Photolysis of Hydrogen Peroxide, an Effective Disinfection System via Hydroxyl Radical Formation

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Received 2 June 2010/Returned for modification 28 July 2010/Accepted 27 September 2010

The relationship between the amount of hydroxyl radicals generated by photolysis of H2O2 and bactericidal activity was examined. H2O2 (1 M) was irradiated with laser light at a wavelength of 405 nm to generate hydroxyl radicals. Electron spin resonance spin trapping analysis showed that the amount of hydroxyl radicals produced increased with the irradiation time. Four species of pathogenic oral bacteria, Staphylococcus aureus, Aggregatibacter actinomycetemcomitans, Streptococcus mutans, and Enterococcus faecalis, were used in the bactericidal assay. S. mutans in a model biofilm was also examined. Laser irradiation of suspensions in 1 M H2O2 resulted in a >99.99% reduction of the viable counts of each of the test species within 3 min of treatment. Treatment of S. mutans in a biofilm resulted in a >99.99% reduction of viable counts within 3 min. Other results demonstrated that the bactericidal activity was dependent on the amount of hydroxyl radicals generated. Treatment of bacteria with 200 to 300 μM hydroxyl radicals would result in reductions of viable counts of >99.99%.

Oral infectious diseases such as dental caries, periodontitis, and endodontic infections, all of which affect teeth and periodontal tissue, are caused by bacteria that inhabit the oral cavity. It is estimated that 150 or more different species of bacteria inhabit the human oral cavity (27). Of these bacteria, it is suggested that specific species play an important role in the etiology of diseases. For instance, it is known that Streptococcus mutans generates acid, which causes a loss of mineral from teeth, so-called dental caries (18). Similarly, Aggregatibacter actinomycetemcomitans is known as one of periodontal pathogenic species (ROS), has one unpaired electron in its structure, so that it is apt to deprive other substances of an electron; e.g., it easily oxidizes other substances (8). It is known that the hydroxyl radical is generated in the immunological response in our laboratory. The hydroxyl radical, one of the reactive oxygen species (ROS), has one unpaired electron in its structure, so that it is apt to deprive other substances of an electron; e.g., it easily oxidizes other substances (8). It is known that the hydroxyl radical is generated in the immunological response in the body to kill invading bacteria (6, 9). Therefore, it is believed that the disinfection system based on the bactericidal effect of artificially generated hydroxyl radicals can be used for clinical dentistry. There are several hydroxyl radical generation systems, such as the Fenton reaction (14), the Haber-Weiss reaction (12), sonolysis of water (15), and photolysis of H2O2 (4). Since the Fenton reaction and the Haber-Weiss reaction involve some chemicals with transition metals such as ferrous compounds, it is likely difficult to terminate the generation of hydroxyl radicals in the oral cavity after treatment. On the other hand, sonolysis of water and photolysis of H2O2 are
simple reaction systems, each including one chemical, water or H$_2$O$_2$, and it is possible to terminate the generation of hydroxyl radicals by means of cessation of ultrasound or light irradiation. Furthermore, photolysis of H$_2$O$_2$ may be more applicable to a hydroxyl radical generation system at narrow lesion sites in the oral cavity than sonolysis of water because recent technology makes it possible for H$_2$O$_2$ and laser light to be delivered to the lesion site.

The purpose of the present study was to evaluate the bactericidal effect of hydroxyl radicals generated by photolysis of H$_2$O$_2$ on four species of oral bacteria. In addition, we discuss the amount of hydroxyl radicals required to disinfect these oral bacteria based on a quantitative analysis of hydroxyl radicals using the electron spin resonance (ESR) spin trapping technique.

Materials and Methods

Reagents. Reagents were purchased from the following sources: 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) from Labote (Tokyo, Japan), H$_2$O$_2$ from Santoku Chemical Industries (Tokyo, Japan), and 4-hydroxy-2,2,6,6-tetramethylpiperidine (TEMPOL) from Sigma Aldrich (St. Louis, MO). All of the other reagents used were of analytical grade.

