ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 2010, p. 5086–5091 Vol. 54, No. 12
0066-4804/10/$12.00 doi:10.1128/AAC.00751-10

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

Hiroyo Ikai,[1] Keisuke Nakamura,[1,2]* Midori Shirato,[1] Taro Kanno,[1] Atsuo Iwasawa,[2,3] Keiichi Sasaki,[1] Yoshimi Niwano,[2] and Masahiro Kohno[2]

Division of Fixed Prosthodontics, Department of Restorative Dentistry, Tohoku University Graduate School of Dentistry, Seiryou 4-1, Aoba-ku, Sendai 980-8575, Japan[1]; New Industry Creation Hatchery Center, Tohoku University, Aoba 6-6-10, Aramaki, Aoba-ku, Sendai 980-8579, Japan[2]; and Tissue Culture Laboratory, Showa University Fujigaoka Hospital, Fujigaoka 1-30, Aoba-ku, Yokohama 227-8501, Japan[3]

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 H_2O_2 and bactericidal activity was examined. H_2O_2 (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 H_2O_2 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 >99.999% 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 pathogens and is often isolated from patients suffering from destructive periodontal disease (3, 27). Thus, prevention and treatment of such oral infectious diseases depend on how effectively the dental plaque containing pathogenic bacteria is removed or disinfected.

Conventionally, treatments of oral infectious diseases consist of mechanical removal and chemical disinfection of pathogenic bacteria. The former includes removal of caries lesions using a low-speed round burr (16), reaming and filing of infected root canals (26), and scaling and root planning of infected root surfaces (5). The latter includes systemic antibiotic therapy (32), local antibiotic therapy (1), and mouth rinsing with a chemical solution. In general, mechanical removal of pathogenic bacteria is preferable to chemical disinfection because mechanical removal is more reliable in terms of plaque removal. However, mechanical removal techniques frequently remove not only plaque or bacteria but also the normal tooth substance and it is also difficult to put the instruments on the lesion site appropriately, especially in a narrow space such as apical root canal sites and molar and premolar furcation sites. On the other hand, chemical disinfection is not accompanied by removal of tooth material and a chemical solution can access lesion sites that are difficult to access with instruments. Conventional chemical disinfection, however, sometimes causes other problems, such as emergence of drug-resistant bacteria and accidental injury by leaked chemicals. In addition, many kinds of commensal bacteria constitute biofilm, which often defeats the efficacy of antimicrobial agents (21, 23). Hence, dental infectious diseases have been treated mainly by mechanical removal of dental plaque and bacteria.

A novel disinfection treatment technique in which artificially generated hydroxyl radicals can kill bacteria has been developed to make use of the advantages of chemical disinfection 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 H_2O_2 (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 H_2O_2 are

* Corresponding author. Mailing address: New Industry Creation Hatchery Center, Tohoku University, Aoba 6-6-10, Aramaki, Aoba-ku, Sendai 980-8579, Japan. Phone: 81-22-795-3976. Fax: 81-22-795-4110. E-mail: keisuke1@mail.tains.tohoku.ac.jp.

▽ Published ahead of print on 4 October 2010.

TABLE 1. Pathogens related to major oral infectious diseases

Oral infectious disease (reference[s])	Pathogens
Caries (18)	*S. mutans*, *Streptococcus sobrinus*, *Actinomyces* spp.
Periodontal disease (3, 13)	*A. actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythus*
Periapical disease (29)	*E. faecalis*, streptococci, *Porphyromonas* spp.
Aspiration pneumonia[a] (28)	*S. aureus*, *Candida albicans*

[a] Although aspiration pneumonia is not an oral infectious disease, it is sometimes caused by oral inhabitant microorganisms in the elderly.

simple reaction systems, each including one chemical, water or H_2O_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_2O_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_2O_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_2O_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 Labotec (Tokyo, Japan), H_2O_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_2O_2. A continuous-wave laser device (RV-1000; Ricoh Optical Industries, Hanamaki City, Japan) was used to photolyze H_2O_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_2O_2. In brief, H_2O_2 and DMPO were mixed in the wells of a 96-well microplate to final concentrations of 1.0 M for H_2O_2 and 0 to 400 mM for DMPO. One molar H_2O_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, 330.50 to 340.50 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. TEMPOL (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 ESR cavity was equipped, was used as an internal standard. The concentration of hydroxyl radicals was determined using digital data processing (JEOL, Tokyo, Japan) and expressed as the concentration of DMPO-OH. All experiments were performed at room temperature.

