Investigation of the Efficacy Results of Atmospheric Cold Plasma Against Multi-Resistant Bacterial Strains

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Abstract

Aim: To investigate the efficacy of Atmospheric Cold Plasma (ACP)-treated phosphate buffered saline (PBS) against Gram positive and Gram negative multidrug resistant bacteria.

Methods: A total of 50 carbapenem-resistant *Klebsiella pneumoniae* and 10 vancomycin-resistant *Enterococcus faecium* strains were included in the study. 100 µl (1/2), 300 µl (3/4), 700 µl (7/8), 1500 µl (15/16), 3100 µl (31/32), 6300 µl of ACP-treated PBS was added to 100 µl of bacterial suspension (10^7 CFU/ml bacterial suspension). After pipetting, the suspension was incubated at room temperature for 30 minutes, inoculated onto sheep blood agar and incubated overnight (16-18 hours) at 37°C.

Results: All strains studied were inhibited by ACP-treated PBS solution. The dilutions given are those in which growth was completely inhibited. 45 of *K. pneumoniae* strains were completely inhibited by ACP-treated PBS solution at 3/4 concentration, while 5 of *K. pneumoniae* strains were completely inhibited by ACP-treated PBS solution at 7/8 concentration. Vancomycin-resistant *E. faecium* strains were inhibited by higher amounts of plasma than *K. pneumonia* strains. Three of *E. faecium* strains 15/16, three of *E. faecium* strains 31/32, four of *E. faecium* strains 63/64 were completely inhibited by ACP-treated PBS solution.

Conclusions: ACP-treated PBS solution was found to be effective against both Gram-positive and Gram-negative bacteria resistant to important antibiotics. A difference in the concentration of ACP-treated PBS required for inactivation was observed in the selected Gram-negative and Gram-positive bacteria. However, this method is hopeful as the available treatment options are limited day by day in both Gram-negative and Gram-positive infections. Future studies are needed for the use of ACP-treated PBS fluids as a treatment modality.

Keywords: *Plasma, multidrug resistance, efficacy, anti-bacterial agents*

1. Introduction

Gram-negative, multidrug-resistant bacteria from the Enterobacterales family are rapidly increasing in our country as well as throughout the world. These isolates may be resistant to antibiotics such as carbapenems, which are the last choice in the treatment of serious infections, and this resistance development may lead to treatment failures by limiting clinical treatment options[1]. Although enterococci, which are Gram-positive bacteria, are members of the intestinal and vaginal flora, they have developed high-level resistance to glycopeptide-derived antibiotics, especially beta-lactam and aminoglycoside groups, because of intensive and faulty antibiotic use in hospitals[2,3]. The decline in treatment options for both Gram-negative and Gram-positive infections has led to the search for other treatment modalities.

Atmospheric Cold Plasma (ACP) has gained increasing importance in recent years due to its strong antimicrobial activities on bacteria, viruses, fungi, and prions[4]. Reactive oxygen radicals (ROS), reactive nitrogen radicals (RNS) and other free radicals formed during the process play a role in the antimicrobial activity of ACP[5]. Recently, it was reported that ACP-treated phosphate buffered saline (PBS) solution gained antimicrobial activity and eventually became effective on multidrug resistant bacteria[6]. Joshi et al[4] demonstrated that a significant amount of ROS is produced by ACP and leads to antibacterial effect through lipid peroxidation and DNA damage in *Escherichia coli* strains. The efficacy of ACP-treated fluids in preventing bacterial infections has been demonstrated in animal studies, as in the study by Oztan et al[7]. It is thought to be a potential new treatment alternative

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for the treatment of multidrug resistant bacteria, which have an important place in nosocomial infections. Therefore, it is important to demonstrate the efficacy of ACP-treated fluids against multidrug resistant bacteria. In this study, we aimed to investigate the efficacy of ACP against Gram positive and Gram negative multidrug resistant bacteria.

2. Materials and methods

2.1. Preparation of bacterial suspension:

Multidrug resistant *Klebsiella pneumoniae* and vancomycin-resistant enterococci (VRE) isolated from various clinical samples were revived from bacterial stocks in the Izmir Tepecik Training and Research Hospital Medical Microbiology Laboratory. bacterial strains were cultured on sheep blood agar and incubated overnight (16-18 hours) at 37°C. After incubation, colonies collected from the resuscitated strains were prepared in phosphate buffered saline (PBS) at a density of 0.5 McFarland (10^8 CFU/ ml) and diluted to 10^7 CFU/ml according to the recommendations of Oztan et al.[7]. Standard bacterial strains known as American Type Culture Collection (ATCC), *S. aureus* ATCC 29213, *E. faecalis* ATCC 29212, *E. coli* ATCC 25922 were tested with ACP-treated PBS.

