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NEÜ YAYINEVİ

Could Ozone Threapy be a Novel Strategy for Combating with Multi-Drug Resistant Bacteria?

Ozon Tedavisi Çoklu İlaca Dirençli Bakterilerle Mücadelede Yeni Bir Strateji Olabilir mi?

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Öz

Amaç: Çoklu ilaca dirençli (MDR) bakteriler ile oluşan enfeksiyonları kontrol etmek, klinisyenler için büyük bir zorluk haline geldi. Bu nedenle, ana yaklaşım alternatif tedavilerin de gözden geçirilmesidir. Bu çalışmada, gaz formundaki medikal ozonun MDR patojenler üzerindeki antibakteriyel etkisini zamana bağlı olarak değerlendirilmesi hedeflenmiştir.
Gereçler ve Yöntem: Çalışma 21 Mart-15 Nisan 2021 tarihleri arasında Necmettin Erbakan Üniversitesi Meram Tıp Fakültesi Hastanesi Tıbbi Mikrobiyoloji laboratuvarı'nda gerçekleştirildi. On MDR bakteri, yedi farklı bakteriyel konsantrasyonunda (102-108 bakteri/petri) petri kaplarında in vitro olarak değerlendirildi. 10, 20 ve 40 dakikalık 40 ug/ml dozda ozon maruziyeti ve kontrol grubunun MDR bakterileri üzerinde etkinliğini araştırıldı. Plakların 24 saatlik inkübasyonundan sonra, ozonun her maruz kalma süresi için ortalama koloni sayıları belirlendi ve Log10'a dönüştürüldü.
Bulgular: Ortalama bakteriyel azalma (log10) dikkate alındığında, ozonun bakterisidal etkisi 10 dakikaklık maruziyette sadece metisiline dirençli Staphylococcus aureus, VIM-1 üreten Klebsiella pneumoniae ve Karbapenem dirençli Acinetobacter baumannii üzerinde saptandı. Optimum etki, test edilen izolatlarda genellikle 20 dakika içinde gözlendi. Bununla birlikte, MDR-Pseudomonas aeruginosa, ozon maruziyetinden nispeten daha az etkilendi. Ancak 40 dakikalık maruziyet sonunda tüm bakteriler %99'luk seviyenin üzerinde inaktive edildi.
Sonuçlar: Ozon gazı, MDR patojenleri üzerinde etkili bakterisidal aktivite gösterdi ve etkisinin, maruz kalma süresine ve bakteri tipine bağlı olduğu tespit edildi. Pandemi dünyasında mikrobiyal enfeksiyonların kontrolü için yeni yaklaşımlara duyulan ihtiyaç dikkate alındığında, ozon tedavisinin optimizasyonu yüksek öncelikli olarak gerçekleştirilmelidir ve ozonun MDR-bakterilerin inaktivasyonu üzerindeki etkisinin derinlemesine anlaşılmasını desteklemek için daha fazla in vivo çalışmaya ihtiyaç vardır.

Anahtar Kelimeler: Ozon terapi, çoklu ilaca dirençli bakteri, tamamlayıcı tıp

Abstract

Aim: This study evaluated the antibacterial effect of gaseous ozone on multi-drug resistant (MDR) pathogens with regard to time dependency. Controlling infections with MDR bacteria became a big challenge for health care professionals. Therefore, the major corcern should be altered to alternative therapies.
Materials and Methods: The study was performed in Necmettin Erbakan University Meram Faculty of Medicine Hospital, Department of Medical Microbiology Laboratory between 21 March to 15 April 2021. Ten MDR bacteria were evaluated in vitro using in the Petri dishes at seven bacterial concentrations (10^2-10^8 bacteria/dish). The ozone showed its efficacy on these MDR bacteria under the following conditions applied: 40 µg/ml at 10, 20 and 40 minutes and control (no gas was used). After 24 hours incubation of plates, the average colony counts for each exposure time of ozone were figured out and transformed to Log10.
Results: Taking acccount the mean bacterial reduction (log10), the bactericidal effect of ozone was determined on only methicillin-resistant *Staphylococcus aureus*, VIM-1 producing *Klebsiella pneumoniae* and Carbapenem resistant-*Acinetobacter baumannii* in 10 minutes exposure. The optimum effect was generally observed within 20 minutes on tested isolates. However, the MDR-*Pseudomonas aeruginosa* showed a relatively lower response to ozone. All bacteria was inactivated over the level at 99% at the end of the 40 min exposure to ozone.
Conclusions: The gaseous ozone showed satisfactory bactericidal activity on MDR pathogens and its effect depens on exposure time and type of bacteria. Taken into account, the need for new approaches for the control of microbial infections in the pandemic world, the optimization of ozone therapy should be undertaken high priority and more in vivo studies are needed to support in depth understanding of the ozone effect on the inactivation of MDR bacteria.