Optimization of ESR analysis of hydroxyl radicals generated by photolysis of H$_2$O$_2$. A continuous-wave laser device (RV-100; Ricoh Optical Industries, Hanamaki City, Japan) was used to photolyze H$_2$O$_2$ in this study. The optimal concentration of DMPO, a spin trap agent, for the ESR spin trapping technique was examined according to the method described in our previous report (19) to accurately quantify the amount of hydroxyl radicals generated by photolysis of H$_2$O$_2$. In brief, H$_2$O$_2$ and DMPO were mixed in the wells of a 96-well microplate to final concentrations of 1.0 M for H$_2$O$_2$ and 0 to 400 mM for DMPO. One molar H$_2$O$_2$ corresponds to 3.4% (wt/wt), which is a concentration used in the oral cavity as a disinfectant. Immediately after mixing, the mixture was irradiated with light at a wavelength of 405 ± 5 nm and an output power of 300 mW from an indium gallium nitride laser diode for 30 s. The diameter of the irradiation field was set to equal that of the well (6.4 mm) so that almost all of the light could pass through the test solution. Thus, the energy density was calculated to be 940 mW/cm$^2$. After irradiation, the sample was transferred to a quartz cell for ESR spectrometry and the ESR spectrum was recorded on an X-band ESR spectrometer (JES-FA-100; JEOL, Tokyo, Japan). The measurement conditions for ESR were as follows: field sweep, 330 to 540 mT; field modulation frequency, 100 kHz; field modulation width, 0.1 mT; amplitude, 80; sweep time, 2 min; time constant, 0.03 s; microwave frequency, 9.420 GHz; microwave power, 4 mW. TEMPO (20 µM) was used as a standard sample to calculate the concentration of DMPO-OH, and the ESR spectrum of manganese (Mn$^{2+}$), with which the concentration of DMPO-OH was saturated within a few minutes of laser irradiation in the experiment described above, further investigation was performed to verify whether the rate of hydroxyl radical generation from 1.0 M H$_2$O$_2$ was constant or decreased with irradiation time. In brief, 180 µl of 1.1 M H$_2$O$_2$ was added to a well without DMPO. After laser irradiation for 180 s, 20 µl of DMPO was added to make final concentrations of 1.0 M for H$_2$O$_2$ and 300 mM for DMPO. Immediately after the addition of DMPO, the sample was further irradiated with a laser for 30 s and the ESR measurement was performed. The amount of DMPO-OH was compared to that from 30 s of laser irradiation of 1.0 M H$_2$O$_2$ containing 300 mM DMPO without the prior laser irradiation. ESR measurement and data analysis were performed as described above.

Hydroxyl radical generation from water with or without laser irradiation and from autolysis of H$_2$O$_2$. The amount of hydroxyl radicals generated by photolysis of 1.0 M H$_2$O$_2$ by 30 s of laser irradiation at an output power of 300 mW was compared to that generated from pure water without laser irradiation, from photolysis of pure water using the same laser condition (output power of 300 mW and irradiation time of 30 s), and from autolysis of 1.0 M H$_2$O$_2$. The autolysis of 1.0 M H$_2$O$_2$ was measured up to 30 min. To further confirm if hydroxyl radical generation continues after cessation of laser irradiation, DMPO-OH was determined following the addition of DMPO to the reaction system after 180 s of laser irradiation of 1.0 M H$_2$O$_2$. DMPO was used at 300 mM. ESR measurement and data analysis were performed as described above.

Bactericidal test. Table 1 shows four major oral infectious diseases and the pathogens relevant to the diseases. A representative bacterial species selected from each oral infectious disease was used for bactericidal testing. The stock culture strains of four bacterial species were obtained from the American Type Culture Collection (Manassas, VA) and the Japan Collection of Microorganisms, RIKEN BioResource Center (Wako, Japan). That is, Staphylococcus aureus ATCC 29232, Enterococcus faecalis ATCC 29212, S. mutans JCM 5705, and A. actinomycetemcomitans JCM 2343 were used in this study. All of the bacterial species were cultured on brain heart infusion (BHI) agar (Becton Dickinson Labware, Franklin Lakes, NJ). S. aureus was cultured aerobically, and the other bacteria were cultured anaerobically using AnaeroPack (Mitsubishi Gas Chemical Company, Tokyo, Japan) at 37°C.

