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Original article

Degradation of *Fusobacterium nucleatum* biofilm and quantity of reactive oxygen species due to a combination of photodynamic therapy and 2.5% sodium hypochlorite

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ABSTRACT

Background: *The persistence of microorganisms in the root canal system is one of the leading causes of root canal treatment failure. Biofilms of putative pathogens hidden inside dentin tubules and other root canal ramifications may limit current disinfection protocols. Photodynamic therapy (PDT) with a wavelength of 628 nm can be used as an antimicrobial strategy that uses low-power laser energy to activate a non-toxic photosensitizer to produce singlet oxygen with the ability to kill microorganisms in root canals. Fusobacterium nucleatum was used because this bacterium is one of the bacteria involved in root canal infection.* ***Purpose:*** *The aim of this study was to compare the bactericidal efficacy of sodium hypochlorite (NaOCl) 2.5%, PDT, and a combination of PDT and NaOCl 2.5% against Fusobacterium nucleatum.* ***Methods:*** *Mature biofilm Fusobacterium nucleatum was divided into four groups according to the protocol of decontamination: K1 (negative control – biofilm), K2 (NaOCl 2.5%), K3 (PDT), and K4 (NaOCl 2.5% + PDT). Biofilm degradation was observed using optical density (OD) at 570 nm using a microplate reader. A reactive oxygen species quantity check was carried out using a nitroblue tetrazolium test, and OD observation was done with a microplate reader at 540 nm.* ***Results:*** *Group 4 (NaOCl 2.5% + PDT) showed more biofilm bacteria elimination than the other groups.* ***Conclusion:*** *A combination of PDT and NaOCl 2.5% can be considered an effective protocol for the elimination of Fusobacterium nucleatum. There is a potentiation relationship between NaOCl 2.5% and PDT FotoSan. Biofilm degradation occurs because of the effect of antibacterial NaOCl 2.5% and the irradiation effect of the Toluidine blue O photosensitizer.*

Keywords: *dentistry; Fusobacterium nucleatum; medicine; photodynamic therapy; sodium hypochlorite*
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INTRODUCTION

Biofilms are matrices of polysaccharides that cover populations of bacteria that are attached to each other or attached to surfaces or between surfaces. A biofilm is a thin layer in microorganisms that can consist of bacteria, fungi, and protozoa. Floating bacteria are also known as planktonic bacteria, which are prerequisites for the formation of biofilms. Bacteria in planktonic form are found inside and outside the biofilm. The composition of the biofilms consists of microorganism cells, extracellular products, and polysaccharides as adhesive materials, and water is the main constituent material of biofilms with a content of up to 97%. Biofilm matrices are quite complex and can contain a variety of non-biofilm materials such as mineral crystals, blood components, or soil components. The main component of biofilms other than microbial cells is extracellular polysaccharide substances that constitute up to 50–90% of biofilms.[1] The microbial cells in the biofilm communicate using a system called quorum sensing. Quorum sensing is the ability of microbes to measure cell density (the number of microbes) by measuring the amount of accumulated secretion of molecular signals produced by cells. This quorum sensing ability can provide bioluminescent capabilities, biofilm formation, or exoenzyme production in bacteria.[2,3] Polysaccharides produced by microbes to form biofilms include extracellular matrix polymers

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(EMP), i.e., polysaccharides removed from within cells. EMP synthesized by microbial cells differ in composition and chemical and physical properties. The physiology of biofilms is currently characterized using a system that has been simplified to single, dual, and multi-species bacterial-community-containing organisms.[4]

Microorganisms are the cause of pulp necrosis in 98.5% of cases while 1.5% are caused by trauma and chemical irritation.[5] The treatment indicated for cases of pulp necrosis is endodontic treatment.[6] Microorganisms in root canals can cause endodontic infections.[7] In general, various types of anaerobic bacteria predominate in endodontic infections. Lee et al.[8] have found that 70.3% of the bacteria in root canals are anaerobic bacteria and 29.7% are aerobic bacteria.