Key words: Ozone therapy, multi-drug resistant bacteria, complementary medicine.

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INTRODUCTION

Antimicrobial Resistance (AMR) is one of the most critical public health threats of the 21st century as well as it continues being a significant burden for world economy (1,2). However, it is now likely to be hidden by coronavirus disease 2019 (COVID-19) pandemic for some time (1,3). Since the beginning of the antibiotic era, that were considered as novel drugs and saved millions of lives (1,2). Nevertheless, the prolonged and inappropriate use of antimicrobials cause a selective pressure on microorganisms and driving of bacterial resistance (4). The rapid emerge and dissemination of antibiotic resistant pathogens lead to failure in clinical outcomes and also associate with high morbidity and mortality, particulary, in hospital acquired infections (2,5). It was estimated that by 2050, AMR-related deaths would access 10 million (6).

As long as the current pandemic, there are probable threats that could pressure on antimicrobial stewardship policies and cause AMR (5). In the COVID-19 pandemic, it is of vital importance to recover lives of coronavirus disease 2019 patients although this signify appeal to common overmedicate of extensive-spectrum initial antibiotics for threapy or protection of complications such as secondary bacterial infections (5,7). Nevertheless, the extensive use of antibiotics (80%-100%) and antifungals (7.5%-15%) in severe ill COVID-19 patients accepted to intensive care units have been reported by several studies (7,8). It is worried that present mistakes and excesses could increase the advance of the eventual global public health problem by resistance of a great diversity of pathogens to a widespectrum of antimicrobials (5).

Similiar to COVID-19, AMR has been reportedly defined as a significant treat to global public health that “knows no bordes”. Therefore, it is likely to become the current crisis facing all countries across the world (3). In fact, with regard to many specialist, included those from from the World Health Organization, people are now in the verge of post antibiotic era. For this reason, the global neglected issue of AMR requires urgent action and attention (5,9). There is hence a increasing need for both the discovery of new classes of antibiotics, the development of alternative and natural products with pharmacoogical properties to defeat antibiotic resistant bacteria (2,5). Furthermore, once a new drug introduced to the clinic, antibiotic resistance can emerge rapidly by way of intense selective pressure soon after introduction (2,4). In additon, the long term treatment which chemical antimicrobials may have side effect such as nephrotoxicity and neurotoxicity (2,4).

One of the alternative treatment option is ozone threapy. Ozone (O_3) is an unstable triatomic from of oxygen which rapidly convert into water releasing a reactive form of oxygen (11). Ozone has been used for a long time for its antioxidant features, antimicrobial activities as well as its benefical effects on rapid tissue and wound healing (10). In many previous study have also shown that ozone has antibacterial, antiparasitic, fungacidal activities (10-13). For this purpose, in ozone threapy, oxigen-ozone (O_2-O_3) gas mixture called as “medical ozone” (5% ozone in 95% oxygen) has been increasingly utilizied for severe or cronic soft tissue and skin infections as a complementary treatment (14). So that, gaseous ozone (O_3) may be a favorable option in the threapy of infections induced by multi-drug resistant microorganisms and that a compounded treatment has the possible to extend the life of convenient antibiotics as well as to decrease the side effects of chemical antimicrobials depending on intensive usage.