Relationship between laser irradiation time and hydroxyl radical generation. The relationship between the laser irradiation time and the amount of hydroxyl radicals generated was investigated using the optimal concentration of DMPO (300 mM) obtained from the experiment described above. The laser irradiation times were set for 10, 20, 30, 60, 120, and 180 s. In addition, the effect of energy density on the photolysis of H_2O_2 was investigated using output powers of 100 and 200 mW, which correspond to 310 and 630 mW/cm^2, respectively. The ESR measurement and data analysis were performed as described above.

Since 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_2O_2 was constant or decreased with irradiation time. In brief, 180 μl of 1.1 M H_2O_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_2O_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_2O_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_2O_2. The amount of hydroxyl radicals generated by photolysis of 1.0 M H_2O_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_2O_2. The autolysis of 1.0 M H_2O_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_2O_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 25923, *Enterococcus faecalis* ATCC 29212, *S. mutans* JCM 5705, and *A. actinomycetemcomitans* JCM 2434 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 adjusted to 2×10^7 cells/ml. In a microplate well (96 well), 150 μl of the suspension was mixed with 150 μl of 2.0 M H_2O_2 diluted with sterile physiological saline to make final concentrations of 1×10^7 cells/ml for bacteria and 1.0 M for H_2O_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_2O_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_2O_2 [expressed as H(+)L(+)] was compared to the effects of (i) 1.0 M H_2O_2 alone [H(+)L(−)], (ii) laser irradiation alone [H(−)L(+)], and (ii) no treatment [H(−)L(−)]. For the L(−) condition, the samples were kept without laser irradiation on a clean bench to avoid contamination. For the H(−) condition, sterile physiological saline was added to the reaction system instead of H_2O_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 H_2O_2. Immediately after the addition of H_2O_2, 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(−). H_2O_2 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 BHI 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 fixed with 2.5% (wt/vol) glutaraldehyde 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 specimens were postfixed with 2% (wt/vol) OsO_4 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 CO_2 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 DS701 scanning electron microscope at 8 kV.

Effect of H_2O_2 concentration on hydroxyl radical generation and bactericidal effect. Since it was believed that the generation of hydroxyl radicals depends on the concentration of H_2O_2, an effective concentration range of H_2O_2 for a bactericidal effect was investigated in relation to the generation of hydroxyl radicals. Samples containing different concentrations of H_2O_2 (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 H_2O_2 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 H_2O_2. When 1.0 M H_2O_2 was irradiated with a laser light with a wavelength of 405 nm, DMPO-OH ($a_N = a_H = 1.49$ mT [where a_N and a_H are the hyperfine coupling constants arising from nitrogen and hydrogen atoms, respectively, in the structure of DMPO-OH]) 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 H_2O_2 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 H_2O_2 which was exposed to laser irradiation for 180 s in advance was not reduced compared to that from H_2O_2 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.

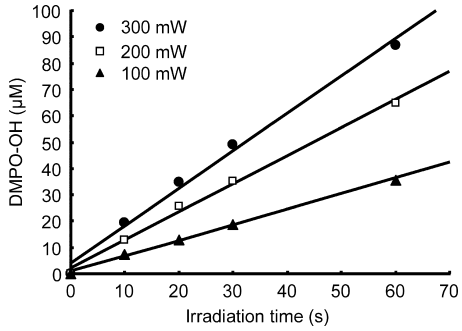

FIG. 1. Relationship between laser irradiation time and the concentration of DMPO-OH at different laser powers. The lines represent theoretical hydroxyl radical generation patterns, and the equations of the lines are as follows: $y = 1.41x + 4.08$ for 300 mW, $y = 1.07x + 2.15$ for 200 mW, and $y = 0.58x + 0.94$ for 100 mW. Each value represents the mean of duplicate determinations.

