Effect of Ozone-induced Diode Laser of Photodynamic Inactivation on Pseudomonas aeruginosa

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ORIGINAL ARTICLE

Effect of Ozone-induced Diode Laser of Photodynamic Inactivation on *Pseudomonas aeruginosa*

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ABSTRACT

Introduction: Pseudomonas aeruginosa bacteria can form biofilms in body tissues and cause many infectious diseases. This study aims to determine the effect of exposure to ozone and laser diode 403 nm at various ozone concentrations and energy density on the inactivation effectiveness of Pseudomonas aeruginosa ATCC 27853 biofilm. **Methods:** Samples were divided into 4 groups, namely C0 control group without treatment, T1 ozone treatment group. , T2 diode laser treatment group and T3 ozone treatment group with diode laser. Treatment used a 403 nm diode laser with a biofilm age of 48 hours. **Results:** The results of statistical tests showed that there was a significant photoinactivation effect on the percentage reduction of Pseudomonas aeruginosa bacterial biofilm. Optimal treatment in the 0.011 mg / L ozone exposure group with a flow time of 80 s resulted in a 42.77% reduction in biofilm. Optimal laser treatment at an energy density of 4.99 J / cm^2 for 300s long radiation time with 57.80% reduction percent. The combination of a 0.008 mg / L ozone treatment and a diode laser with an energy density of 4.99 J / cm^2 resulted in a 78.74% reduction in percentage, which was significantly different from the other treatment groups. So the combination of ozone treatment with a diode laser is effective in reducing the biofilm Pseudomonas aeruginosa bacteria.

Keywords: Ozone, Diode laser, Pseudomonas aeruginosa, Biofilms, Energy density

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INTRODUCTION

Infectious disease is a disease of concern, especially in developing countries. Pseudomonas aeruginosa bacteria can form biofilms in body tissues and cause many nosocomial infectious diseases in humans (1). The biofilm protects it against the penetration of antibiotics, antibodies, complement and phagocyte cells so that resistance to aeruginous antibiotics such as ampicillin with a sensitivity of 4%, septran by 9.2%, augmentin by 9.6% and lomefloxacin 25.8% (2). A biofilm is a collection of microbial cells attached irreversibly to a surface and encased in a matrix of extracellular polymeric substances (EPS) that it produces itself and shows phenotypic changes such as changes in growth rates and changes in gene transcription from planktonic cells or free cells (3). So it is necessary to do an alternative antimicrobial method that does not cause resistance, namely photodynamic inactivation (PDI) (4). PDI is part of photodynamic therapy, which is a therapeutic modality that utilizes light and photo sensitizing agents to obtain inactivation effects (PDI) (5) and cell modulation (6).

The combination of light and certain photosensitizers in PDI will cause photoinactivation of bacteria, thereby inhibiting cell metabolic activity due to damage to cytoplasmic membranes, due to peroxidation by reactive oxygen. Some bacteria naturally contain porphyrin compounds which act as photosensitizer molecules that are sensitive to light (7). Photoinactivation is the process of absorption of light by photosensitizer (porphyrin) in bacteria, which is then activated,

causing a reaction on the substrate. The success of photosensitation depends on the type and quantity of photosensitizer (8). The problem with photodynamic inactivation in biofilms is the low oxygen levels in the lower layer of the biofilm (9). So it is necessary to do another alternative to inactivate the bacteria Pseudomonas aeruginosa by adding ozone. According to Borelli and Bocci (2010) ozone and unsaturated fatty acids, antioxidants, thiol compounds, glutathione and albumin can be reacted. Thiol and glutathione compounds are one of the compounds contained in biofilms (10).

The addition of ozone is carried out to increase damage to biofilms because ozone has the ability to kill biofilms, kill viruses and fungi, improve tissue circulation, accelerate tissue epithelialization, and stimulate cell regeneration (11) which can diffuse the disytoplasmic biofilms so that biofilms experience erosion. Ozone is also able to provide a continuous reaction to produce reactive oxygen by producing H2O2 (12). Effective ozone concentration is at a concentration of 0.010 mg / L with a flow time of 60s and a percentage reduction of biofilm of 37.78. The combination of ozone and photodynamics provides a greater reduction potential for Staphylococcus aureus biofilm, namely at an ozone concentration of 0.010 mg / L, laser energy dose 28, 45 J / cm2 and 71.96% effectiveness compared to other treatment groups with the same energy dose (13).