It was aimed to investigate the effectiveness of gaseous ozone on various MDR clinical and reference bacterial strains according to diffirent time interval that need to be optimisation and also highlight the use of local ozone application as the therapeutical alternative to cure infections with MDR pathogens in the present study

MATERIALS AND METHODS

The study was performed in Necmettin Erbakan University Meram Faculty of Medicine, Department of Medical Microbiology Laboratory between 21 March to 15 April 2021. The nature of the study was a prospective, experimental research.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Institution (decision number: 2021/3161).

Bacteria cultures and growth conditions

Antibacterial effect is evaluated on three MDR clinical isolates *(Acinetobacter baumannii, Pseudomonas aeruginosa* and *Klebsiella pneumoniae)* and seven reference bacterial strains. Of the six Gram negative strains (OXA-48 producing *Klebisella pneumoniae* (NCTC 13442), VIM-1 producing *K. pneumoniae* (NCTC 13440), KPC producing *K. pneumoniae* (CCUG 56233), NDM-1 producing *K. pneumoniae* (NCTC 13443), IMP producing *E. coli* (NCTC 13476), mcr-1 producing *E. coli* (NCTC 13846) and one

Gram positive strain (MRSA, ATCC BAA-1720) were provided from the bacteriology culture collection of the our Laboratory of Microbiology. The identification of the clinical isolates and antibiotic susceptibility testing were applied via conventional technicals and automated system (Vitek 2, bioMerieux, Marcy lâ€™Etoile, France).

Plating method; All isolates were plated onto blood agar (bioMerieux, Marcy lâ€™Etoile, France) and incubated in aerobic conditions at 37 °C for 24 hours, then suspended in distilled water. Bacteria suspensions were adjusted to 0.5 McFarland standard turbidity corresponding to approximately 1-2 × 10^8 colony forming units (CFUml^{-1}) via a densitometer (DENSICHEK® PLUS, bioM erieux, Marcy lâ€™Etoile, France). Prepared decrimal serial dilutions are transferred to sterile U tubes with a final concentration equaling to 1×10^2 CFU ml^{-1} The sample taken from each of the tubes was inoculated onto blood agar and then the inoculum was spread on each plate surface.

Ozone application

A commercially available ozone (O_3) generating system device (Hyper-Medozon Comfort; Herrmann Apparatebau GmbH, Kleinwallstadt, Germany) was used by the manufacturer's protocol to obtain medical ozone. The ozone dose and the gas flow were checked simultanously recommended by the Standards Committee of the International Ozone Association (IOA). In order to secure reproducibility of the findings, this study was performed in an environment with checked temperature at 25 °C and each experiment was performed in triplicate. The agar-blood in Petri dishes, which is optimum material under laboratory conditions, was used as the culture medium in this study. The plates were divided into two main groups-control (CG: not gas applied) and treated (TG). The TG plates with opened covers were inserted into the sterilized ozone-resistant plastic bags on flat cardboard suface. After fixation and sealing of the pastic bag with a special strap, the air is completely removed from the bag and later the bag is fiiled with the ozone gas mixture at 40 µg/ml concentrations and three exposure times (10, 20, 40 min). The flow of O3 was kept fixed at 1L/min in all tests. After ozone application, the TG plates were removed with the CG plates for incubation in aerobic conditions at 37 °C for 24 hours. Then the bacterial colony count in each plate was evaluated and the rate of remaning colonies was statistically analyzed. Colony-forming units on blood agar were counted. Also the colony number of the petri dishes which was not ozone gas applied was used to calculate killing rate. Log_{10} bacterial reduction factor (RF) and kill percentage (% kill) was calculated by using an equation presented in ASTM E2315[15]. RF= Log_{10} (control) − Log_{10} (treated) (where control is the number of colonies recovered from the unexposed and treated is the number of colonies recovered from the exposed to O_3) Killing rate (%) = (CFU of the control − CFU of the test) /CFU of the control)

Statistical analysis

The frequencies, ratios, mean and standard deviations of the bacteria in the groups in terms of different variables are presented with descriptive statistics. Whether the distributions of the research variables encounter the normality assumption was examined by using both skewness and kurtosis values and histograms. The evaluation of results showed that the research variables provide the normality assumption. The Kruskal-Wallis H test and the Mann-Whitney U test were used for intergroup comparisons. The changes in continuous variables measured in different time periods were tested with the Friedman F Test and Wilcoxon. The significance level for all analysis results was determined as $p < 0.05$. In this study, data analysis was performed using SPPS 25 (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.) program.

