

Original Article

Efficacy and safety of a therapeutic apparatus using hydrogen peroxide photolysis to treat dental and periodontal infectious diseases

Hirohisa Sato, Yoshimi Niwano, Keisuke Nakamura, Takayuki Mokudai, Hiroyo Ikai,
Taro Kanno and Hiroshi Egusa

Tohoku University Graduate School of Dentistry, 4-1 Seiryomachi, Aoba-ku, Sendai 980-8575, Japan

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ABSTRACT — The present study aimed to evaluate the acute locally injurious property of our most current hydroxyl radical generation system by hydrogen peroxide (H_2O_2) photolysis. This system, which releases 3% H_2O_2 with a 405-nm laser, was developed in our laboratory for the treatment of dental and periodontal infectious diseases. First, the hydroxyl radical yield generated by H_2O_2 photolysis was examined by applying an electron spin resonance-spin trapping technique. Second, the bactericidal effect of the device was examined under a simulant condition in which *Streptococcus mutans*, a pathogenic bacterial species that causes caries, was irrigated with running 3% H_2O_2 concomitantly with laser irradiation. Finally, the acute topical effect of the model apparatus on rat palatal mucosa was evaluated by histological examination. We found that the hydroxyl radical yield was dependent upon laser output power. The bacterial count was substantially reduced within as little as 3 min. No abnormal findings were observed in the palatal mucosa, even when rats received three treatments of 3% H_2O_2 with laser irradiation at an output power of 40 mW. These results suggest that our apparatus has the ability to kill bacteria via hydroxyl radical generation and is safe to use at the lesion site of dental and periodontal infectious diseases.

Key words: Hydroxyl radical, Photolysis of H_2O_2 , Efficacy and safety evaluation, Disinfection apparatus, Dental and periodontal infectious diseases

INTRODUCTION

A novel disinfection technique utilizing artificially generated hydroxyl radicals by hydrogen peroxide (H_2O_2) photolysis has been developed in our laboratory (Ikai *et al.*, 2010; Oyamada *et al.*, 2013; Shirato *et al.*, 2011; Toki *et al.*, 2015). We previously showed that hydroxyl radical ($\cdot OH$) yield from H_2O_2 photolysis is dependent upon the irradiation time and laser output power. Additionally, this technique can kill pathogenic bacteria effectively through radical generation and is unlikely to induce bacterial resistance, possibly because of non-specific oxidative damage of cell structures by hydroxyl radicals (Ikai *et al.*, 2013).

Regarding the concentration of H_2O_2 , 3% H_2O_2 is typically used in the oral cavity as a disinfectant. Moreover, a subcommittee of the U.S. Food and Drug Administration (Food and Drug Administration, 2003)

concluded that H_2O_2 is safe at concentrations of up to 3%. Thus, the H_2O_2 concentration used in the disinfection system was fixed at 3%. To apply this disinfection technique in the field of dentistry, especially for dental and periodontal infectious disease treatment, we generated several test models. In these models that were basically equipped with an ultrasound scaler, a continuous-wave laser is incorporated and 3% H_2O_2 is released from the tip of the apparatus to the lesion concomitantly with laser irradiation at 405 nm through an optical fiber. Regarding a comparable technique, photodynamic therapy (PDT) that generates singlet oxygen has been tried to treat periodontal infectious diseases. The PDT consists of mainly three components, light, oxygen, and a photosensitizer. Once the photosensitizer is irradiated with light of a specific wavelength, it transfers the excitation energy to molecular oxygen resulting in singlet oxygen formation (Cló *et al.*, 2007). Since the PDT utilizes molecular oxygen, it

would be hard to treat infectious diseases under anaerobic conditions. Indeed, it was recently reported that PDT was more effective as an adjunctive treatment to scaling and root planning (SRP known as conventional periodontal therapy) than SRP alone; however, no distinct differences were found between both treatment modalities regarding the effect on certain obligate anaerobic bacteria including *Porphyromonas gingivalis*, a major pathogen bacterial species in periodontitis (Talebi *et al.*, 2016). In our technique, exogenous H_2O_2 is used as a source of hydroxyl radicals so that it would be effective even under anaerobic conditions.

