Comparison of the Antibacterial Effects of Photodynamic Therapy and Cold Atmospheric Plasma on Methicillin-Resistant Staphylococcus aureus

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***Abstract*— Antibiotics are the drugs that are used to treat bacterial infection by killing bacteria or inhibiting their proliferation mechanisms. However, some infections cannot be treated with antibiotics because of the frequent, incorrect, and unconscious use of them. Alternative treatment methods such as antibacterial photodynamic therapy (aPDT) and cold atmospheric plasma (CAP) are being developed to solve these problems. Both alternative treatment methods aim to show the antibacterial effect and control infections on clinically isolated methicillin-resistant *Staphylococcus aureus* (MRSA). In this study, MRSA, a type of gram-positive bacteria that causes skin and dental infections, has been tried to be inactivated *in vitro*. 808 nm laser light at 84 J/cm^2 energy density and indocyanine green in different concentrations between 10-200 µM were used for aPDT. CAP was produced with 20 kHz and 30 kV and used for the CAP treatment. 3.52 logarithmic reduction of MRSA obtained in 100 µM ICG-aPDT and 3.61 logarithmic reduction of MRSA obtained 300 seconds CAP treatment.**

Keywords*— *MRSA; Antibacterial photodynamic therapy; 808nm; Indocyanine green; Cold atmospheric plasma

I. INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a gram-positive bacteria that can be found on the flora of external skin and mucosal surfaces [1]. These bacteria can cause infectious diseases via multiplication and colonization [2]. *S. aureus* developed resistance against methicillin which is a β-lactam antibiotic acting by blocking proteins that take the role in cell wall production via producing β-lactamase after clinical usage of it [1]. MRSA is the resistance strain of *S. aureus* to methicillin and the main pathogen that causes superficial skin, community, and hospital-acquired infections worldwide [1, 3-5]. Infections can cause critical problems if they are not treated so they should be cleared out via suitable treatment methods. Together with the disadvantages of antibiotic resistance due to overuse, incorrect, and frequent use and other conventional treatment methods bring into the requirement of different and alternative antibacterial treatment methods to overcome and solve the inactivation problem of bacteria especially for the elimination of antibiotic-resistant bacteria such as MRSA [6-8].

aPDT is a non-invasive therapy that aims to kill pathogenic microorganisms to control infections by interacting photosensitizer (PS) with low-power light via producing reactive oxygen species (ROS) [3, 9]. ROS form via type I and II photochemical mechanisms which may occur singly or simultaneously and cause bacterial death by damaging the macromolecules of bacteria [3, 10]. There are examples of clinical usage of aPDT for solving bacterial infection problems in dentistry such as dental caries, and root canal disinfection [11]. In addition to dental applications, aPDT is very promising for curing problematic and chronic wound infections which are generally formed because of surgery, trauma, burns, and diseases by showing antibacterial effect and improving the wound healing process [9, 12].

Plasma or ionized gas is the fourth state of matter that contains various components such as electrons, cations, and ultraviolet radiation [13, 14]. CAP forms ozone, superoxide, hydrogen peroxide, and hydroxyl radicals that do not create any thermal effects. CAP is effective against microorganisms by producing ROS like aPDT and reactive nitrogen species (RNS) [14]. There are examples of CAP treated bacterial infections of dental tissue that express the antibacterial effect on different bacteria in the literature [15, 16]. In addition to dental tissue infections, there are laboratory and clinical studies that have shown that the CAP application provides also positive effects on wound healing [14, 17].

In general, aPDT and CAP applications were examined independently. These two different methods revealed

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significantly positive results in antibacterial treatment in certain conditions. Until now, there is not any study that compare these applications according to their specific parameters such as energy, frequency, application time, etc. In this study, the antibacterial efficacy of an aPDT with ICG which shows high light absorption at 800 nm in the near-infrared (NIR) spectrum [3, 9, 18] and CAP treatment was examined *in vitro* against MRSA that is the main source of hospital-acquired, wounds and dental infections [3, 14, 19]. The results of the two methods were compared and their superiority against each other was determined including their advantages and disadvantages.

II. MATERIAL AND METHODS

A. Bacterial Strain

A single colony of MRSA (clinically isolated) was selected and inoculated in tryptic soy broth (TSB) overnight at 37°C in an orbital shaker. The pellet was dissolved in phosphate-buffered saline (PBS) to get about 10^8colony-forming units (CFU/mL).

B. Photosensitizer

ICG, an anionic synthetic cyanine dye, was used as a PS in this study. Stock ICG solution was diluted using PBS to obtain 10 to 200 µM concentrations before each experiment. Bacteria were incubated with ICG for 15 minutes in the dark.