RESULTS

All tested groups showed the reduction of bacterial colonies and there was significant different between ozone treated and control groups (p=0.000). It was determined that there was significant different between ozone treated groups (10 min p=0.000; 20 min p=0.000; 40 min p=0.000, exposure time) by Kruskal Wallis test. Especially at the concentration of 10^8 mL^{-1}, the greatest decrease was detected in the first 10 min. (Table 1). However, the same rate of decrease was not observed in continued exposure times at the concentration of 10^8 CFU mL^{-1} The lowest difference between the control and 10 min was at the concentration of 10^5 cfu/mL. Besides, the difference was greatest in the group with the 10^5 CFU mL^{-1} concentration between 10 min. and 20 min. ozone exposure. In addition, it was found that the bacterial reduction at the 10^8 and 10^7 CFU mL^{-1} concentrations were significantly higher than all other concentrations between 20 min- 40 min ozone exposure (Table 1).

Similarly, the bacterial log_{10} reduction was observed greater at high initial concentrations than

Table 1. Evaluation of bacterial reduction (log_{10}) according to exposure times and bacterial concentrations

CONC	n	C-10 min		10 min. - 20 min.		20 min.- 40 min.	
		Mean	SD	Mean	SD	Mean	SD
10^2	30	2.72	0.75	0.20	0.76	0.00	0.00
10^3	30	3.30	1.12	0.30	0.92	0.76	0.14
10^4	30	3.33	1.51	1.02	1.48	0.92	0.17
10^5	30	2.17	1.42	2.25	1.61	0.85	0.16
10^6	30	2.74	1.24	1.73	1.67	1.58	0.29
10^7	30	3.07	0.83	1.19	1.66	1.77	0.32
10^8	30	3.26	0.11	0.74	0.90	2.09	0.38
Total	210	2.94	1.15	1.06	1.49	1.53	0.11
		p < 0.001		p < 0.001		p = 0.01	

C: Control, CONC: Concentration, n: number, SD: Standard deviation

lower concentrations at the end of 40 min. For instance, the avareage reduction was detected at 10^2 initial concentration approximately 2.75 log CFU mL^{-1} in within 40 min for MRSA isolates whereas it was detected at 108 concentration nearly 3.18 log CFU mL-1 in first 10min. and ~ 8.18 log CFU mL^{-1} within 40 min. However, the killing rates of MRSA were similar both high and low concentrations at the level of nearly over> 99.9% in all exposure times. Furthermore, the killing rate was detected higher within 10 min. at greater bacterial concentrations than lower concentrations in MDR-*P. aeruginosa*. The average killing rate at 10^8 CFU mL-1 was 99.9% whereas it was determined as 33.3% for this bacteria. The mean log_{10} reduction of all bacteria with times of exposure was illustrated in Figure 1a. Even though the aveage bacterial reduction was over 5 log units in which meant revealed the decent bactericidal activity, the whole of bacteria could not inactivate in high inoculum concentrations (Figure 1b.)