To our knowledge, few studies have examined the direct involvement of hydroxyl radicals in localized damage *in vivo*, possibly because of its extremely short lifetime, approximately 10^{-9} sec (Halliwell and Gutteridge, 1986; Pryor, 1986; Roots and Okada, 1975; Sies *et al.*, 1992). Thus, as the first step in safety evaluation, the acute locally injurious property of our hydroxyl radical generation system was evaluated by examining the oral mucosa and healing of full thickness skin wounds in rats (Yamada *et al.*, 2012). No abnormal findings were observed in the buccal mucosal region after three treatments utilizing this disinfection technique. However, the output power of the laser in the initial model apparatus was limited to 7 mW. Thus, a safety evaluation under more powerful conditions is warranted.

After continual improvement, a test model with the ability to emit laser light with an output power of 40 mW or more was generated. Therefore, the aim of the present study was to evaluate the acute locally injurious property of this latest model apparatus in relation to hydroxyl radical generation and bactericidal activity under a simulant condition for clinical use.

MATERIALS AND METHODS

Test model of a therapeutic apparatus

A test model of a therapeutic apparatus for the treatment of dental and periodontal infectious diseases was fabricated by AZ Co., Ltd. (Miyagi, Japan). Figure 1 shows images of the apparatus in which a continuous-wave laser was incorporated. Three percent H_2O_2 was released from the tip of the apparatus to the lesion concomitantly with laser irradiation at 405 nm through an optical fiber.

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

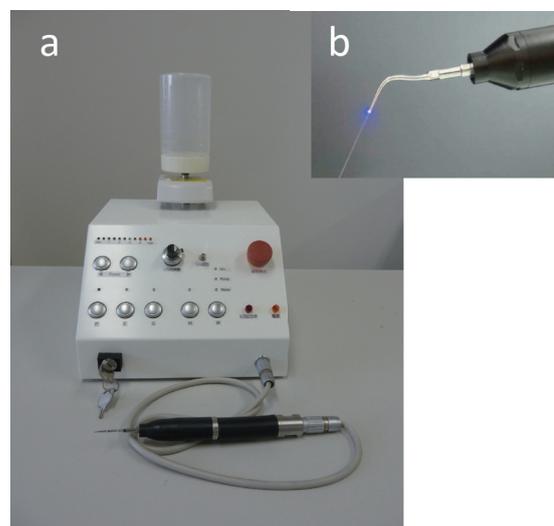


Fig. 1. (a) Photograph of the apparatus in which a bottle container for 3% H_2O_2 and a continuous-wave laser were incorporated. (b) Three percent H_2O_2 is released from the tip of the apparatus with laser irradiation at 405 nm through an optical fiber.

(Tokyo, Japan), and 4-hydroxy-2,2,6,6-tetramethylpiperidine (TEMPO) from Sigma-Aldrich (St. Louis, MO, USA). All of the other reagents used were of analytical grade.

Electron spin resonance (ESR) determination of hydroxyl radicals generated by the model apparatus

Hydroxyl radical yield generated by the model apparatus was evaluated in two manners. First, we evaluated the effect of laser light on static 3% H_2O_2 . An aliquot (300 μ L) of 3% H_2O_2 with 300 mM DMPO in a well of a 96-well black microplate was irradiated with laser light emitted from the tip for 5-40 sec at an output power of 14 or 28 mW. The tip was placed at approximately 2 mm from the surface of the H_2O_2 solution. Second, we evaluated the effect of running 3% H_2O_2 . A reservoir containing 3% H_2O_2 with 300 mM DMPO was installed in the model apparatus. The H_2O_2 solution was then released from the tip for 1 min at a flow rate of 12 or 24 mL/min with laser light at the output power of 14 or 28 mW. The released solution was collected in a beaker for ESR determination, in which the distance between the top of the tip and the side of the beaker was set to be approximately 1 cm. Following each procedure, an aliquot of the reaction mixture 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 meas-

urement conditions for ESR were as follows: field sweep, 332.07-342.07 mT; field modulation frequency, 100 kHz; field modulation width, 0.1 mT; amplitude, 200; sweep time, 2 min; time constant, 0.03 sec; microwave frequency, 9.420 GHz; and microwave power, 4 mW. TEMPOL (2 μ M) was used as a standard to calculate the concentration of DMPO-OH (a spin adduct of DMPO and hydroxyl radical), and the ESR spectrum of manganese (Mn^{2+}) held in the ESR cavity was used as an internal standard.