In this study, *Fusobacterium nucleatum* was used because this bacterium is one of the bacteria involved in root canal infection. *F. nucleatum* is an anaerobic bacterium in the form of non-spore, non-motile, and gram-negative bacteria. These bacteria are associated with spontaneous pain, tenderness to percussion, tenderness to palpation, gum swelling, hemorrhagic exudates, tooth mobility, inadequate restorations, and inadequate obturation.[9]

The irrigation material that is often used in endodontic treatments is sodium hypochlorite (NaOCl) 2.5%. Sodium hypochlorite neutralizes amino acids to form water and salt. Hypochlorous acid is a component contained in the solution of sodium hypochlorite. When in contact with organic tissue, hypochlorous acid will act as a solvent and will free chlorine. The liberated chlorine will join the amino protein group and form chloramine. Hypochlorous acid ($HOCl^-$) and hypochlorite ions (OCl^-) induce amino acid degradation and hydrolysis. The chloramination reaction between chlorine and the amino group forms chloramine that disrupts cell metabolism. Chlorine is a powerful oxidizing agent that provides antibacterial properties that inhibit bacterial enzymes by forming irreversible oxidation of sulfhydryl groups, essential enzymes of bacteria.[10,11] At a pH between 4 and 7, most of the chlorine will take the form of HOCl, the active and responsible part in bacterial inactivation, whereas, at a pH above 9, it will be dominated by OCl^-, whose nature is less active.[12]

Saponification, neutralization of amino acids, and chloramine reactions that occur in microorganisms and organic tissues will provide antimicrobial effects and tissue dissolution processes.[11] In addition, hypochlorite preparations are sporicidal and virucidal in nature, thus will produce a greater dissolving effect on necrotic tissues than in vital tissues. This underlies the use of sodium hypochlorite solution as the irrigation material.[12]

The photosensitizer is a cation (positively charged) that will bind to the bacterial cell wall that is an anion (negatively charged). From this bond, there will be an electrostatic interaction between the photosensitizer and the bacterial cell wall, namely the release of Ca^{2+} and Mg^{2+} ions from the cell so that the cell wall is weaker and its permeability increases. The increase in the permeability of the bacterial cell wall causes the photosensitizer cation to enter the cytoplasmic membrane of the bacteria so that there is a deeper disorganization of the permeability barrier. This will increase the absorption and binding of photosensitizer cations with bacterial plasma membranes so that photosensitizer bonds occur with bacterial plasma membranes.[13,14] The irradiation in the photosensitizer will be absorbed, which produces two types of mechanisms. In mechanism type I, electron transfer occurs between the photosensitizer and the substrate so that it will produce radical ions called reactive oxygen species (ROS) that consist of superoxide anions, hydroxyl radicals, and hydrogen peroxide. These ions are oxidative to cells. In mechanism type II, there is an electron transfer between the photosensitizer and the oxygen receptor that produces a singlet of oxygen, which is a reactive form of oxygen and a powerful oxidative agent.[13,14] The results of both mechanisms can cause several effects, including crosslink lengthening of plasma membrane proteins, inactivation of the enzyme Nicotinamide adenine dinucleotide + hydrogen succinate, and lactate dehydrogenase, damaging the balance of K^+ ions and other ions and damaging the DNA of bacterial cells. As a result of some of these effects, the growth of bacteria can be inhibited so that the target bacteria will die.[13,15,16]

ROS is one of the free radicals derived from oxygen.[17] ROS is a radical form of an unpaired atom. ROS is often used in biomedical free radical terms. Included in the ROS category are not only free radicals carrying oxygen but also molecules that do not have paired electrons such as hydrogen peroxide, hypochlorous acid, and peroxynitrite anion acid (ONOO-). Such ROS, especially superoxide radicals, are constantly produced by the body.[18] Superoxide radicals are the most widely produced free radicals in the body and are derived from the reduction of one unpaired free electron in the outer shell layer.[19] These radicals are produced by phagocytic cells and serve to kill bacteria. In addition to the formation of superoxide radicals in macrophage and neutrophil cells, production of extracellular also occurs in small quantities as intercellular signaling molecules by several other cell types such as endothelial cells, lymphocytes, and fibroblasts.[18]

Photodynamic inactivation is a therapy modality that uses a photosensitizer agent, a light source, and oxygen to produce ROS.[20] Photodynamic therapy (PDT) can be used as an antimicrobial strategy that uses low-power laser energy to activate a non-toxic photosensitizer to produce singlet oxygen with the ability to kill microorganisms in root canals.[21] Research conducted by Neves et al. stated that the combination of PDT with irrigation agents was more effective in killing bacteria than either NaOCl alone or PDT alone.[22] PDT has significant effectiveness in the elimination of bacterial biofilms when combined with a disinfecting agent. PDT can help reach root canal areas of teeth that are not touched by mechanical preparation of endodontic instruments or NaOCl irrigating