2.2. Preparation of ACP-treated PBS solution:

Atmospheric cold plasma was obtained from the Izmir Katip Çelebi University, Department of Biomedical Engineering Plasma Laboratory. Sterile PBS solution (0.9% NaCl) was processed under normal atmospheric pressure to flow through the set-up (see Figure 1). The setup consisted of a copper electrode whose surface is covered with a 1-mm glass act as dielectric barrier connected to a high voltage cable to obtain dielectric barrier discharge plasma. The microsecond alternating current (AC) power supply was operated at a peak-to-peak voltage of 24 kV, a discharge current of 3.5 mA and a frequency of 25 kHz, giving approximately 9 W in terms of power output. 3 ml of PBS was transferred into a petri dish made of glass with a diameter of 40 mm and treated for 5 min at a constant discharge gap of 1.5 mm. After ACP treatment, the PBS solution was collected and stored at +4°C.

Figure 1

Image of the plasma setup for the treatment of phosphate buffered saline solution.

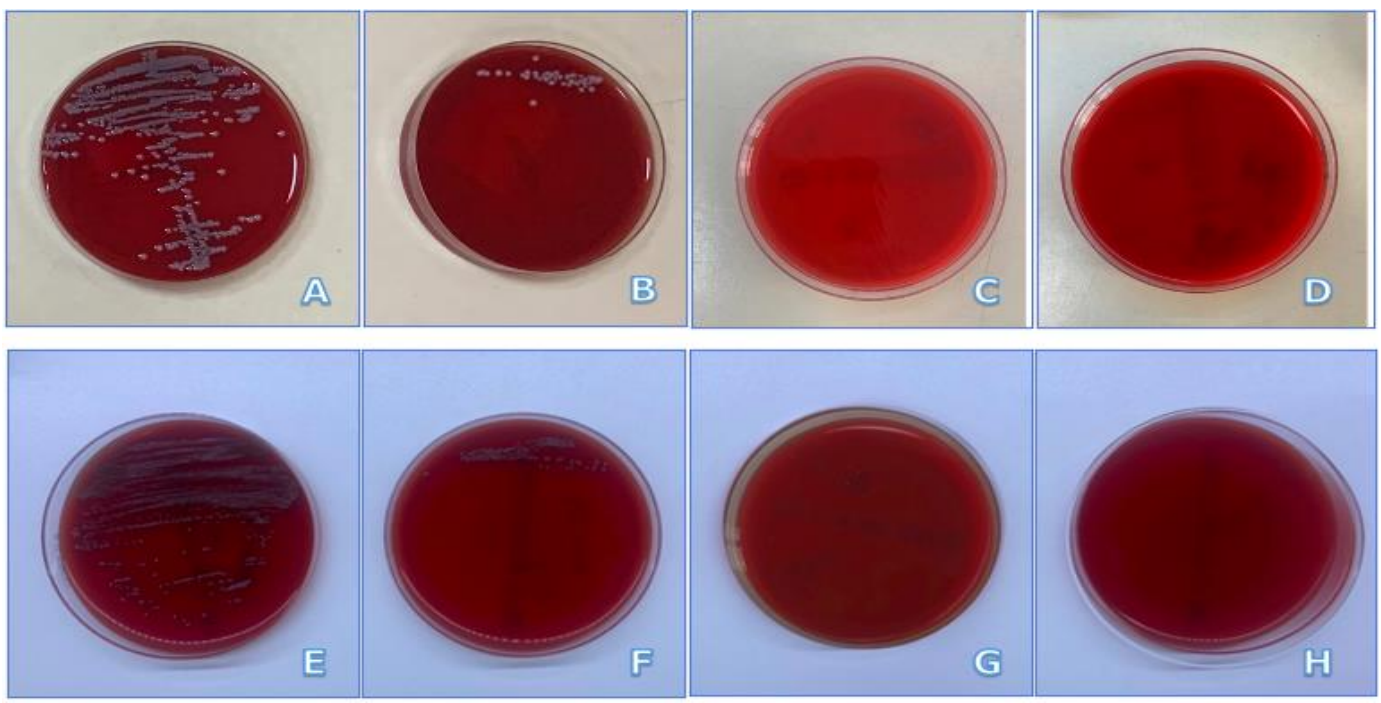

Figure 2

Efficacy samples of ACP-treated PBS solution at different concentrations. A, B, C, D figures are examples for K. pneumoniae; E, F, G, H figures are examples for E. faecium. A: Positive control for K. pneumoniae. B:100 bacterial suspension/100 ACP-treated PBS (1/2). C:100 bacterial suspension /300 ACP-treated PBS (3/4). D:100 bacterial suspension /700 ACP-treated PBS (7/8). E: Positive control for E. faecium. F: 100 bacterial suspension /300 ACP-treated PBS (1/4). G:100 bacterial suspension /700 ACP-treated PBS (7/8). H:100 bacterial suspension /1500 ACP-treated PBS (15/16).

2.3. Testing the effectiveness of ACP treated PBS solution:

Plasma treated PBS solution was not diluted since the plasma treated liquids lose their antimicrobial effect[7]. Therefore, 100 µl, 300 µl, 700 µl, 1500 µl, 3100 µl, 6300 µl of ACP-treated PBS was added to 100 µl of bacterial suspensions. The final solutions used contained ACP-treated PBS at a ratio of 1/2, 3/4, 7/8, 15/16, 31/32, 63/64 respectively. After pipetting, the suspension was incubated at room temperature for 30 minutes, inoculated on sheep blood agar with sterile loop (10 µl) and incubated overnight (16-18 hours) at 37°C. For the interpretation of efficacy, 10^7 CFU/ml was considered as the effective concentration inhibiting bacterial growth (Fig.2).