A bacterial suspension of each species was prepared in sterile physiological saline from cultures grown on BHI agar at 37°C for 1 to 4 days. The suspension was diluted to a concentration of 1 to 2 × 10$^8$ cells/ml and a microplate well (96 well) was added to a microplate test tube containing 200 µl of 1.1 M H$_2$O$_2$ diluted with sterile physiological saline to make final concentrations of 1 × 10$^7$ cells/ml for bacteria and 1.0 M for H$_2$O$_2$. Immediately after mixing, the suspension was irradiated with a laser light with an output power of 300 mW (940 mW/cm$^2$) for 1, 2, or 3 min. After irradiation, 50 µl of the sample was mixed with an equal volume of sterile catalase solution (5,000 U/ml) to terminate the bactericidal effect of the remaining H$_2$O$_2$. A 10-fold serial dilution of the mixture was then prepared using sterile physiological saline and 10 µl of the dilution was seeded onto BHI agar to evaluate the number of viable microorganisms in the suspension. The agar medium was cultured for 48 h under the conditions described above for each bacterial species, and then the number of CFU/ml was determined. The bactericidal effects of the hydroxyl radicals generated by laser irradiation of 1.0 M H$_2$O$_2$ [expressed as H$_2$O$_2$ (L)] was compared to the effects of (i) 1.0 M H$_2$O$_2$ alone [H$_2$O$_2$ (L)], (ii) laser irradiation alone [H$_2$O$_2$ (L)], and (ii) no treatment [H$_2$O$_2$ (L)]. For the L condition, the samples were kept without laser irradiation on a clean bench to avoid contamination. For the H$_2$O$_2$ (L) condition, sterile physiological saline was added to the reaction system instead of H$_2$O$_2$. All tests were performed in triplicate.

The bactericidal effect of hydroxyl radicals was also evaluated using an experimental biofilm model. In a microplate well, 20 µl of a bacterial suspension of S. mutans adjusted to 2 × 10$^7$ cells/ml was mixed with 180 µl of BHI broth containing 0.5% sucrose. The microplate was then incubated anaerobically as described above for 24 h to allow biofilm to form on the bottom and side wall of the well. After the incubation, the medium containing unattached bacteria was removed. The well was gently washed three times using sterile physiological...
saline and filled with 200 μl of 1.0 M H2O2. Immediately after the addition of H2O2, the well containing the biofilm was irradiated with a laser with an output power of 300 mW for 1 to 5 min. Control groups were set up in the same way as described above, i.e., H+L(−), H−L(+), and H−L(−). H2O2 was removed after treatment, and 200 μl of sterile physiological saline and 10 μl of 5,000-U/ml sterile catalase solution were mixed in the well. The biofilm was scraped using a sterile cotton swab and suspended in the solution. A 10-fold serial dilution of the solution was prepared, and 10 μl of the dilution was plated on BH agar. Agar plates were incubated anaerobically as described above to determine the number of CFU/well.

The condition of the biofilm before the bactericidal treatment was observed by scanning electron microscopy (SEM). A portion of the microplate wells were postfixed with 2% (wt/vol) OsO4 in 0.2 M cacodylate buffer (pH 7.4) at 4°C overnight. After washing with 0.1 M cacodylate buffer (pH 7.4), the sample fixed with 2.5% (wt/vol) glutaraldehyde in 0.2 M cacodylate buffer (pH 7.4) at 4°C for 2 h and then routinely processed for SEM observation. In brief, the postfixed specimens were dehydrated through an ethanol series, replaced with isoamyl acetate, and dried with liquid CO2 by using an ID-2 critical-point dryer (Eiko Ltd., Tokyo, Japan). Each dried specimen was coated with gold by using an SC7640 sputter coater (Quorum Technologies Ltd., Hailsham, United Kingdom) and observed by using a Topcon DS801 scanning electron microscope at 8 kV.