C. The Optical Setup and Light Source

An 808 nm fiber laser device that is in the NIR spectrum was used as a light source (Teknofil İstanbul) for aPDT experiments. The distance between the tip of the laser probe and the optical plate surface was kept constant at 10 cm and the output power kept at 1 W. To obtain the antibacterial effect, optimum ICG concentrations were determined by keeping the energy dose and application time constant at 84 J/ cm^2 and 593 seconds, respectively.

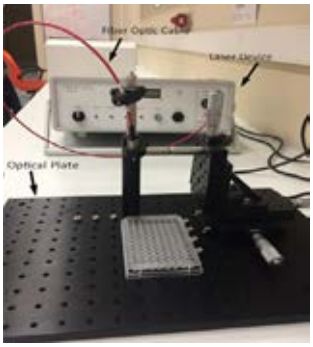

Fig.1. 808nm laser device

D. Cold Atmospheric Plasma Setup

CAP was formed with a repetition rate of 20 kHz and a voltage of 30 kV using the custom made plasma device shown in Figure 2. The distance between the electrode and the metal stage was arranged to 2 mm. To obtain the antibacterial effect, optimum exposure times were determined by changing exposure time and keeping frequency and voltage constant.

Fig.2. Plasma device

E. Photodynamic Therapy Experiments

The effects of aPDT with different ICG concentrations were studied *in vitro* on MRSA strain. The groups were; control group that received no application of laser nor ICG, laser group that received only laser application without ICG, ICG group that was incubated with ICG, and aPDT group that received laser light in the presence of ICG. Each group was repeated with 3 samples on 96-well plates. 50 µL diluted bacterial suspension for each well was transferred to 96-well plate. 50 µL ICG with a certain concentration was added to each well and incubated at room temperature in dark for 15 minutes to prevent photobleaching for ICG and aPDT groups. 50 µL PBS was added into the wells of control and laser groups to provide equal conditions. After incubation, bacterial suspensions in laser and aPDT groups were irradiated with laser light at 84 J/cm^2 energy dose for 593 seconds Bacterial suspensions of all groups were diluted in PBS using the serial dilution method after applications. 100 µL of diluted bacterial samples were spread on tryptic soy agar (TSA) and incubated overnight at 37°C for colony counting.

F. Cold Atmospheric Plasma Treatment Experiments

The effects of CAP with different application times were studied on MRSA *in vitro*. The groups were control group that received no application of CAP and CAP group that received the CAP treatment with specific parameters. For each group, 1 mL of diluted bacteria were transferred to the liquid holder chamber and treated with CAP. Each group repeated in 3 samples. Exposure time and frequency was selected as 300 seconds and 20 kHz as a result of

optimization studies based on our previous researches. Bacterial suspensions of all groups were diluted in PBS using the serial dilution method after applications. 100 µL of diluted bacterial samples were spread on tryptic soy agar and incubated overnight at 37°C for colony counting.

G. Statistical Analysis

After each experiment was conducted, the number of CFU/mL was determined by the naked eye and multiplied by the dilution factor in order to normalized data according to control groups. Test groups were compared by the Student's t-Test with the control group and the results were evaluated as significant when the p-value (p) was < 0.05.

III. RESULTS

A constant energy density, 84 J/cm^2, was applied to MRSA in order to determine the effect of laser light on the bacteria. When the laser group was compared with the control group, it was observed that the laser application did not cause an antibacterial effect on MRSA (Figure 3).

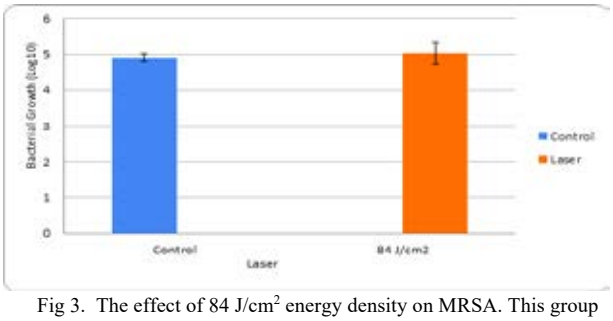

Fig 3. The effect of 84 J/cm^2 energy density on MRSA. This group irradiated with laser light only and bacterial viability were compared with the control group after 808 nm - 84 J/cm^2 light was applied

The control and ICG groups have similar bacterial viability which can be observed in Figure 4. Five different ICG concentrations between 10-200 µM were used and they did not cause any reduction in bacterial viability when they were used alone. Results in Figure 3 and Figure 4 indicate that ICG and laser treatment did not express any cytotoxic effect on MRSA when using alone.