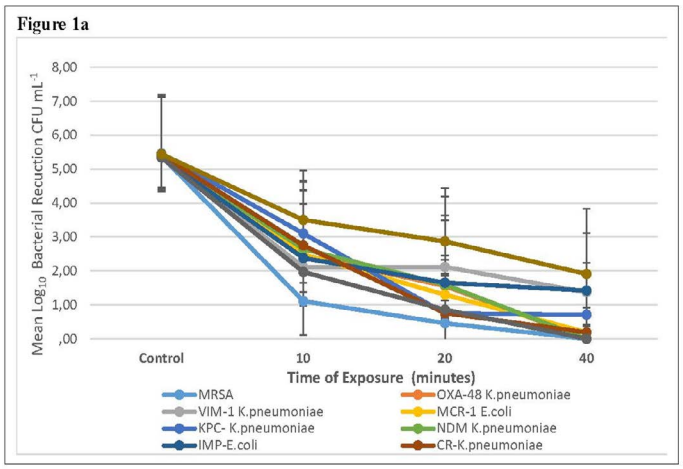
The greatest decrease was detected in methicillin-resistant *S. aureus* (MRSA) between control and 10 min. ozone treated group (TG) (Table 2). The decrease in MRSA was significantly higher than the decrease in all other bacteria (the statistical significance between *OXA-48 K. pneumoniae p=0.001, VIM-1 K. pneumoniae p=0.036, mcr-1 E.coli p=0.001, KPC-K. pneumoniae p=0.000, NDM- K. pneumoniae p=0.000, IMP E. coli p=0.001, CR-K. pneumoniae p=0.000, MDR-P. aeruginosa p=0.000 except CR-A. baumannii (p=0.110)* after first 10min ozone exposure. Nevertheless, the decrease in *CR-A. baumannii* was significantly higher than only the reduction in *KPC-K. pneumoniae* and *MDR-P. aeruginosa*. It was observed that the decreases in *KPC-K. pneumoniae* between 10 and 20 min were significantly higher than the decreases in MRSA, OXA-48 *K. pneumoniae*, VIM-1 *K. pneumoniae*, *IMP E. coli* and MDR-*P. aeruginosa*. The reduction in *CR-K. pneumoniae* was significantly higher than the decrease in VIM-1 *K. pneumoniae*.

In the measurement of the 10 min. ozone treatment, *KPC-K. pneumoniae* (p= 0.038) and *MDR-P.*

Table 2. Comparison of logarithmic bacterial reduction according to bacterial species depending on exposure time to ozone gas

Type of Bacteria	n	C- 10 min.		10 min.- 20 min.		20 min. – 40 min.	
		Mean	SD	Mean	SD	Mean	SD
MRSA	21	4.27	1.34	0.65	1.59	0.46	1.45
OXA-48 *K.pneumoniae*	21	2.92	1.08	0.91	1.47	1.56	2.07
VIM-1 *K.pneumoniae*	21	3.25	1.03	0.00	0.00	0.75	1.40
mcr-1 *E.coli*	21	2.94	0.95	1.17	1.53	1.11	1.83
KPC-*K.pneumoniae*	21	2.36	0.93	2.35	1.81	0.03	0.15
NDM-*K.pneumoniae*	21	2.73	0.90	1.06	1.33	1.59	1.90
IMP-*E.coli*	21	2.96	0.92	0.74	1.17	0.23	0.39
CR-*K.pneumoniae*	21	2.67	0.99	1.99	1.53	0.57	1.43
CR-*A.baumannii*	21	3.37	0.79	1.11	1.40	0.86	1.59
MDR-*P.aeruginosa*	21	1.94	0.99	0.64	1.03	0.95	1.39
Total	210	2.94	1.15	1.06	1.49	0.81	1.53
p	210	p< 0.001		p< 0.001		p= 0.008	

C: Control, CONC: Concentration, n: number, SD: Standard deviation



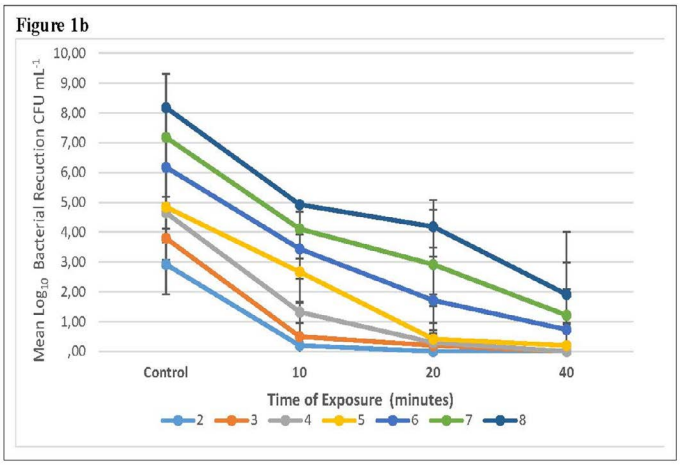


Figure1a. The Mean ± SD $\log_{10}$ reduction in bacterial cell counts in the culture medium was give in Figure1a. belonging to each different bacteria. Colony-forming units: CFU