This study aims to determine the effectiveness of photodynamic inactivation combined with ozone at various laser exposure times for the reduction of Pseudomonas aeruginosa biofilm.

MATERIALS AND METHODS

Biofilm Development Assay

The pure culture of Pseudomonas aeruginosa ATCC 27853 approximated 108 CFU/mL or 1.0 McFarland Standard was used for this study. 100µL bacteria culture was placed in 96-well microplate and was added 20 µL 20% sucrose solutions. The samples were placed to a shaker for 4 hours until appeared suspense. The samples were incubated for 44 hours on 39°C in the incubator.

The samples were treated accordance with each groups. The samples were treated with a crystal violet assay for measuring the survival biofilm. The samples were rinsed by posphat buffer saline (PBS) with pH 7.4 three times. The samples were added 100 µL 1% crystal violet solutions and rinsed by saline water. The samples were added 50µL 33% Glacial Acetic Acid (GAA) solution and measured using micro plate reader 595 nm (14).

Light Source

The light source used is laser diode from Thorlabs with an output wavelength of 401.4 nm, power output 2.49 mW and spot area 0.15 cm^2. The output power and wavelength of laser were measured with PM100D Powermeter (Thorlab) and JASCO CT10 monochromator.

Sample Treatments

The samples were divided into 4 groups, namely C0 untreated control group, T1 ozone treatment group, T2 diode laser treatment group and T3 ozone treatment group with diode laser. Treatment uses a 403 nm diode laser with a biofilm life of 48 hours. Samples was treated with variation of irradiation time of laser 60 s, 120 s, 180 s, and 240 s. The variation of ozone flow time is 20s, 40s, 60s, 80s.

The results of the data were the percentage of ratio of the dead deaths and the survival biofilm or the biofilm reduction (% CFU.ml-1). The data would be tested statistically using ANOVA one-way test. The significant value p = 0.05 was used as a determinant of statistical conclusion results. The response of biofilm reduction used T-test and ANOVA two-way test.

RESULTS

Laser characterization and ozone flow time

The output light source was important for determining the effectiveness of the treatment. Figure 1 was the output power of light sources. The output power of laser was (2.49724 ± 0.01) mW. The output power was used to determine the dose of light energy. The spectral output test aimed to obtain the diameter of spectral output that is suitable with the bacteria in the microplate. The microplate that was used for bacterial treatment has a diameter of 5 mm. The wavelength of light sources was shown in Figure 2. Based on the fitting result on Figure 2, the wavelength of laser was (401.4 ± 0.09984) nm. The energy density of light was shown on Table I.

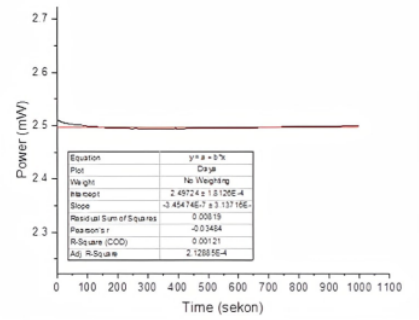

Figure 1 : The Output Power of the Diode Laser.

Table I : The Energy Density of Diode Laser

Laser Power Output (mW)	Laser beam area (cm^2)	Wavelength (nm)	Irradiation time (s)	Energy density (J/cm^2)
(2.497±0.01)	(0.15 ±0.01)	(401.4 ± 0.05)	60	0.998
			120	1.998
			180	2.996
			240	3.995
			300	4.994

Characterization of ozone concentration aims to determine the effective concentration of ozone gas to reduce Pseudomonas aeruginosa biofilm. Measurement of ozone concentration was carried out using the iodometric titration technique with $Na_2S_2O_3$ solution as the titration solution. The results of this ozone concentration measurement include measurements by calculating the volume value of $Na_2S_2O_3$ calculated during the iodometric titration. After obtaining the volume of Na2S2O3, the concentration value is sought using the formula:

O3 concentration = (mass O3) / (volume O3)
With mass O3 = 24 x V1 x Nt. The volume of O3 is obtained from experimental results, a color change occurs when the volume of ozone flowing is 240 mL.