Bactericidal assay

A stock culture strain of *Streptococcus mutans* JCM 5705, a pathogenic bacterial species that causes caries, was purchased from the Japan Collection of Microorganisms, RIKEN BioResource Center (Saitama, Japan). Bacteria were anaerobically cultured on brain heart infusion (BHI) agar (Becton Dickinson Labware, Franklin Lakes, NJ, USA) using the Anaero Pack (Mitsubishi Gas Chemical Company, Tokyo, Japan) at 37°C for 2 days. A bacterial suspension was prepared in sterile physiological saline, and the optical density at 590 nm measured by a colorimeter (CO 7500 Colorimeter, Biochrom Ltd., Cambridge, UK) was adjusted to 1.5, which corresponded to approximately 1.5×10^8 colony forming units (CFU)/mL. A schematic illustration of the bactericidal assay is shown in Fig. 2. An aliquot (100 μ L) of the bacterial suspension was placed on a cellulose nitrate membrane filter (pore size = 0.45 μ m) followed by aspiration at 70 kPa. Bacteria on the membrane were irrigated with each test solution (physiological saline or 3% H_2O_2) with or without

laser irradiation at an output power of 40 mW released from the test apparatus at a flow rate of 24 mL/min for 3 min under aspiration at 70 kPa. Bacteria on the membrane were swabbed with a cotton swab for 10 sec, and the swab was agitated for 30 sec in 1 mL of physiological saline with 100 U catalase, which was used to terminate the bactericidal effect of remnant H_2O_2 . A 10-fold serial dilution of the solution was prepared and 10 μ L of the dilution were plated on BHI agar. Agar plates were incubated anaerobically as described above to enumerate CFU/membrane. In the case of treatment with 3% H_2O_2 with laser irradiation, 100 μ L of the recovered solution were also plated on BHI agar to set a detection limit of < 10 CFU/membrane. The agar plates were cultured for 2 days at 37°C to determine CFU/membrane. The following test conditions were evaluated: H(-)L(-), physiological saline alone; H(-)L(+), physiological saline with laser irradiation at 40 mW; H(+L)(-), 3% H_2O_2 alone; and H(+L)(+), 3% H_2O_2 with laser irradiation at 40 mW.

In addition, the effect of laser output power on *S. mutans* was similarly examined. In the experiment, the following test conditions were evaluated: H(+L)(-), 3% H_2O_2 alone; H(+L)(10), 3% H_2O_2 with laser irradiation at 10 mW; H(+L)(20), 3% H_2O_2 with laser irradiation at 20 mW; and H(+L)(40), 3% H_2O_2 with laser irradiation at 40 mW. In this experiment, *S. mutans* JCM 5705 cultured anaerobically in BHI broth overnight was centrifugally harvested and suspended in physiological saline. The suspension whose optical density was adjusted to 1.5 was used as the inoculum.