Figure1b. Each experiment was repeated 3 times. The Mean ± SD $\log_{10}$ reduction in bacterial cell counts in the culture medium. The average $\log_{10}$ bacterial reduction at the different concentrations 10^2-10^8 CFU mL^{-1} with gaseous ozone of 40 µg/ml for 10, 20 and 40 minutes exposure. Colony-forming units: CFU

aeruginosa (p= 0.004) were found to be more resistant than MRSA. VIM-1 *K. pneumoniae* was also obtained to be more resistant than MRSA (p= 0.010), *OXA-48 K. pneumoniae* (p= 0.010), *NDM- K. pneumoniae* (p= 0.010) and *CR-A. baumannii* (p= 0.010). It was determined that some bacterial species did not show time-dependent killing continuity. For example, 3.24 $\log_{10}$ bacterial reduction was observed in the first 10 min of ozone exposure in VIM-1 *K. pneumoniae*, while no difference was observed between 10 min and 20 min. Additionally, approximately 1 $\log_{10}$ bacterial reduction was also appointed at the end of the 40 min (Table 3).

Gaseous ozone was showed bactericidal effect (>=3$\log_{10}$ bacterial reduction) on MRSA, VIM-1 *K. pneumoniae* and *CR-A. baumannii* in 10 min exposure time and the bacterial decresases were detected as 4.27 $\log_{10}$ CFU mL^{-1}, 3.24 $\log_{10}$ CFU $mL^{-1}$and 3.37 $\log_{10}$ CFU mL^{-1}, respectively. The least bacterial decrease (1.93 $\log_{10}$) in 10 min ozone exposure was observed in *MDR-P. aeruginosa*. At the 20 min ozone exposure, the bactericidal activity was detected on all tested bacteria except MDR-*P. aeruginosa* (2.57

Table 3. Determination of logarithmic bacterial inactivation at the different time exposure to ozone for each bacteria

Type of Bacteria	n	Control		10 min.		20 min.		40 min.	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
MRSA	21	5.38	1.82	1.11	2.05	0.46	1.45	0.00	0.00
OXA-48 *K.pneumoniae*	21	5.39	1.79	2.47	2.25	1.56	2.07	0.00	0.00
VIM-1 *K.pneumoniae*	21	5.36	1.81	2.12	2.29	2.12	2.29	1.37	2.01
mcr-1 *E.coli*	21	5.42	1.76	2.48	2.06	1.30	1.91	0.19	0.87
KPC- *K.pneumoniae*	21	5.46	1.74	3.10	1.86	0.75	1.58	0.71	1.52
NDM *K.pneumoniae*	21	5.38	1.81	2.65	2.00	1.59	1.90	0.00	0.00
IMP-*E.coli*	21	5.34	1.78	2.38	2.00	1.65	1.98	1.42	1.70
CR-*K.pneumoniae*	21	5.42	1.75	2.75	1.72	0.76	1.61	0.19	0.87
CR-*A.baumannii*	21	5.34	1.83	1.97	2.00	0.86	1.59	0.00	0.00
MDR-*P.aeruginosa*	21	5.44	1.75	3.50	1.12	2.87	1.57	1.91	1.92
Toplam	210	5.39	1.75	2.45	2.01	1.39	1.91	0.58	1.36
p	210	p = 1.000		p = 0.017		p = 0.001		p < 0.001	

CONC: Concentration, n: number, SD: Standard deviation

log_{10} CFU mL^{-1} bacterial reduction). Finally, it was determined that the 40 min ozone exposure showed bactericidal effect on all tested isolates including *MDR-P. aeruginosa*.

DISCUSSION

As more antibiotics are losing their activity to MDR microorganisms, the major corcern should be altered to alternative therapies. It is essential to enhance reseach into new and natural strategies to deal with infectious disease. As well as it should be aimed at decreasing the expand and transmission ratio for these pathogens, via contact either between people or between people and settings /surfaces/medical equipments by new research in this pandemic world (16,17). The bactericidal impact of gaseous ozone is well recognized so far (10). Meanwhile, there is a lack of studies aiming the effectiveness of ozone against MDR pathogens, particularly carbapenemase-producing bacteria. In this assay, the antimicrobial activity of gaseous ozone against ten multi-drug resistant bacteria was investigated. The gaseous ozone showed its efficacy on MDR Gram negative and positive bacteria under the following conditions applied: 40 µg/ml at 10 min, 20 min and 40 min. The results demostrated that ozone was effective against all tested bacteria. Nevertheless, the optimal effect was observed with a dose of 40 µg/ml and within 20 min except *MDR-P. aeuginosa*.