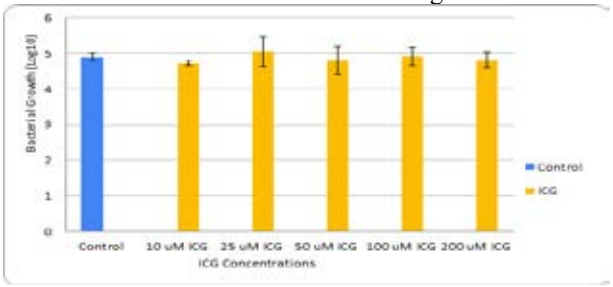

Fig.4. The effect of 10, 25, 50, 100, and 200 µM ICG concentrations on MRSA. These groups were incubated with the photosensitizer (ICG) only, did not receive any laser light application.

The laser light of 84 J/cm^2 energy dose in the presence of five different ICG concentrations which range from 10 to 200 µM and CAP that was produced by 20 kHz and 30 kV were applied to the MRSA and significant changes were observed in the meaning of reduction in bacterial cell viability. The change was statistically significant when the results of aPDT and CAP groups compared with the control group. 3.5 logarithmic reduction was obtained in 100 µM ICG-aPDT. These results indicated that the application of these five ICG concentrations and 84 J/cm^2 laser light together had a lethal effect on MRSA. The most effective one was the application with 100 µM ICG upon irradiation. 3.61 log reduction was obtained in 300 seconds CAP treatment. The result of 300 seconds CAP treatment also had a lethal effect on MRSA (Figure 5).

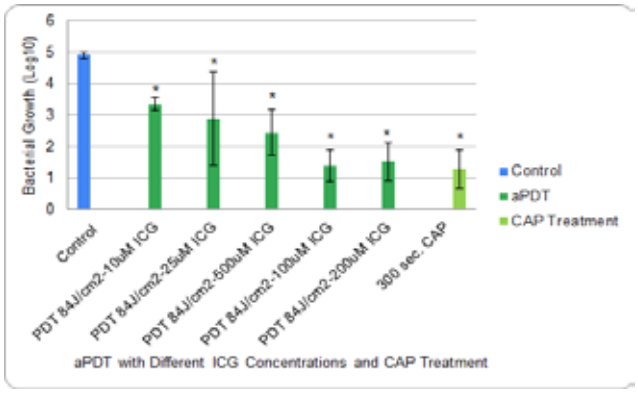

Fig.5. The effect of 10, 25, 50, 100, and 200 µM ICG concentrations together with 84 J/cm^2 energy density of 808 nm laser light and 300 seconds CAP treatment on MRSA. Statistically significant results were obtained when compared with the control group. * indicates the statistical significance (p <0.05) comparing to the control group.

IV. DISCUSSION

Inactivation of bacteria with aPDT and CAP is based on the formation of ROS production. Oxidative stress causes cell membrane damage via attacking macromolecules found on the membrane or causes intracellular damages inside bacterial cells [14, 18]. In this study, aPDT was conducted using ICG as a PS and 808 nm laser light, and CAP treatment was conducted via CAP device at 20 kHz and 30 kV. When the results of aPDT and CAP groups were evaluated according to the control group, reduction of bacterial number was observed in all aPDT and CAP groups. Maximum inactivation in which 3.5 log reduction of MRSA was obtained in 100 µM ICG-aPDT while 3.61 log reduction was obtained in 300 seconds CAP treatment. Other logarithmic inactivation values are 1.55 log, 2.01 log, 2.45 log, and 3.35 log for 10, 25, 50, and 200 µM ICG respectively. aPDT application with 200 µM ICG showed slightly decreased logarithmic reduction than 100 µM-aPDT but there is no critical difference between these two. It can be concluded that 100 µM ICG concentration at 84 J/cm^2 energy density of 808

nm laser light and 300 seconds CAP that was produced with 20 kHz and 30 kV treatment is enough to observe approximately 3.5 logarithmic reductions of MRSA.

In practice, CAP treatment may offer a more practical, quicker, and cheaper application than aPDT since CAP treatment does not require the usage and incubation time of a PS. Also, CAP treatment may enable higher logarithmic bacterial inactivation in defined exposure time than aPDT does. On the other hand, it can be difficult to adjust the plasma device to receive appropriate discharge while optical fiber of laser device adjusting is easier. In conclusion, aPDT and CAP treatment offer alternative treatment methods when suitable PS and its concentrations, the wavelength of the light, and energy density for aPDT and frequency, voltage and exposure time for CAP treatment. In this *in vitro* study, usage of 808 nm laser light at 84 J/cm^2 with 100 µM ICG for 593 seconds and CAP that was produced with 20 kHz and 30 kV for 300 seconds offers a suitable inactivation of MRSA for aPDT and CAP treatment respectively. Also, our results may light further *in vivo* and clinical trials.

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