2.4. Statistical Analysis

Descriptive information will be obtained by giving distribution and frequency percentages in obtaining the data of the study.

3. Results

All strains studied were affected by ACP-treated PBS solution. The results of the efficacy of ACP-treated PBS solution against multidrug-resistant bacterial strains at different concentrations are given in Table 1. 45 of *K. pneumoniae* strains were completely inhibited by ACP-treated PBS solution at 3/4 concentration, while 5 of *K. pneumoniae* strains were completely inhibited by ACP-treated PBS solution at 7/8 concentration. Vancomycin-resistant *E. faecium* strains were inhibited by higher amounts of plasma than multidrug resistant *K. pneumonia* strains. 3 of *E. faecium* strains 15/16, 3 of *E. faecium* strains 31/32, 4 of *E. faecium* strains 63/64 were completely inhibited by ACP-treated PBS solution. The inhibition concentration for *E. coli* ATCC 25922 was inhibited with 3/4 ACP-treated PBS solution. On the other hand, the inhibition concentration for *E. faecalis* ATCC 29212 and *S. aureus* ATCC 29213 was 15/16. Thus, in the case of standard bacterial strains, higher amounts of inhibition by plasma were achieved in gram-positive as well as in the bacteria included in the study.

Table 1

The number of concentrations at which ACP-treated PBS is effective against multidrug-resistant bacterial strains.

Bacteria suspension	100µl	100µl	100µl	100µl	100µl	100µl
ACP treated PBS	100µl	300µl	700µl	1500µl	3100µl	6300µl
Bacteria strains	(1/2)	(3/4)	(7/8)	(15/16)	(31/32)	(63/64)
K. pneumoniae (n=50)	0	45	5	0	0	0
E. faecium (n=10)	0	0	0	3	3	4
S. aureus ATCC 29213 (n=1)	0	0	0	1	0	0
E. faecalis ATCC 29212 (n=1)	0	0	0	1	0	0
E. coli ATCC 25922 (n=1)	0	1	0	0	0	0

ACP, Atmospheric Cold Plasma; PBS, phosphate buffered saline; ATCC, American Type Culture Collection.