Ozone is a potent biocidal agent, capable of inactivating several pathogens including Gram (-) and Gram (+) bacteria, fungi or viral capsids. The Ozone (O3) can be applied as a bactericidal agent in the forms of ozonized water or oil, ozone associated with other substances and more principally the gaseous O_3/O_2 gas mixture. The inactivation or reduction of microorganisms depends on ozone concentration, type of pathogens, initial bacterial load and time of exposure (18).

It is immensely significant to underline that the greater activity detected of the action of gaseous ozone in higher concentrations of bacterial inoculum in the presented study. This situation can be clarified that the greater is the inoculum, the higher is the colony forming unit in the control plates. Therefore, Log_{10} is greater at higher inoculum concentrations of microorganisms and is minor at lower concentrations. For this reason, it is required to interpret the results of Log_{10} and killing ratio together, pointing that the reduction was mostly 100%, regardless of the inoculum used (16).

Although the sensitivity of bacteria to ozone gas at the same concentration varies, it has been determined that meticilin resistant *S. aureus*, which is a Gram-positive bacterium, is more sensitive than Gram-negative bacteria, and the inactivation is provided faster and at a higher rate in this study. These findings are similar to those determined by Giuliani et al. (19) and Hirai (20) who, in their studies on the effect of ozonized water on various types of bacteria, described that the effect of the ozone applications was greater in the action on Gram-positive bacteria. Komanapalli et al. (21) notified that O_3 affects proteins easier and faster than lipids. Therefore, Gram positive bacteria may be more likely sensitive to ozone. The MRSA was inactivated at the level of >99% within 10 min in this study. On the contrary, Azuma et al. (22) reported that the MRSA was inactivated gradually: 36% after.1 min, 79% after 5 min, and 83% after 10 min. contact to ozone.

The mean colony counts for each exposure time of gaseous ozone were figured out and transformed to Log_{10}. The logarithmic inactivation of *MDR-P. aeruginosa* was lower than the results detected for other Gram negative bacteria at the all tested exposure time.

The bactericidal activity was generally observed within 20min. on tested isolates. However, the *MDR-P. aeruginosa* showed a relatively lower response to ozone and it was observed that the bacterial reductions within 10 min., 20min., 40min. as 1.93 Log_{10} CFU mL^{-1}, 2.57 Log_{10} CFU mL^{-1}, 3.53 Log_{10} CFUmL^{-1}, respectively. So, it was required more than 20min. exposure to ozone for the bactericidal effect. Although the bactericidal efficiency was reached to relatively adequate level, an average ~1.91 log CFU mL^{-1} *MDR-P. aeruginosa* population could survived after the 40 min. ozone application. These results suggested different mechanisms of pathogens to deal with the bactericidal effects of gaseous ozone. A previous assay indicated a selection of a robust bacterial population through ozonation, which is defined by a high guanine-cytosine (GC) content of their genomes (23). The weaker results obtained for MDR-*P. aeruginosa* in the current study may be related to high GC-contents >60% belonging to this species (24). Similiar to this work, Andreani et al. reported that *S. aureus, E. faecalis E. coli, S. mutans* and *S. typhi* were highly sensitive to ozone at a concentration of $1x10^2$ CFU mL^{-1}, presenting a decrease of viable cells varing from 45 to 80 % within 30 min of exposure to ozone. On the other hand, *P.*

aeruginosa was inactivated in the same conditions by only 25 % of the initial bacterial load (25).