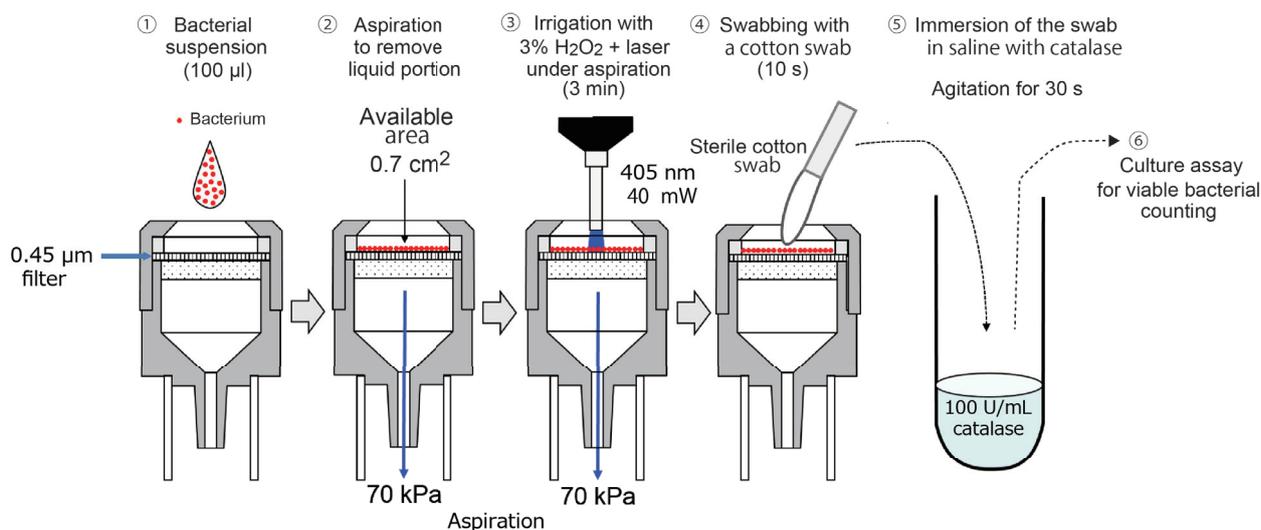


Fig. 2. Schematic illustration showing the bactericidal assay. Irrigation was performed with running 3% H_2O_2 with laser irradiation.

Animals

The experiments reported here were conducted in accordance with the guidelines for animal experiments and animal care adopted by Tohoku University. Eight-week-old male Wistar rats were purchased from Charles River Laboratories Inc. (Kanagawa, Japan) and used after acclimatization for 1 week. During the acclimatization and experimental periods, animals were given access to food pellets (Labo MR Stock, Nosan Corp., Tokyo, Japan) and tap water *ad libitum*. The rats were housed at $23 \pm 3^\circ\text{C}$ under a 12-hr light/12-hr dark cycle.

Topical treatment of oral cavity and histological examination

Three to five rats were allocated to each group. Under isoflurane and pentobarbital anesthesia, a small region of the palatal mucosa on the left side of the oral cavity (Fig. 3) was irrigated once or once a day for three consecutive days with each treatment for 7 min at a flow rate of 10 mL/min. The following treatments were tested: (1) pure water, (2) 3% H_2O_2 and laser irradiation at 5 mW, (3) 3% H_2O_2 and laser irradiation at 10 mW, (4) 3% H_2O_2 and laser irradiation at 20 mW, and (5) 3% H_2O_2 and laser irradiation at 40 mW. Immediately following the last treatment, animals were sacrificed by cervical dislocation, and the irrigated portion with surrounding area was excised and fixed in 10% neutral formalin. Following fixation, the tissues were trimmed, dehydrated by an ethanol series, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for microscopy.

Statistical analyses

Statistical significances in the DMPO-OH yield in the experiment with the running 3% H_2O_2 , and the CFU/membrane obtained in the bactericidal assay were assessed by the Tukey-Kramer HSD multi-comparison test. The analysis for the bactericidal assay was performed following logarithmic conversion. In the case of treatment with 3% H_2O_2 with laser irradiation, when colonies were not detected, the value of the detection limit (10 CFU/membrane) was used for the statistical analysis. P values of < 0.05 were considered significant.

RESULTS

ESR determination of hydroxyl radicals generated by the test model of therapeutic apparatus

Figure 4a shows representative ESR spectra obtained by laser irradiation with static 3% H_2O_2 , and Fig. 4b summarizes the quantitative analysis of DMPO-OH.

The spin adduct DMPO-OH was assigned using hyperfine coupling constants (hfcc). The hfcc of DMPO-OH were $a\text{H}=a\text{N}=1.49$ mT, which coincided with those of the DMPO-OH adduct reported previously (Buettner, 1987). The signal intensity of DMPO-OH increased linearly with laser irradiation time and output power, so that similar results were obtained for DMPO-OH concentrations. Regarding the slope of the regression line, which indicates the generation rate of hydroxyl radicals, the slope of 28 mW output power was double that of 14 mW.

Figure 5 summarizes the quantitative analysis of DMPO-OH under the condition of running 3% H_2O_2 with 300 mM DMPO. The yield of DMPO-OH from the specimen treated with [24 mL/min, 14 mW] was approximately 1.3 μM , almost half the yield of the specimen treated with [24 mL/min, 28 mW]. The yield of DMPO-OH from the specimen treated with [12 mL/min, 28 mW] was 1.9 μM , which was between the yields of specimens treated with [24 mL/min, 14 mW] and [24 mL/min, 28 mW].