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solutions in conventional standard root canal preparation procedures.[21–23] Absorption by photosensitizer is a photophysical process to produce ROS and singlet oxygen. The laser energy absorbed by the photosensitizer molecule will then activate the occurrence of photochemical reactions, resulting in a radical product that damages the bacterial cell. The larger the photon intensity and the longer the exposure, the more photosensitizer will be activated to produce various ROS that has an effect on the number of bacterial deaths.[24]

One of the most common and frequently used root canal irrigation agents to date is 2.5% NaOCl. Bacteria can penetrate the root dentinal tubules to a depth of 1000 µm, while the irrigation disinfection material only reaches a depth of 100 µm. This allows re-infection and causes root canal treatment failure.[25,26] Therefore, new methods in endodontic treatment are needed to eliminate pathogenic bacteria to achieve successful root canal treatment.[27,28] Based on the description above, this research was conducted to determine the biofilm degradation and the quantity of ROS in the *F. nucleatum* biofilm due to the combination of PDT and 2.5% NaOCl irrigation.

MATERIALS AND METHODS

Ethical Clearance Certificate: 027/HRECC.FODM/I/2021. This study was approved by the Ethics Committee of the Faculty of Dental Medicine, Universitas Airlangga. The culture of *F. nucleatum* ATCC 25586 was obtained from the *F. nucleatum* bacterial stock at the Faculty of Dental Medicine Research Center, Airlangga University, Surabaya. The bacterial preparations were incubated at 37°C in an anaerobic atmosphere for 24–48 hours. The bacterial culture was diluted into Tryptic Soy Broth (TSB) media and equated with the McFarland standard of 1.5 x 108 CFU/ml, then 200 µl was placed into a 96-well microtiter plate. The ROS was calculated using a nitroblue tetrazolium (NBT) test with a microplate reader.[29] Group 1: The untreated control group contained only the *F. nucleatum* biofilm. Group 2: 100 µl of 2.5% NaOCl irrigation solution was dripped into the well containing the *F. nucleatum* biofilm. 100 µl (1 mg mL^{-1}) of NBT solution was then dripped into the well, and incubation was carried out for 30 minutes at a temperature of 37°C. Next, 100 µl of TSB was dripped into the well, followed by 20 µl of hydrochloric acid (HCl) (0.1 M), and finally, 50 µl of dimethyl sulfoxide (DMSO) was dripped into the well. The 96-well microtiter plate was inserted into a microplate reader with a wavelength of 570 nm for OD observations. Group 3: A photosensitizer in the form of 100 µl of Toluidine blue O liquid was dripped into the well for 60 seconds and then irradiated with PDT using FotoSan for 50 seconds. 100 µl (1 mg ml^{-1}) of NBT solution was then dripped into the well, and incubation was carried out for 30 minutes at a temperature of 37°C. 100 µl of TSB was dripped into the well, followed by 20 µl of HCl (0.1 M), and finally, 50 µl of DMSO was dripped into the well. The 96-well microtiter plate was then inserted into a microplate reader with a wavelength of 570 nm for OD observations.

A total of two 96-well microtiter plates were used to observe the biofilm degradation and quantities of ROS. The 96-well microtiter plates were grouped into four groups, with each group containing eight samples. Group 1 was the control group and contained only the *F. nucleatum* biofilm. Group 2 contained *F. nucleatum* biofilm irrigated with 2.5% NaOCl. Group 3 contained *F. nucleatum* biofilm and was given a Toluidine blue O photosensitizer and PDT FotoSan irradiation. Group 4 contained *F. nucleatum* biofilm, and 100 µl of 2.5% NaOCl irrigation solution was dripped into the well. A photosensitizer in the form of 100 µl of Toluidine blue O liquid was dripped into the well for 60 seconds and then irradiated with PDT FotoSan for 50 seconds. 100 µl (1 mg mL^{-1}) of NBT solution was dripped into the well, and incubation was carried out for 30 minutes at a temperature of 37°C. Next, 100 µl of TSB was dripped into the well, followed by 20 µl of HCl (0.1 M), and finally, 50 µl of DMSO was dripped into the well.

