gradually increasing. Antibacterial agents make up a significant portion of this budget. In hospital-acquired infections, the duration of antibiotic use is quite prolonged, and wide-spectrum antibiotics are used due to high resistance. Nosocomial infections increase both cost and labor loss. Prevention of nosocomial infections should be the goal instead of treating them after they occur. Various chemicals used in disinfection–sterilization applications in hospitals have various disadvantages such as cost, toxicity, waste, and contribution to resistance development. It has been determined that ozonated water we used in our study can provide a very high antibacterial effect *in vitro* at a very low cost. In other studies, the antiviral activity of ozone including SARS-CoV-2 has also been shown. In addition, ozone is an environmentally friendly product that breaks down quickly after showing its effect and does not leave chemical residues. The data we obtained suggest that ozone can be used in various disinfection–sterilization processes in hospitals. We believe that a cost-effective solution can be produced by supporting such studies with clinical research.

Ethics Committee Approval

This study approved by the Marmara University Faculty of Medicine Clinical Research Ethics Committee (Date: 08.01.2021, Decision No: 09.2021.45).

Informed Consent

Retrospective study.

Peer-review

Internally peer-reviewed.

Authorship Contributions

Concept: Ö.Y., B.A.; Design: Ö.Y., B.A.; Supervision: Ö.Y., B.A.; Fundings: Ö.Y.; Materials: Ö.Y., B.A.; Data: Ö.Y.; Analysis: Ö.Y., B.A.; Literature search: Ö.Y., B.A.; Writing: Ö.Y.; Critical revision: Ö.Y., B.A.

Conflict of Interest

None declared.

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Dirençli Klinik İzolatlar Üzerinde Ozonun İnhibisyon Etkisi

Amaç: Günümüzde hastane kaynaklı dirençli bakterilerin neden olduğu enfeksiyonların tedavisi çok zor hale gelmiştir. Uygun sterilizasyon-dezenfeksiyon yöntemlerinin kullanılmasıyla hastane enfeksiyonlarının sınırlandırılabileceği ve minimumda tutulabileceği bilinmektedir. Çalışmamızda düşük maliyetli ve toksik olmayan, kimyasal kalıntı ve atık bırakmayan ozon gazının nozokomiyal enfeksiyonlara neden olan dirençli mikroorganizmalar üzerindeki inhibisyon etkisini araştırmayı amaçladık.

Gereç ve Yöntem: Çalışmamıza 80 adet çeşitli direnç paternine sahip, hastane enfeksiyonu etkeni olan bakteri kökeni dahil edildi. Her bir bakteriden onar adet olmak üzere; MRSA, VRE, MDR *P. aeruginosa*, ESBL (+) *K. pneumoniae*, karbapenemaz(+) *K. pneumoniae*, kolistin dirençli *K. pneumoniae*, kolistin dirençli *A. baumannii* complex ve kolistin hassas *A. baumannii* complex kullanıldı. Bir litre steril distile su (DS) bir saat boyunca ozon ile doyuruldu. 9.9 mL ozonlanmış DS üstüne 0.1 mL bakteri süspansiyonu eklendi (son bakteri konsantrasyonu 10^6 KOB/ml). Oda sıcaklığında bekletilen süspansiyonlardan 10. ve 30. dakikalarda 10 µL'lik kalibre öze ile koyun kanlı agara sayım plağı şeklinde ekim yapıldı. Yirmi dört saatlik inkübasyon sonrasında petriler değerlendirilerek üreyen koloni sayısı hesaplandı.

Bulgular: Ozonlanmış su ile 10. ve 30. dakikada elde edilen bakteri inhibisyon oranları sırasıyla Gram pozitif nozokomiyal dirençli patojenler için %97.29 ve %100; Gram negatif nozokomiyal dirençli patojenler için ise %94.76 ve %99.99 olarak saptandı.

Sonuç: Çalışmamızda kullandığımız ozonlanmış suyun *in vitro* olarak çok düşük bir maliyetle çok yüksek antibakteriyel etki sağlayabildiği tespit edilmiştir. Diğer çalışmalarda, SARS-CoV-2 dahil ozonun antiviral aktivitesi de gösterilmiştir. Elde ettiğimiz veriler, ozonun hastanelerde çeşitli dezenfeksiyon-sterilizasyon işlemlerinde kullanılabileceğini göstermektedir. Bu tür çalışmaların klinik araştırmalarla desteklenerek uygun maliyetli/etkin bir çözüm üretilebileceğine inanıyoruz.

Anahtar Sözcükler: Bakteri; dezenfeksiyon; inhibisyon; ozon.