Effect of H2O2 concentration on hydroxyl radical generation and bactericidal effect. Since it was believed that the generation of hydroxyl radicals depends on the concentration of H2O2, an effective concentration range of H2O2 for a bactericidal effect was investigated in relation to the generation of hydroxyl radicals. Samples containing different concentrations of H2O2 (0, 0.25, 0.5, and 1.0 M) and 300 mM DMPO were irradiated with a laser for 30 s. ESR analysis of the sample was performed as described above. E. faecalis, which showed the highest resistance to the hydroxyl radical disinfection system in the above-described experiments, was used in the bactericidal test. The bacterial suspension and H2O2 were mixed and irradiated with a laser for 3 min. Total viable counts of bacteria were evaluated as described above.

RESULTS

Optimization of ESR analysis of hydroxyl radicals generated by photolysis of H2O2. When 1.0 M H2O2 was irradiated with a laser light with a wavelength of 405 nm, DMPO-OH (aH = aH = 1.49 mT) was detected. The amount of DMPO-OH increased with the concentration of DMPO to a certain extent and then was saturated at a DMPO concentration of around 300 mM, indicating that the optimal concentration of DMPO for quantification of hydroxyl radicals in this generation system was 300 mM.

Relationship between laser irradiation time and hydroxyl radical generation. It was observed that DMPO-OH generated by the photolysis of 1.0 M H2O2 increased linearly with the irradiation time up to 60 s (Fig. 1). Then the actually measured DMPO-OH was gradually saturated. However, hydroxyl radical generation from the photolysis of 1.0 M H2O2 was exposed to laser irradiation for 180 s in advance was not reduced compared to that from H2O2 without the prior laser irradiation. Since the laser irradiation for 180 s did not affect the DMPO-OH generation rate per unit of time, hydroxyl radicals are generated in accordance with a linear proportion, as shown in Fig. 1, even though the amount of DMPO-OH is saturated.

The output power of the laser affected the rate of hydroxyl radical generation (Fig. 1). The slope values of lines indicate the rates of DMPO-OH generation (μM s−1). When the equations of the lines were calculated by least squares using plots of up to 30 s, the slope values were 0.58 for 100 mW, 1.07 for 200 mW, and 1.41 for 300 mW, indicating that the hydroxyl radical generation rate was in proportion to the output power of the laser. Therefore, a power output of 300 mW was used in the bactericidal test.

Hydroxyl radical generation from water with and without laser irradiation and from autolysis of H2O2. Contrary to the photolysis of H2O2, only small amounts of DMPO-OH from pure water without laser irradiation, photolysis of pure water, and autolysis of 1.0 M H2O2 were detected. The mean concentrations of DMPO-OH detected in pure water, pure water irradiated with a laser, and 1.0 M H2O2 without laser irradiation were 0.1, 0.1, and 0.2 μM, respectively. The DMPO-OH generated by autolysis of 1.0 M H2O2 increased in a time-dependent manner. The amount of hydroxyl radicals (1.2 μM) generated by autolysis of H2O2 even after 30 min was, however, much smaller than that produced by photolysis of H2O2. The photolysis of H2O2 could be terminated by cessation of irradiation as described below. H2O2 (1.1 M) without DMPO was irradiated with a laser for 180 s. Immediately after the irradiation, DMPO was added to the H2O2 to make final concentrations of 1.0 M H2O2 and 300 mM DMPO, and DMPO-OH was determined by ESR analysis. Only a trace amount of DMPO-OH (0.2 μM) was detected under this condition.