For the results of the study, the means and standard deviations of each group were calculated. The Shapiro–Wilk test for normality was used to determine the population data distribution of each group. After concluding that the data were normally distributed, Levene's test for homogeneity was carried out to determine the similarity of the variations in the sample groups. To compare the differences across each group, Tukey's HSD test was followed using an independent T-test for the differences in the two group tests.

RESULTS

The data obtained come from the OD observations through a microplate reader of each bacterium in each group. The bar chart for the means and standard deviations of biofilm degradation and the quantities of ROS in each group can be seen in Figure 1.

In the degradation biofilm group before data analysis, normality and homogeneity tests were performed. The Shapiro–Wilk normality test was performed and a p-value of $p = 0.674$ ($p > 0.05$) was found for the control treatment group, $p = 0.958$ for the NaOCl treatment group, $p = 0.821$ for the PDT FotoSan treatment group, and $p = 0.940$ for the combination treatment group, meaning that all data were normally distributed. Levene's test was then followed to determine the homogeneity of the data. The results of Levene's test showed $p = 0.001$ ($p < 0.05$). This shows that the treatment group had unequal homogeneity of variance (not homogeneous). From the results above, it was found that all treatment groups were normally distributed and had unequal variances (not homogeneous), thus the independent sample statistical test was carried out. As shown in Table 1, the biofilm degradation of all treatment groups had a p-value of $p < 0.05$. This indicates that there was a

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significant difference in the biofilm degradation across all treatment groups.

In the ROS quantity group, normality and homogeneity tests were carried out. The Shapiro–Wilk normality test was performed and a p-value of p = 0.767 (p > 0.05) was found for the control treatment group, p = 0.734 for the NaOCl treatment group, p = 0.317 for the PDT FotoSan treatment group, and p = 0.340 for the combination treatment group, meaning that all data were normally distributed. Next, Levene's test was followed to determine the homogeneity of the data. The results of Levene's test showed p = 0.999 (p > 0.05). This indicates that the treatment groups had the same homogeneity of variance (homogeneous).

To determine the difference in the quantities of ROS across treatment groups, the ANOVA statistical test was performed. The results of the ANOVA test obtained a p-value of p = 0.001 (p < 0.05). This indicates that there was a difference in the quantities of ROS across the treatment groups. To find out the differences across treatment groups, Tukey's HSD test was carried out statistically. The results of Tukey's HSD statistical test can be seen in Table 2. The quantities of ROS across all treatment groups had a p-value of p < 0.05. This indicates that there was a significant difference in the quantities of ROS across all treatment groups.

DISCUSSION

In this study, Fusobacterium nucleatum was used because this bacterium is one of the bacteria involved in root canal infection. Fusobacterium nucleatum is an anaerobic bacterium in the form of non-spore, non-motile, and gram-negative bacteria. These bacteria are associated with spontaneous pain, tenderness to percussion, tenderness to palpation, gum swelling, hemorrhagic exudates, tooth mobility, inadequate restorations, and inadequate obturation.[9]

The results of the statistical analysis show that the average biofilm degradation of the NaOCl-only group (0.68) was lower than the control group (1.06). This is in accordance with the results of the research conducted by Canga and Subashi,[30] which stated that 2.5% NaOCl has a better antibacterial effect than 2% chlorhexidine and can denature bacterial toxins and dissolve organic tissue. In addition, Sahebi et al.[31] also stated that NaOCl had better bacterial inhibition than aloe vera and normal saline.

A study conducted by Janani et al.[32] showed that 2.5% NaOCl was more effective in eliminating bacteria from infected root canals than PDT. The results of this study showed a significant decrease in the number of bacteria in the NaOCl group compared to the control group, and almost no bacteria were detected in the NaOCl group after using the polymerase chain reaction technique.[32]