Where, 24 = convention factor $\left(\frac{24000\ \frac{me}{L}}{1000\ \frac{mL}{L}}\right)$

VI = volume of $Na_2S_2O_3$ (mL)

Nt = normality of $Na_2S_2O_3$ (mg/me) = 0.035

The relationship between ozone concentration and ozone flow time (second) can be seen in Figure 3.

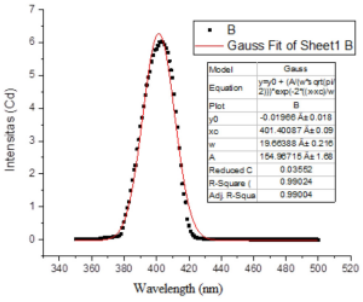

Figure 2 : The Wavelength of The Diode Laser.

PDI Laser and ozon treatment

Figure 4 shows the results of the absorption spectrum test for the Pseudomonas aeruginosa bacteria located at (403 ± 0.05) nm.

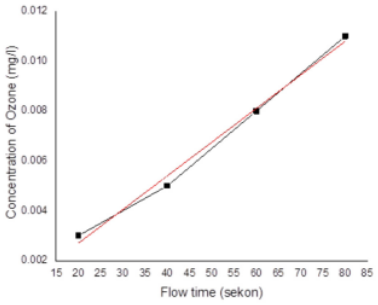

Figure 3 : Graph of the relationship between ozone concentration and ozone flow time.

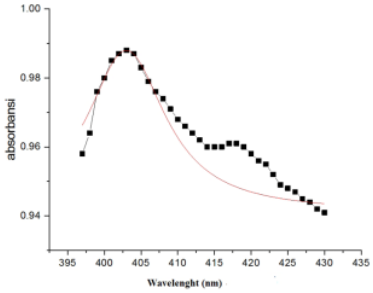

Figure 4 : Graph of Pseudomonas aeruginosa bacteria absorption spectrum test results.

Figure 5 shows the combined effect of diode laser irradiation and ozone on the viability of bacterial biofilms. The combination of laser with ozone with flow times of 60 s and 80 s showed a greater inactivation effect than diode laser treatment alone.

Figure 6 shows the effect of combined laser therapy with ozone on the percent reduction in bacterial biofilm.

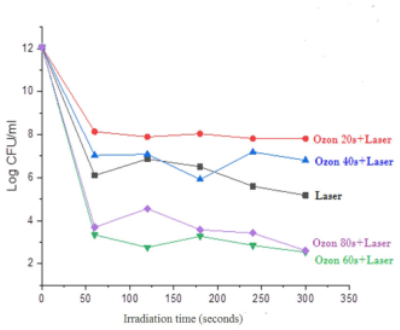

Figure 5 : Combination treatment of Laser with ozone to bacterial biofilm viability.

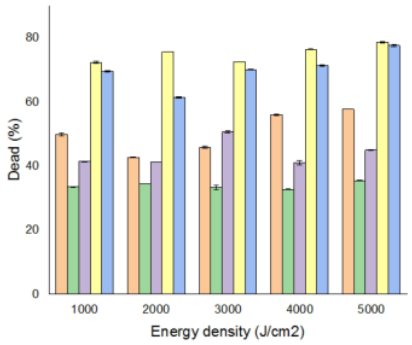

Figure 6 : Combination treatment of Laser with ozone to percentage of biofilm reduction.

DISCUSSION

Photodynamic inactivation (PDI) is a non-invasive optical treatment modality that is influenced by 3 main factors, namely light as an ionizer for chemical reaction processes, photosensitizer as a light-absorbing molecule, and free radicals that are reactive to biological systems such as cells [15].