4. Discussion

In this study, the antimicrobial effect of ACP-treated PBS solution on carbapenem-resistant *K. pneumoniae* and vancomycin-resistant *E. faecium* was investigated. Antimicrobial resistance (AMD) is one of the most important items on the global health agenda in recent years due to both its public health problem and economic cost. According to surveillance studies, there is an increase in resistance in antibiotics used against *K. pneumoniae* and *E. faecium*, in addition to *Acinetobacter* species, which are resistant to many antibiotics in Turkey[8]. Therefore, the decrease in treatment options in infections caused by both Gram-positive and Gram-negative bacteria leads to the search for different treatment options. In 2017, the World Health Organization prepared a list of priority pathogens to guide and encourage research and development of new antibiotics. According to this list, carbapenem-resistant, extended spectrum beta-lactamases-producing *Enterobacterales* critical and vancomycin-resistant *E. faecium* are among the high priority bacteria[9].

In an era when it is becoming increasingly difficult to find new antimicrobial drugs against these resistant bacteria, it is important to understand how antimicrobial effects occur and their potential clinical implications. Various non-antibiotic agents exhibit antimicrobial activity through multiple and distinct mechanisms of action. Numerous studies have reported the antimicrobial activity of some non-steroidal anti-inflammatory drugs, local anesthetics, anti-psychotics, anti-depressants, antiplatelets and statins[8]. Non-traditional approaches are also being developed to combat antibiotic resistance with agents with antimicrobial effects such as antimicrobial peptides targeting the bacterial cell membrane, efflux pump inhibitors, phage therapies, antibodies with antibiotic effects and immunomodulators[10].

Physical plasma and plasma treated liquids have gained increasing importance in recent years due to its strong antimicrobial activities on bacteria, viruses, fungi, and prions[4]. Physical plasma is used especially in sterilization units under different temperatures and pressures. Apart from the studies in which it is applied directly to the skin, there are also studies in which ACP-treated liquids (such as N-acetylcysteine solution, PBS) were used for their antimicrobial properties[7,11]. In our study, we found ACP-treated PBS to be effective against 50 strains of *K. pneumonia* and 10 strains of *E. faecium*. The effect of ACP is mainly due to oxidative and nitrosative stress induced by ROS and RNS produced during its interaction[12,13]. ROS, RNS and free radicals are thought to cause damage to various cellular structures and eventually microbial inactivation[4,14,15,16].

In this study, the amount of ACP-treated PBS with complete inhibition was found to be different between Gram-positive and Gram-negative bacteria and more amount was needed in Gram-positive bacteria. This situation is similar to the study that use quality control bacteria for *E. coli* ATCC 12900 and S. aureus ATCC 25923[17]. According to this study, more plasma treated liquid was used for the inhibition of *Enterococcus faecalis* ATCC 29212 and *S. aureus* ATCC 25923 strains than for the inhibition of *E. coli* ATCC 12900 strains. The ROS produced by ACP damage the thin peptidoglycan and thicker lipopolysaccharide layer of the cell wall in Gram-negative bacteria but cause less DNA damage than in Gram-positive bacteria. In Gram-positive bacteria, serious damage to intracellular components such as DNA damage is more prominent, and the cell wall is intact[17]. Since cell wall structures are different in Gram-positive and Gram-negative bacteria, it was thought that this may be the reason for the difference in the need for ACP-treated PBS. In a study in which *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 strains were used to model perforated appendicitis in rats, no significant bacterial inactivation was observed with ACP-treated PBS at 15 minutes, whereas no significant bacterial inactivation was observed at 30 minutes and 45 minutes of treatment with E. coli and S. aureus strains by approximately 3.5 log at 30 minutes and 7 log at 45 minutes, and these inactivation values demonstrated the in vivo efficacy of ACP-treated PBS[7]. On the other hand, this is the first study from Turkey to demonstrate the efficacy of ACP-treated PBS in resistant Gram-positive and Gram-negative bacteria in vitro.

As a result, ACP-treated PBS solution was found to be effective against both Gram-positive and Gram-negative bacteria resistant to important antibiotics. A difference in the concentration of ACP-treated PBS required for inactivation was observed in the selected Gram-negative and Gram-positive bacteria due to two possible inactivation mechanisms. These findings are critical for the successful development of plasma applications for in vivo experiments for treatment.

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Statement of ethics
This study was conducted in accordance with the ethical principles of the Declaration of Helsinki and was approved by Non-Interventional Clinical Research Ethics Committee of University of Health Sciences, Tepecik Training and Research Hospital (Date: 13.07.2023 and Decision no: 2023/06-58).

Conflict of interest statement
The authors declare that they have no financial conflict of interest with regard to the content of this report.

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