Hydroxyl radical generation from water with and without laser irradiation and from autolysis of H_2O_2. Contrary to the photolysis of H_2O_2, only small amounts of DMPO-OH from pure water without laser irradiation, photolysis of pure water, and autolysis of 1.0 M H_2O_2 were detected. The mean concentrations of DMPO-OH detected in pure water, pure water irradiated with a laser, and 1.0 M H_2O_2 without laser irradiation were 0.1, 0.1, and 0.2 μM, respectively. The DMPO-OH generated by autolysis of 1.0 M H_2O_2 increased in a time-dependent manner. The amount of hydroxyl radicals (1.2 μM) generated by autolysis of H_2O_2 even after 30 min was, however, much smaller than that produced by photolysis of H_2O_2. The photolysis of H_2O_2 could be terminated by cessation of irradiation as described below. H_2O_2 (1.1 M) without DMPO was irradiated with a laser for 180 s. Immediately after the irradiation, DMPO was added to the H_2O_2 to make final concentrations of 1.0 M H_2O_2 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(+). *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

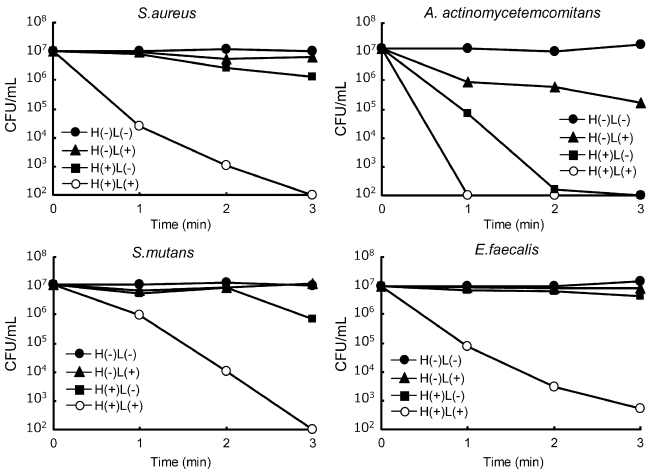

FIG. 2. Number of viable microorganisms in the suspension after each treatment. The H(+)L(+) condition (hydroxyl radial disinfection system) could kill all of the four bacterial species used in this study with a ≥4-log reduction within 3 min. Each bar represents the mean of triplicate determinations.

a ≥4-log reduction was observed. H(+)L(−) and H(−)L(+) had little or no bactericidal effect on *E. faecalis*.

SEM images demonstrated that the experimental biofilm of *S. mutans* composed of extracellular matrix and multiple cell layers was formed on the whole plate within 24 h (Fig. 3A). The scattered clusters of bacterial cells covered and surrounded by extracellular matrix were observed. The bacteria in the biofilm were also disinfected by hydroxyl radicals generated by photolysis of H_2O_2 (Fig. 3B). The number of bacteria treated with H(+)L(+) showed a ≥5-log reduction, while H(+)L(−) killed the bacteria with only a 1-log reduction.

According to the equation of a linear relationship between DMPO-OH and irradiation time, the hydroxyl radical concentrations generated by 1, 2, and 3 min of photolysis of 1.0 M H_2O_2 were 89, 173, and 258 μM, respectively. The results indicated that 89 μM hydroxyl radicals was enough to kill *A. actinomycetemcomitans* (10^7 CFU/ml), and 173 μM hydroxyl radicals was enough to kill both *S. aureus* and *S. mutans* (10^7 CFU/ml). Similarly, 258 μM hydroxyl radicals was enough to kill *E. faecalis* (10^7 CFU/ml) with a ≥4-log reduction. Furthermore, 258 μM hydroxyl radicals was enough to kill *S. mutans* in the experimental biofilm (10^8 cells/well) with a 5-log reduction.