Bactericidal assay

Figure 6 summarizes the number of *S. mutans* recovered from specimens. No or slight changes in the recovered viable bacterial count were observed following H(-) L(-) and H(-) L(+) treatments. Whereas treatment with H(+) L(-) resulted in a 3.5-log reduction of viable bacteria, an approximately 6-log reduction was achieved by treatment with H(+) L(+) in which laser was irradiated at the output power of 40 mW. Figure 7 shows the effect of laser output power on *S. mutans*. The recovered viable bacterial count was reduced with the output power, and even treatment with H(+) L(10) in which laser was irradiated at the output power of 10 mW tended to be lower than that of H(+) L(-).

Topical treatment of oral cavity

Figure 8 shows representative histological images of the palatal mucosa region of the eight groups (treated once or thrice with pure water alone or 3% H_2O_2 with laser irradiation at 10, 20, or 40 mW). No abnormal findings were observed in any of the 3% H_2O_2 with laser irradiation groups compared with the two pure water groups. Moreover, neither disintegrated stratified epithelium nor infiltration of inflammatory cells in the connective tissue was observed.

DISCUSSION

In this study, ESR analysis clearly showed that laser irradiation of 3% H_2O_2 generated hydroxyl radicals (expressed as the spin adduct DMPO-OH). As shown

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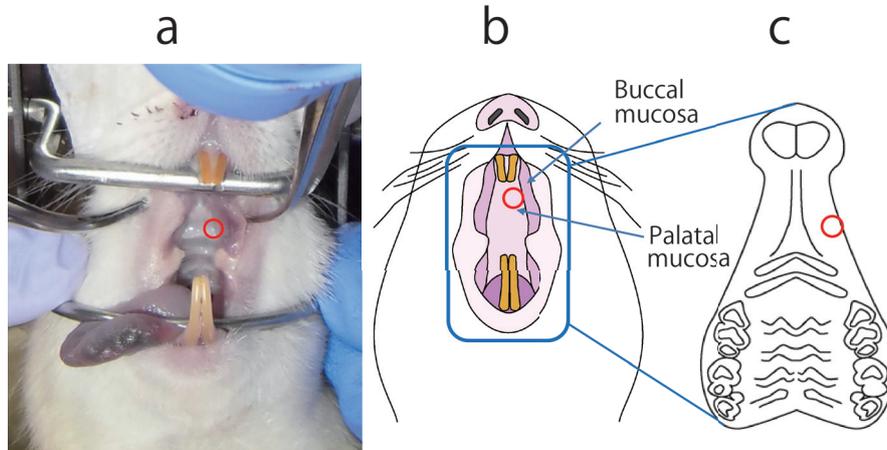


Fig. 3. Schematic illustration showing our *in vivo* rat model to evaluate the acute locally injurious property of our hydroxyl radical generation system. A small region of the palatal mucosa (as indicated by red circles) on the left side of the oral cavity was irrigated with running 3% H_2O_2 with laser irradiation. (a): Photograph of the oral cavity of rat retained on a fixator. (b): Diagram of the photograph shown in A. (c): Enlarged illustration of the upper jaw.