The choice of wavelength of the light source used is very important in the process of absorbing light by photosensitizer molecules. The photosensitizer molecule used in this study is a porphyrin photosensitizer which is naturally produced by bacteria. Porphyrins are sensitive to light with a wavelength in the blue visible light area (16). The light emitted by the blue diode laser will be absorbed by the Pseudomonas aeruginosa bacteria because it has a wavelength spectrum that is close to the absorption spectra of these bacteria. The interactions that can occur are photochemical. The result of the photochemical process is ROS which is reactive so that it can damage the target (17).

The addition of ozone was carried out to optimize the reduction of Pseudomonas aeruginosa biofilm. Ozone has toxic or toxic properties when it interacts with microorganisms. ozone produces H_2O_2 and destroys the thin film on the biofilm. Treatment with ozone at an exposure time of 40 seconds caused a greater decrease in biofilm due to the formation of H_2O_2 (10). H_2O_2 molecules are oxidant molecules that strengthen immunity by producing free radicals. The formation of free radicals is obtained from the oxidative process of H2O2 electron transfer through the Fenton reaction. Ozone does not penetrate into the tissue but can spread to the cytoplasm of bacterial cells (17), resulting in damage to the bacterial cell membrane resulting in cell lysis. Bocci said, ozone can react with polyunsaturated fatty acids (PUFA), antioxidants, thiol compounds (-SH), glutathione (GSH) and albumin. The mechanism of inactivation occurs when biofilms bind to thiol protein (-SH) species due to their high nature and are influenced by denaturation, so enzymes, carbohydrates, DNA and RNA can be affected depending on the ozone dose (10).

The combination of laser with ozone flow time of 60s resulted in a reduction effectiveness of the biofilm by 78.74%. This is because the amount of reactive oxygen produced increases if there is H_2O_2 on the target which occurs during type II photochemical reactions, causing the reduction of biofilms to be even greater. The results of ozone treatment with a concentration of 0.011 mg / L and a flow time of 80s resulted in a biofilm reduction presentation of 42.77%. While laser irradiation treatment at a laser energy density of 4.99 J / cm2 with 300s irradiation time resulted in a bacterial biofilm presentation of 57.80%.

The mechanism of inactivation in bacterial biofilms involves an ozone effect and a laser photodynamic effect. Cell damage caused by the formation of ROS including singlet oxygen, hydrogen peroxide, and superoxide anion radicals. Ozone produces H_2O_2 in biofilms for diffusion in the cytoplasm resulting in the presence of toxic molecules that reduce biofilms while laser photodynamics will produce free radical products (ROS) which will damage biofilms and cause lysis of bacterial cells (18, 19). Pseudomonas aeruginosa is a Gram-negative, rod-shaped, flagella-shaped bacteria with a smooth surface. After reatment, normal cells experience various damages starting from the cell membrane. This causes the cytoplasm and cell organelles to react directly with toxic molecules (20, 21). Grisham uses the fluorescent method to detect H_2O_2 formation in the nucleus, mitochondria, endoplasmic reticulum and plasma membrane (18) Grisham. The combination of laser and ozone will increase the production of ROS thereby increasing the ability for bacterial inactivation and reduction of bacterial biofilms.

CONCLUSION

The results of statistical tests showed that there was a significant photoinactivation effect on the percentage reduction of Pseudomonas aeruginosa bacterial biofilms. Optimal treatment in the 0.011 mg / L ozone exposure group with a flow time of 80 s resulted in a 42.77% reduction in biofilm. Optimal laser treatment at an energy density of 4.99 J / cm^2 for 300s long radiation time with 57.80% reduction percent.

The combination of a 0.008 mg / L ozone treatment and a diode laser with an energy density of 4.99 J / cm^2 resulted in a 78.74% reduction in percentage, which was significantly different from the other treatment groups. So the combination of ozone treatment with a diode laser is effective in reducing the bacterial biofilm Pseudomonas aeruginosa.

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