The potential of our results is interesting. It was investigated whether the antibacterial activity of ozone was affected by the types of resistance genes carried by the bacteria. It was determined minimal difference in the time dependent inactivation among the three different type of carbapenemase producing (blaOXA-48, blaKPC or blaNDM^{-1}) *K. pneumoniae* isolates. No distinct differences were noted at the first 10min for these bacteria. However, the net log reduction of *KPC-K. pneumoniae* was significatly higher OXA-48 *K. pneumoniae* within 10 min-20 min. In addition that the bacterial decrease of OXA-48 *K. pneumoniae* was significantly higher than both of them within 20min-40min. Nevetheless, all of the three isolates were in activated at the level of >90% and the bactericidal effectiveness was detected within 20 min. A consistently a longer exposition time might conceivably end in a higher inactivation ratio (26). However, some bacterial species could not represent time-dependent inactivation contionusly (27). Taking the results of VIM-1 *K. pneumoniae* strains, no distinct differences were noted between the effects of exposure to gaseous ozone for 10 vs. 20 min.

No significant differences occured between IMP-1 producing *E. coli* and mcr-1 carring *E. coli* the effects of exposure to gaseous ozone; the average log reduction within 10 min was 2.93 and 2.96 log units, respectively. Meanwhile, the bactericidal activity was observed within 20 min both of them. The carbapenemase-producing *A. baumannii* isolates were also found considerably sensitive to ozone, reduction rates greater than 4 log units were revealed at first 20 min. Similiarly, Mark et al (28) reported that ozone could be a promising agent to perform disinfection of surfaces contaminated with carbapenemase producing *A. baumannii* under room conditions. Inactivation rates higher than 5 log units were observed on all stainless steel and ceramic carriers after ozon contact (80 ppm ozone; 60 min.). Song et al. (29) investigated the clinical safety and efficacy of topical ozone in two patients with MRSA skin infection. These authors reported that almost 100% MRSA and 100% *S. aureus* a were inactivated by ozonated water in 1 min. Yasheng et al. (30) perfomed a combination of ozonated water and conventional treatment on eighteen patients with chronic osteomyelitis and gained good clinical outcomes. In a study of Oh et al. (31) the ozone could decrease antibionicrobial resistant bacteria and their resistance genes by more than 90% even at 3 mg/L ozone concentration. In another study, All of carbapenem resistant *Enterobacteriaceae,* MRSA, vancomycin-resistant *Enterococcus spp., MDR Acinetobacter spp.* and *MDR P.aeruginosa* were inactivated at the level of >90% only within 10 min. Interestingly, antimicrobial sensitive bacteria (AMSB) represented similar patterns to ozonation in the same study (21). On the contrary, in a study of Lüddeke showed that antibiotic resistant *E. coli* and staphylococci virtually survived ozone exposure better than AMSB (32).

Overall, the bactericidal effects of ozone strongly depend on the bacterial species. Some facultative bacteria are capable of different levels of resistance to ozone oxidative stress to survive. Therefore, the factors affecting the sensitivity to ozone should be clarified by futher deep studies.

In general, the diversity of the available literature data, in addition to the various methodological strategies performed in the different assays, complicate the exact assesment of the efficiency of the ozone implementations. This study includes the following limitations: during the experiment ozone dose was stable, so we could not evaluate the ozone dose dependent efffect. The nature of the study, it is not clear evident how well our results may convert into clinical practice in which parameters such as variable blood flow, necrotic tissue, and great bacterial loads may play a significant role, especially in the soft tissue infections.

CONCLUSIONS

Given the results exposed, gaseous ozone showed adequate bactericidal activity on MDR bacteria and its effect increased dependently exposure time. The results of these studies clearly underline the necessity of properly optimizing the ozone practices (e.g. specific ozone dose, exposure time) considering both the bacterial species and related antibiotic resistance profiles, as well as physico-chemical properties, safety corcern to combat infections with multi-drug resistant bacteria. Bearing in mind the necessity for novel approaches for the control of microbial infections, the optimization of ozone treatment should be taken high priority and more further in vivo studies are needed to provide in depth understanding of the ozone effect on the inactivation of antibiotic resistant bacteria in the current pandemic world.

Informed consent

Nature of the study, it is not required patient

consent form.

This study conformed to the Helsinki Declaration. The study was approved by the ethic review board from Necmettin Erbakan University Faculty of Medicine (decision number: 2021/3161)

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