Bactericidal test. All of the bacterial species used in the present study were effectively killed with a ≥4-log reduction under the H+(+)L(+) condition within 3 min (Fig. 2). There were, however, differences in susceptibility to hydroxyl radical disinfection among the species. A. actinomycetemcomitans was the most susceptible to hydroxyl radical disinfection, followed by S. aureus and S. mutans. E. faecalis showed the highest resistance to the disinfection method. A. actinomycetemcomitans was completely killed within 1 min by H+(+)L(+) (Fig. 2A). In addition, A. actinomycetemcomitans was completely killed even by H+(+)L(−) within 3 min and was also killed somewhat effectively even by H−L(+) (Fig. 2A). S. aureus and S. mutans responded similarly to the different treatments. They were almost completely killed by H+(+)L(+) within 3 min, while H+(+)L(−) had only a limited bactericidal effect and H−L(+) did not kill them. On the other hand, E. faecalis was not completely killed within 3 min even by H+(+)L(+), though
A laser diode with a wavelength of 405 ± 5 nm, which is the boundary wavelength between visible light and UV light, was used as an energy source for photolysis of H₂O₂ in the present study. For safety reasons, visible light is preferable to UV light because UV irradiation might not only photolyze H₂O₂ but also damage normal tissues. Although it has been known that UV irradiation can photolyze H₂O₂ effectively (4, 22), visible light with a wavelength of 405 nm also has an ability to photolyze H₂O₂ (25). In addition, visible light is much safer than UV light for operators when the disinfection system is applied in a clinical setting. As for another aspect of the safety of this system, 1.0 M H₂O₂, which is almost equal to the 3% H₂O₂ used as an oral disinfectant (30, 31), was used as a substrate for hydroxyl radical generation. Even if H₂O₂ remains in the oral cavity, it quickly decomposes to water and oxygen. Furthermore, the ESR analysis demonstrated that the generation of hydroxyl radicals stopped immediately after the cessation of laser irradiation. Thus, the generation of hydroxyl radicals is controllable and the disinfection system can be used safely in the oral cavity. However, there is concern that hydroxyl radicals generated during treatment using this system might damage normal tissue in the oral cavity. Because it is suggested that the ROS, including hydroxyl radicals, cause oxidative damage at cellular and tissue levels which leads to some diseases such as Parkinson’s disease, rheumatoid arthritis, and ischemic attacks (10). Although it is believed that this disinfection system could be used safely if hydroxyl radicals were generated at restricted lesion sites for a short time, the safety of artificially

Effect of H₂O₂ concentration on hydroxyl radical generation and bactericidal effect. The yield of DMPO-OH generated by photolysis of H₂O₂ linearly increased with the concentration of H₂O₂ (Fig. 4A). E. faecalis was killed dependently on the concentration of H₂O₂ (Fig. 4B). In particular, laser irradiation of 1.0 M H₂O₂ could kill the bacteria with an approximately 4-log reduction.

DISCUSSION

A laser diode with a wavelength of 405 ± 5 nm, which is the boundary wavelength between visible light and UV light, was used as an energy source for photolysis of H₂O₂ in the present study. For safety reasons, visible light is preferable to UV light because UV irradiation might not only photolyze H₂O₂ but also damage normal tissues. Although it has been known that UV irradiation can photolyze H₂O₂ effectively (4, 22), visible light with a wavelength of 405 nm also has an ability to photolyze H₂O₂ (25). In addition, visible light is much safer than UV light for operators when the disinfection system is applied in a clinical setting. As for another aspect of the safety of this system, 1.0 M H₂O₂, which is almost equal to the 3% H₂O₂ used as an oral disinfectant (30, 31), was used as a substrate for hydroxyl radical generation. Even if H₂O₂ remains in the oral cavity, it quickly decomposes to water and oxygen. Furthermore, the ESR analysis demonstrated that the generation of hydroxyl radicals stopped immediately after the cessation of laser irradiation. Thus, the generation of hydroxyl radicals is controllable and the disinfection system can be used safely in the oral cavity. However, there is concern that hydroxyl radicals generated during treatment using this system might damage normal tissue in the oral cavity. Because it is suggested that the ROS, including hydroxyl radicals, cause oxidative damage at cellular and tissue levels which leads to some diseases such as Parkinson’s disease, rheumatoid arthritis, and ischemic attacks (10). Although it is believed that this disinfection system could be used safely if hydroxyl radicals were generated at restricted lesion sites for a short time, the safety of artificially
generated hydroxyl radicals on normal tissues must be evaluated before it is applied clinically.