Effect of H_2O_2 concentration on hydroxyl radical generation and bactericidal effect. The yield of DMPO-OH generated by photolysis of H_2O_2 linearly increased with the concentration of H_2O_2 (Fig. 4A). *E. faecalis* was killed dependently on the concentration of H_2O_2 (Fig. 4B). In particular, laser irradiation of 1.0 M H_2O_2 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 H_2O_2 in the present study. For safety reasons, visible light is preferable to UV light because UV irradiation might not only photolyze H_2O_2 but also damage normal tissues. Although it has been known that UV irradiation can photolyze H_2O_2 effectively (4, 22), visible light with a wavelength of 405 nm also has an ability to photolyze H_2O_2 (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 H_2O_2, which is almost equal to the 3% H_2O_2 used as an oral disinfectant (30, 31), was used as a substrate for hydroxyl radical generation. Even if H_2O_2 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

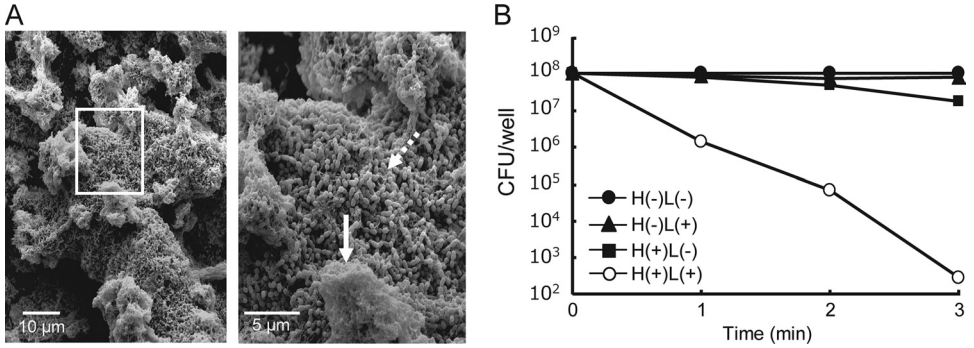

FIG. 3. SEM images of an experimental biofilm and bactericidal effect of hydroxyl radicals on *S. mutans* in the biofilm. (A) It was observed that the biofilm was composed of extracellular matrix and multiple cell layers. The right image shows a higher magnification of the outlined area in the left image. The solid arrow shows extracellular matrix covering the cluster of bacterial cells, and the dotted arrow shows the multiple cell layers. (B) The biofilm contained approximately 10^8 CFU/well, as shown by H(−)L(−). The H(+)L(+) condition (hydroxyl radial disinfection system) could kill *S. mutans* in biofilm with a ≥5-log reduction within 3 min. Each bar represents the mean of triplicate determinations.

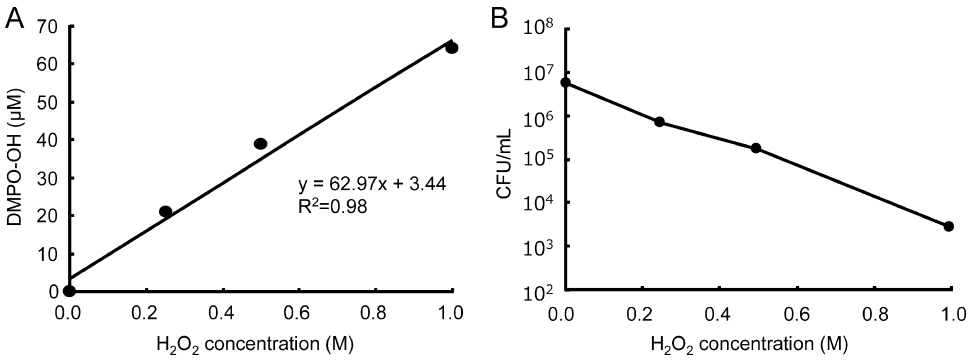

FIG. 4. Effect of H_2O_2 concentration on hydroxyl radical generation and bactericidal effect. (A) The yield of DMPO-OH increased in proportion to the concentration of H_2O_2 when the sample was irradiated with a laser light with an output power of 300 mW for 30 s. Each value represents the mean of duplicate determinations. (B) *E. faecalis* was mixed with different concentrations of H_2O_2 and irradiated with a laser light at an output power of 300 mW for 3 min. Bacterial killing depended on the concentration of H_2O_2. Each value represents the mean of triplicate determinations.

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/cm^2 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 H_2O_2. On the other hand, a single treatment with 1.0 M H_2O_2 or laser irradiation did not kill the bacteria within 3 min except for *A. actinomycetemcomitans*, which was very susceptible to H_2O_2. 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 H_2O_2 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 H_2O_2 irradiated with a laser for 3 min, it was killed to a degree depending on the concentration of H_2O_2. Since ESR analysis demonstrated that the yield of hydroxyl radicals increased with the concentration of H_2O_2, it was confirmed that the bactericidal effect depends on the amount of hydroxyl radicals. A concentration of H_2O_2 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 H_2O_2 (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 H_2O_2 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.