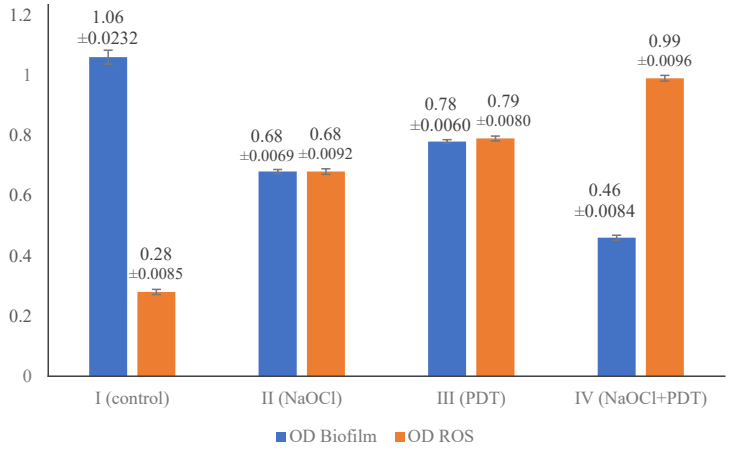

Figure 1. The mean and standard deviation of biofilm degradation and ROS.

Table 1. The results of the independent sample test for biofilm degradation

	NaOCl	PDT FotoSan	Combination
Control	p = 0.001	p = 0.001	p = 0.001
NaOCl		p = 0.001	p = 0.001
PDT FotoSan	p = 0.001		p = 0.001

Table 2. The results of Tukey's HSD test for quantities of ROS

	NaOCl	PDT FotoSan	Combination
Control	p = 0.001	p = 0.001	p = 0.001
NaOCl		p = 0.001	p = 0.001
PDT FotoSan			p = 0.001

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The mean quantity of ROS in the NaOCl-only group (0.68) was higher than the control group (0.28). This is in accordance with the statement by Zhang et al.,[33] who said that exposure to disinfecting agents can induce increased levels of ROS, bacterial membrane damage, ROS-mediated DNA damage, and an increased stress response. In addition, according to Harris[34] and Mohmmed et al.,[35] an increase in ROS causes oxidative stress in cells that causes lipid peroxides, impaired protein synthesis, and DNA damage.

NaOCl is an irrigation agent used in endodontic procedures that has antimicrobial properties and can dissolve organic tissue.[36] NaOCl produces hypochlorous acid, which is an oxidizing agent that acts as a solvent. When NaOCl comes into contact with tissue it will produce hydroxyl ions and hypochlorous acid.[37] In addition, NaOCl has a high pH, which triggers the release of hydroxyl ions.[34–36] The release of hydroxyl ions can cause cell death through two mechanisms, namely by increasing ROS directly or by decreasing adenosine triphosphate.[38]

However, this antimicrobial mechanism of NaOCl becomes ineffective against pathogenic bacteria in the anatomical area that is difficult to reach by irrigation solutions or mechanical preparation by endodontic instruments at the root canal cleaning stage. In addition, dentinal tubules have a narrow lumen (1–2 um) and are 2–3 mm in length, making them a challenge for disinfection materials. The minimal and maximal bacterial penetration depths into the dentinal tubules were 1 µm and 1480 µm, respectively, with a mean of 167 µm.[39] Thus, a complementary/supportive technique to increase the effectiveness of root canal treatment is needed.[40]

The average biofilm degradation in the PDT-only group (0.78) was lower than the control group (1.06). The results of this study showed that PDT only could eliminate Fusobacterium nucleatum biofilm. This is in accordance with a study conducted by Bibova et al.,[41] which stated that FotoSan can be considered as an additional procedure to kill bacteria in the root canal system after standard endodontic treatment. In this study, PDT FotoSan was used to disinfect root canals. FotoSan uses red light with a wavelength of 630 nm. Photodynamic therapy might be useful as an alternative approach for antimicrobial treatment.

The photosensitizer used in this study was Toluidine blue O. The Toluidine blue O photosensitizer contains phenothiazine. Phenothiazine is a cation that will bind to an anion bacterial cell wall. From this bond, there will be an electrostatic interaction increasing the permeability of bacterial cell walls that causes the Toluidine blue O cation to permeate more into the bacterial cytoplasmic membrane to disorganize the barrier of permeability further. Toluidine blue O research shows that it also has antibacterial power because it can interact with lipopolysaccharides of bacterial cell membranes even without irradiation. When irradiating with a wavelength of 630 nm, there will be a maximum absorption of photosensitizer fluid so that PDT photo cations occur to kill bacteria better compared to the use of photosensitizer fluid without irradiation.