A laser with an output power of 300 mW was used to make an energy density of 940 mW/cm² in this study. However, since the irradiation field size of a lesion site in the mouth, such as a caries cavity, a periodontal pocket, or a root canal, will be much smaller than that of a well (6.4 mm), the same energy density will be obtained with a weaker laser output power. For example, if the irradiation field is 1 mm in diameter, a laser power of 10 mW or less will be enough because the area is reduced to approximately 1/36. Alternatively, the irradiation time will be shortened when a laser is used at an output power of >10 mW. Thus, it is not necessary to use a laser at 300 mW for 3 min and the laser power and the irradiation time can be optimized for each case.

As shown in Table 1, S. mutans and A. actinomycetemcomitans are pathogens that cause dental caries and periodontal disease, respectively (3, 13, 18). E. faecalis is often isolated from infected root canals (29), and S. aureus is isolated from removable-denture plaque and sometimes causes aspiration pneumonia in the elderly (28). In addition, it is well known that S. aureus and E. faecalis acquire resistance to antibiotics in some cases (2, 7). We used these four bacterial species as representative oral pathogens. All of the bacterial species used in this study could be killed by laser irradiation of 1.0 M H₂O₂.

On the other hand, a single treatment with 1.0 M H₂O₂ or laser irradiation did not kill the bacteria within 3 min except for A. actinomycetemcomitans, which was very susceptible to H₂O₂. This finding suggests that the bactericidal effect depends on the amount of hydroxyl radicals and also the time of exposure to hydroxyl radicals.

It has been reported that the bacteria constituting a biofilm show high resistance to disinfectant and antibiotics (21). One reason for this is that the extracellular matrix protects the bacterial cells from chemicals. The present study demonstrated that the hydroxyl radical disinfection system could kill S. mutans even in a biofilm (Fig. 3B) the structure of which was similar to that reported in previous studies (11, 20). Hydroxyl radicals can react with not only bacteria but also organic materials such as extracellular matrix. Thus, the hydroxyl radicals generated by photolysis of H₂O₂ could reach bacterial cells in biofilm and kill them. Similar results were reported for a disinfection system using another ROS, singlet oxygen. The singlet oxygen generated by photodynamic therapy (PDT) can kill bacteria in biofilm (17, 33, 34). Since it is believed that the reactivity and oxidizing power of hydroxyl radicals are higher than those of singlet oxygen (24), the hydroxyl radical disinfection system probably exerts a greater bactericidal effect than PDT. However, since the experimental model of biofilm used in this study was a single-species biofilm and was not dental plaque, the results of the bactericidal test in the present study do not necessarily reflect the clinical situation. Thus, further studies which mimic the clinical situation should be conducted.

When E. faecalis was treated with different concentrations of H₂O₂, it was killed to a degree depending on the concentration of H₂O₂. Since ESR analysis demonstrated that the yield of hydroxyl radicals increased with the concentration of H₂O₂, it was confirmed that the bactericidal effect depends on the amount of hydroxyl radicals. A concentration of H₂O₂ lower than 1.0 M will probably be able to kill oral pathogens when the irradiation time is prolonged. However, a short treatment time would be preferable from a clinical point of view. Therefore, if the safety aspect of the disinfection system is confirmed, it is recommended to use 1.0 M H₂O₂ (about 3%), which is a concentration used in the oral cavity, for the disinfection system.

The results of the present study demonstrated that hydroxyl radicals generated by photolysis of 1.0 M H₂O₂ increased with the laser irradiation time and could kill oral pathogenic bacteria in a short time. In other words, the time-dose relationship of this disinfection system shows that it is very effective. Therefore, it is believed that the disinfection technique using artificially generated hydroxyl radicals could be applied for the treatment of various oral infectious diseases. It is also suggested that 200 to 300 μM hydroxyl radicals would be enough to kill bacteria with a ≥4-log reduction.

ACKNOWLEDGMENT

This research was supported by Ministry of Economy, Trade, and Industry Grant-in-Aid for Regional Innovation Creation R&D Programs 21R2007C, 2010.
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