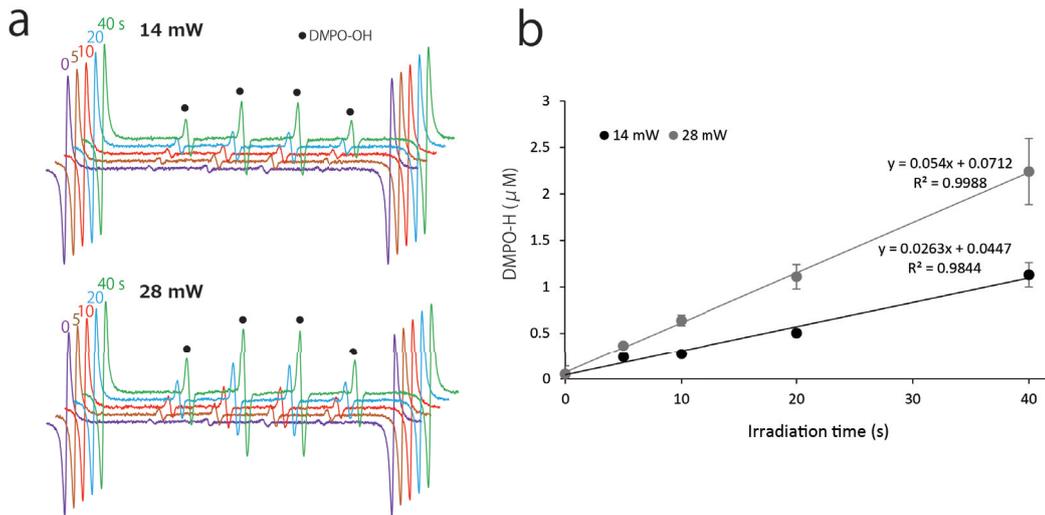


Fig. 4. Hydroxyl radical yields from treatment with static 3% H_2O_2 with laser irradiation. (a) Representative ESR spectra from treatment with static 3% H_2O_2 irradiated with laser light at an output power of 14 or 28 mW. (b) Hydroxyl radical yields. Data are expressed as the mean with standard deviation ($n = 3$).

by the signal intensity of DMPO-OH, the hydroxyl radical yield was dependent on both irradiation time and laser output power. When running 3% H_2O_2 was treated by laser irradiation at a flow rate of 24 mL/min, hydroxyl radical yield was also dependent on laser output power. Moreover, the yield at laser output power of 28 mW was almost double that at 14 mW, indicating that the hydroxyl radical yield could be controlled by laser output power.

The hydroxyl radical yield tended to slightly decrease, but not significantly, at a slower flow rate (12 mL/min). Since the volume of H_2O_2 per unit time irrigated at 24 mL/min was higher than that at 12 mL/min, more H_2O_2 would be exposed to laser irradiation at 24 mL/min than at 12 mL/min. However, when the hydroxyl radical yield was expressed as a concentration, the flow rate did not appear to affect the yield as much, because the laser

evenly irradiates running H_2O_2 unless the flow volume becomes too small.

Under a simulant condition in which bacteria were irrigated with running 3% H_2O_2 concomitantly with laser irradiation, *S. mutans* was effectively killed with an approximately 6-log reduction in CFU within 3 min. However, running 3% H_2O_2 alone resulted in only 3.5-log

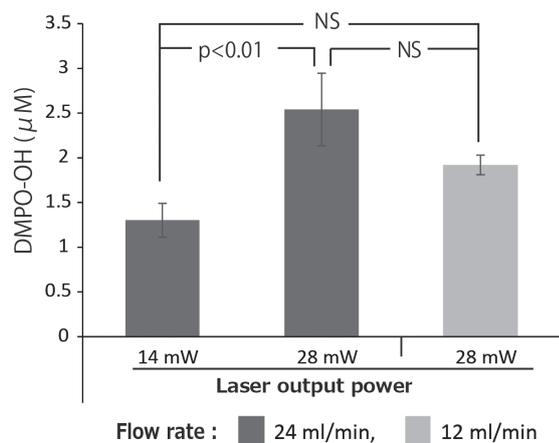


Fig. 5. Hydroxyl radical yields from treatment with running 3% H_2O_2 with laser irradiation for 1 min. The flow rate was 12 or 24 mL/min, and the output power of laser light was 14 or 28 mW.

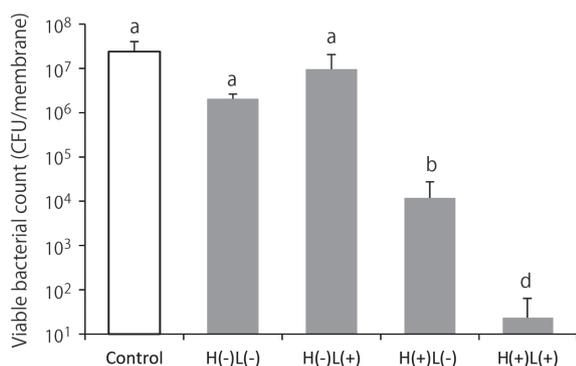


Fig. 6. Number of viable *Streptococcus mutans* recovered after each treatment. Control, no treatment; H(-)L(-), physiological saline alone; H(-)L(+), physiological saline with laser irradiation at 40 mW; H(+L)(-), 3% H_2O_2 alone; and H(+L)(+), 3% H_2O_2 with laser irradiation at 40 mW. Data are expressed as the mean with standard deviation ($n = 3$). Significant differences ($p < 0.05$) in each group are denoted by different alphabetical letters (*i.e.*, bars with the same alphabetical letter are not significantly different).

reduction, indicating that hydroxyl radicals generated by H_2O_2 photolysis contribute to the disinfection process.