REFERENCES

1. **Ainamo, J., T. Lie, B. H. Ellingsen, B. F. Hansen, L. A. Johansson, T. Karring, J. Kisch, K. Paunio, and K. Stoltze.** 1992. Clinical responses to subgingival application of a metronidazole 25% gel compared to the effect of subgingival scaling in adult periodontitis. J. Clin. Periodontol. **19:**723–729.
2. **Amyes, S. G.** 2007. Enterococci and streptococci. Int. J. Antimicrob. Agents **29**(Suppl. 3)**:**S43–S52.
3. **Anonymous.** 1996. Consensus report. Periodontal diseases: pathogenesis and microbial factors. Ann. Periodontol. **1:**926–932.
4. **Anzai, K., T. Aikawa, Y. Furukawa, Y. Matsushima, S. Urano, and T. Ozawa.** 2003. ESR measurement of rapid penetration of DMPO and DEPMPO spin traps through lipid bilayer membranes. Arch. Biochem. Biophys. **415:**251–256.
5. **Badersten, A., R. Nilveus, and J. Egelberg.** 1984. Effect of nonsurgical periodontal therapy. II. Severely advanced periodontitis. J. Clin. Periodontol. **11:**63–76.
6. **Badwey, J. A., and M. L. Karnovsky.** 1980. Active oxygen species and the functions of phagocytic leukocytes. Annu. Rev. Biochem. **49:**695–726.
7. **Chambers, H. F.** 2001. The changing epidemiology of *Staphylococcus aureus*? Emerg. Infect. Dis. **7:**178–182.
8. **Chapple, I. L., and J. B. Matthews.** 2007. The role of reactive oxygen and antioxidant species in periodontal tissue destruction. Periodontol. 2000 **43:** 160–232.
9. **Clifford, D. P., and J. E. Repine.** 1982. Hydrogen peroxide mediated killing of bacteria. Mol. Cell. Biochem. **49:**143–149.
10. **Cross, C. E., B. Halliwell, E. T. Borish, W. A. Pryor, B. N. Ames, R. L. Saul, J. M. McCord, and D. Harman.** 1987. Oxygen radicals and human disease. Ann. Intern. Med. **107:**526–545.
11. **Dong, C., and F. Q. Zhang.** 2009. Effect of denture base materials on mRNA expression of the adhesion-associated genes from the *Streptococcus mutans* biofilms. J. Oral Rehabil. **36:**894–901.
12. **Fridovich, I.** 1986. Biological effects of the superoxide radical. Arch. Biochem. Biophys. **247:**1–11.
13. **Haffajee, A. D., R. P. Teles, and S. S. Socransky.** 2006. Association of *Eubacterium nodatum* and *Treponema denticola* with human periodontitis lesions. Oral Microbiol. Immunol. **21:**269–282.
14. **Halliwell, B., and J. M. Gutteridge.** 1984. Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem. J. **219:**1–14.
15. **Hu, Y., Z. Zhang, and C. Yang.** 2008. Measurement of hydroxyl radical production in ultrasonic aqueous solutions by a novel chemiluminescence method. Ultrason. Sonochem. **15:**665–672.
16. **Kidd, E., O. Fejerskov, and I. Mjor.** 2003. Caries removal and the pulpodentinal complex, p. 267–274. *In* O. Fejerskov and E. Kidd (ed.), Dental caries. The disease and its clinical management. Blackwell Munksgaard, Oxford, United Kingdom.
17. **Konopka, K., and T. Goslinski.** 2007. Photodynamic therapy in dentistry. J. Dent. Res. **86:**694–707.
18. **Marsh, P. D., and B. Nyvad.** 2003. The oral microflora and biofilms on teeth, p. 49–70. *In* O. Fejerskov and E. Kidd (ed.), Dental caries. The disease and its clinical management. Blackwell Munksgaard, Oxford, United Kingdom.
19. **Nakamura, K., T. Kanno, H. Ikai, E. Sato, T. Mokudai, Y. Niwano, T. Ozawa, and M. Kohno.** 2010. Reevaluation of quantitative ESR spin trapping analysis of hydroxyl radical by applying sonolysis of water as a model system. Bull. Chem. Soc. Jpn. **83:**1037–1046.