The mean ROS quantity in the PDT-only group (0.79) was higher than the control group (0.28). This is in accordance with the statement by Abrahamse and Hamblin[42] that said that PDT uses a non-toxic photoactive dye called a Toluidine blue O photosensitizer that is activated with visible light to produce ROS.

Photodynamic mechanisms with Toluidine blue O involve the interaction of light with agents that produce oxygen. The irradiation with light at a certain wavelength according to the absorption peak of the photosensitizer will produce energy.[43] The effectiveness of quantum yield for producing a particular ROS type depends on the photosensitizer, the availability of oxygen, and the reaction environment.[44] The energy transferred from the activated photosensitizer will be forwarded to the available oxygen so that it is transformed into singlet oxygen as a very reactive and toxic oxygen species. Contact between the singlet oxygen and bacterial cell walls will cause oxidative damage to bacterial cells by inducing ROS production. ROS is a free radical of oxygen that can damage the microorganism membrane and accelerate the death of microorganisms.[45] The concentration of radical ions and many oxygen singlets will cause damage to the lysosomes, mitochondria, and plasma membranes of larger bacterial cells, leading to more dead bacterial cells.[42]

However, PDT alone was less effective in eliminating bacteria. This is in accordance with a study conducted by Damasceno and Araújo[46]; PDT is a supporting technique to improve root canal disinfection after biomechanical preparation of endodontic treatment. In addition, according to Souza et al.,[47] the main approach for bacterial elimination is conventional chemomechanical preparation with the addition of chemicals such as NaOCl. Thus, further research was conducted on the combination of PDT and NaOCl.

The average ROS quantity of the combination group (0.99) was higher than the control group (0.28), the single NaOCl group (0.68), and the single PDT group (0.79). Research conducted by Vaziri et al.[40] and Souza et al.[47] said that the combination of PDT and 2.5% NaOCl was the best choice to maximize disinfection. Vaziri et al.[40] conducted a study of 60 single-rooted teeth and found that after the combined treatment of PDT and 2.5% NaOCl, no live bacteria were found.

Research conducted by Ng et al.[48] said that the combination of 6% NaOCl and PDT with Toluidine blue O was better than 6% NaOCl alone. In their study, Ng et al.[48] used 52 necrotic teeth and radiographically showed apical periodontitis. The results showed that 86.5% of the root canals were free of bacteria after the combination of the chemomechanical method and PDT with Toluidine blue O, while in the chemomechanical-only group, only 49% were free of bacteria.[48]

According to Bumb et al.,[49] PDT has the ability to penetrate the dentinal tubules in the root canal wall to a depth of 890–900 µm, while NaOCl was only able to penetrate to a depth of 60–150 µm. Research conducted by Hopp and Biffar[50] showed that exposure to red light

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with a wavelength of 628 nm can activate Toluidine blue O to produce ROS. PDT has significant effectiveness in the elimination of bacterial biofilms when combined with a disinfecting agent. PDT can help reach areas that are not touched by mechanical preparation or NaOCl irrigating solutions in conventional procedures. In conclusion, there is a potentition relationship between NaOCl 2.5% and PDT FotoSan. Biofilm degradation occurs because of the effect of antibacterial NaOCl 2.5%, and the irradiation effect of the Toluidine blue O photosensitizer means that there is a transfer of electrons between the photosensitizer and substrate. ROS increases due to the electron configuration of the oxygen molecule being in an excited (unstable) state. Excited oxygen tends to strive for a stable electron state; therefore, this oxygen will interact with the surrounding biological system. The interaction that occurs between the excited oxygen and biological systems such as bacterial cells will damage these systems and cell structures.

Research conducted by Hopp and Biffar[50] showed that exposure to red light with a wavelength of 628 nm can activate Toluidine blue O to produce ROS. The resulting ROS produced is very reactive, such as superoxide oxygen singlets and hydroxyl radicals that destroy bacteria.[51] In addition, the resulting ROS can target and destroy biomolecules in the bacterial cell wall.[52] PDT has significant effectiveness in the elimination of bacterial biofilm when combined with disinfection. PDT can help reach the root canal area that is not touched by mechanical preparation of endodontic instruments or NaOCl irrigation solutions in the preparation of root canal treatments.

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