Reactive oxygen species (ROS), such as singlet oxygen, superoxide anion radicals, H_2O_2 , and hydroxyl radicals, induce oxidative damage to tissues or cells if not controlled (Fridovich 1986; Slater 1984). Of the ROS, the hydroxyl radical has the highest reactivity and an extremely short lifetime (10^{-9} sec) (Halliwell and Gutteridge, 1986; Pryor, 1986; Roots and Okada, 1975; Sies *et al.*, 1992). Thus, although residual toxicity of the hydroxyl radical would be negligible because of its extremely short lifetime, the hydroxyl radical might cause acute oxidative damage to the oral mucosa. According to the histological examination in the present study, no detrimental effect was observed in the palatal mucosa, even in rats that received three treatments of 3% H_2O_2 and concomitant laser irradiation at an output power of 40 mW. This finding suggests that the acute locally injurious property of the disinfection technique is considerably low.

In conclusion, our latest test model may have sufficient radical disinfection potency and can be safely used to treat dental and periodontal infectious diseases.

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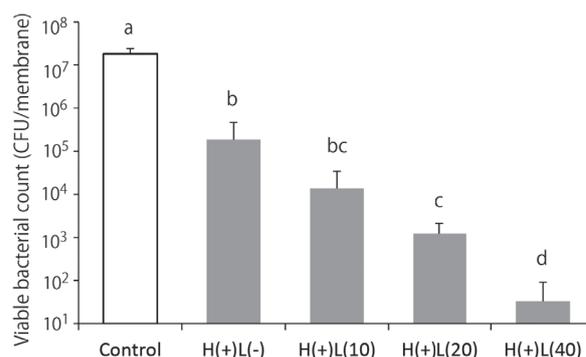


Fig. 7. Number of viable *Streptococcus mutans* recovered after each treatment. Control, no treatment; H(+L)(-), 3% H_2O_2 alone; H(+L)(10), 3% H_2O_2 with laser irradiation at 10 mW; H(+L)(20), 3% H_2O_2 with laser irradiation at 20 mW; and H(+L)(40), 3% H_2O_2 with laser irradiation at 40 mW. Data are expressed as the mean with standard deviation ($n = 3$). Significant differences ($p < 0.05$) in each group are denoted by different alphabetical letters (*i.e.*, bars with the same alphabetical letter are not significantly different).

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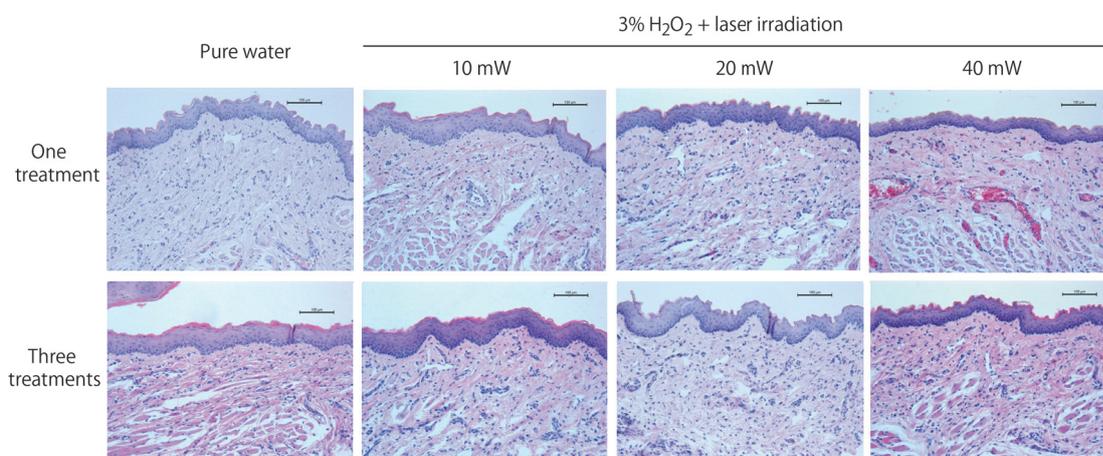


Fig. 8. Representative histological images of palatal mucosal regions treated once or thrice with pure water alone or running 3% H₂O₂ with laser irradiation at 10, 20, or 40 mW. No abnormal findings were observed in any of the 3% H₂O₂ with laser irradiation groups compared with the two pure water groups. Hematoxylin-eosin staining. Scale bar: 100 μ m.