20. **Namba, N., Y. Yoshida, N. Nagaoka, S. Takashima, K. Matsuura-Yoshimoto, H. Maeda, B. Van Meerbeek, K. Suzuki, and S. Takashiba.** 2009. Antibacterial effect of bactericide immobilized in resin matrix. Dent. Mater. **25:**424–430.
21. **Nickel, J. C., I. Ruseska, J. B. Wright, and J. W. Costerton.** 1985. Tobramycin resistance of *Pseudomonas aeruginosa* cells growing as a biofilm on urinary catheter material. Antimicrob. Agents Chemother. **27:**619–624.
22. **Niwano, Y., E. Sato, M. Kohno, Y. Matsuyama, D. Kim, and T. Oda.** 2007. Antioxidant properties of aqueous extracts from red tide plankton cultures. Biosci. Biotechnol. Biochem. **71:**1145–1153.
23. **Overman, P. R.** 2000. Biofilm: a new view of plaque. J. Contemp. Dent. Pract. **1:**18–29.
24. **Redmond, R. W., and I. E. Kochevar.** 2006. Spatially resolved cellular responses to singlet oxygen. Photochem. Photobiol. **82:**1178–1186.
25. **Sakai, K., J. Kato, H. Kurata, G. Nakazawa, A. Akashi, A. Kameyama, and Y. Hirai.** 2007. The amount of hydroxyl radicals generated by titanium dioxide and 3.5% hydrogen peroxide under 405-nm diode laser irradiation. Laser Physics **17:**1062–1066.
26. **Saunders, W. P., and E. Saunders.** 2003. Root canal instrumentation, p. 236–260. *In* G. Bergenholtz, P. Horsted-Bindslev, and C. Reit (ed.), Textbook of endodontology. Blackwell Munksgaard, Oxford, United Kingdom.
27. **Socransky, S. S., and A. D. Haffajee.** 2003. Microbiology of periodontal disease, p. 106–149. *In* J. Lindhe, T. Karring, and N. P. Lang (ed.), Clinical periodontology and implant dentistry, fourth ed. Blackwell, Oxford, United Kingdom.
28. **Terpenning, M. S., G. W. Taylor, D. E. Lopatin, C. K. Kerr, B. L. Dominguez, and W. J. Loesche.** 2001. Aspiration pneumonia: dental and oral risk factors in an older veteran population. J. Am. Geriatr. Soc. **49:**557–563.
29. **Theilade, E.** 2003. The microbiology of the necrotic pulp, p. 111–129. *In* G. Bergenholtz, P. Horsted-Bindslev, and C. Reit (ed.), Textbook of endodontology. Blackwell Munksgaard, Oxford, United Kingdom.
30. **Wennstrom, J. L., G. Dahlen, K. Grondahl, and L. Heijl.** 1987. Periodic subgingival antimicrobial irrigation of periodontal pockets. II. Microbiological and radiographical observations. J. Clin. Periodontol. **14:**573–580.
31. **Wennstrom, J. L., L. Heijl, G. Dahlen, and K. Grondahl.** 1987. Periodic subgingival antimicrobial irrigation of periodontal pockets (I). Clinical observations. J. Clin. Periodontol. **14:**541–550.
32. **Winkel, E. G., A. J. Van Winkelhoff, M. F. Timmerman, U. Van der Velden, and G. A. Van der Weijden.** 2001. Amoxicillin plus metronidazole in the treatment of adult periodontitis patients. A double-blind placebo-controlled study. J. Clin. Periodontol. **28:**296–305.
33. **Wood, S., D. Metcalf, D. Devine, and C. Robinson.** 2006. Erythrosine is a potential photosensitizer for the photodynamic therapy of oral plaque biofilms. J. Antimicrob. Chemother. **57:**680–684.
34. **Zanin, I. C., R. B. Goncalves, A. B. Junior, C. K. Hope, and J. Pratten.** 2005. Susceptibility of *Streptococcus mutans* biofilms to photodynamic therapy: an in vitro study. J. Antimicrob. Chemother. **56:**324–330.