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Conflict of interest---- The authors declare that there is no conflict of interest.

REFERENCES

- Buettner, G.R. (1987): Spin trapping: ESR parameters of spin adducts. *Free Radic. Biol. Med.*, **3**, 259-303.
- Cló, E., Snyder, J.W., Ogilby, P.R. and Gothelf, K.V. (2007): Control and selectivity of photosensitized singlet oxygen production: challenges in complex biological systems. *Chembiochem.*, **8**, 475-481.
- Food and Drug Administration (2003): Oral Health Care Drug Products for Over-the-Counter Human Use; Antigingivitis/Antiplaque Drug Products; Establishment of a Monograph, FR Doc 03-12783 available at <http://www.fda.gov/ohrms/dockets/98fr/03-12783.html> (accessed July 2016).
- Fridovich, I. (1986): Biological effects of the superoxide radical. *Arch. Biochem. Biophys.*, **247**, 1-11.
- Halliwell, B. and Gutteridge, J.M. (1986): Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts. *Arch. Biochem. Biophys.*, **246**, 501-514.
- Ikai, H., Nakamura, K., Shirato, M., Kanno, T., Iwasawa, A., Sasaki, K., Niwano, Y. and Kohno, M. (2010): Photolysis of hydrogen peroxide, an effective disinfection system via hydroxyl radical formation. *Antimicrob. Agents Chemother.*, **54**, 5086-5091.
- Ikai, H., Odashima, Y., Kanno, T., Nakamura, K., Shirato, M., Sasaki, K. and Niwano, Y. (2013): *In vitro* evaluation of the risk of inducing bacterial resistance to disinfection treatment with photolysis of hydrogen peroxide. *PLoS One*, **8**, e81316.
- Oyamada, A., Ikai, H., Nakamura, K., Hayashi, E., Kanno, T., Sasaki, K. and Niwano, Y. (2013): *In vitro* bactericidal activity of photo-irradiated oxydol products via hydroxyl radical generation. *Biocontrol Sci.*, **18**, 83-88.
- Pryor, W.A. (1986): Oxy-radicals and related species: their formation, lifetimes, and reactions. *Annu. Rev. Physiol.*, **48**, 657-667.
- Roots, R. and Okada, S. (1975): Estimation of life times and diffusion distances of radicals involved in x-ray-induced DNA strand breaks of killing of mammalian cells. *Radiat. Res.*, **64**, 306-320.
- Shirato, M., Ikai, H., Nakamura, K., Hayashi, E., Kanno, T., Sasaki, K., Kohno, M. and Niwano, Y. (2011): Synergistic effect of thermal energy on bactericidal action of photolysis of H₂O₂ in relation to acceleration of hydroxyl radical generation. *Antimicrob. Agents Chemother.*, **56**, 295-301.
- Sies, H., Stahl, W. and Sundquist, A.R. (1992): Antioxidant functions of vitamins. Vitamins E and C, beta-carotene, and other carotenoids. *Ann. N.Y. Acad. Sci.*, **669**, 7-20.
- Slater, T.F. (1984): Free-radical mechanisms in tissue injury. *Biochem. J.*, **222**, 1-15.
- Talebi, M., Taliiee, R., Mojahedi, M., Meymandi, M. and Torshabi, M. (2016): Microbiological efficacy of photodynamic therapy as an adjunct to non-surgical periodontal treatment: A clinical trial. *J. Lasers Med. Sci.*, **7**, 126-130.
- Toki, T., Nakamura, K., Kurauchi, M., Kanno, T., Katsuda, Y., Ikai, H., Hayashi, E., Egusa, H., Sasaki, K. and Niwano, Y. (2015): Synergistic interaction between wavelength of light and concentration of H₂O₂ in bactericidal activity of photolysis of H₂O₂. *J. Biosci. Bioeng.*, **119**, 358-362.
- Yamada, Y., Mokudai, T., Nakamura, K., Hayashi, E., Kawana, Y., Kanno, T., Sasaki, K. and Niwano, Y. (2012): Topical treatment of oral cavity and wounded skin with a new disinfection system utilizing photolysis of hydrogen peroxide in rats. *J. Toxicol. Sci.*, **37**, 329-335.