Journal of Oral Science, Vol. 56, No. 1, 35-39, 2014

Original

Passive ultrasonic irrigation in the presence of a low concentration of hydrogen peroxide enhances hydroxyl radical generation and bactericidal effect against *Enterococcus faecalis*

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(Received December 2, 2013; Accepted January 9, 2014)

Abstract: Chemomechanical procedures can be used to eliminate bacteria from root canals. However, detectable bacteria sometimes remain because of the complexity of the root canal system. Endodontic passive ultrasonic irrigation (PUI) with hydrogen peroxide (H_2O_2) may be a promising option for increasing bactericidal hydroxyl radical ($HO^{\cdot}$) generation. In this *in vitro* experiment, we examined the effects of $HO^{\cdot}$ generated using PUI and a low concentration of H_2O_2. An ultrasonic tip was submerged in 0.45 mol/L (1.5%) H_2O_2 in a microfuge tube. H_2O_2 was activated by an ultrasonic unit, the tip of which was kept centered in the tube, to mimic PUI. $HO^{\cdot}$ generation was detected by electron spin resonance spectroscopy. An *Enterococcus faecalis* suspension in H_2O_2 was then prepared and activated as described above. Bactericidal effects were assessed by viable counting. Two-way analysis of variance and Tukey's test were used to assess the statistical significance of differences among groups ($P < 0.05$). $HO^{\cdot}$ generation and bactericidal activity were significantly increased by PUI in H_2O_2 in a time-dependent manner and were significantly higher than with H_2O_2 alone or with PUI in a Tris-HCl suspension. These results suggest that PUI in the presence of a low H_2O_2 concentration is a promising new disinfection strategy.
(J Oral Sci 56, 35-39, 2014)

Keywords: passive ultrasonic irrigation; hydroxyl radical; *Enterococcus faecalis*; reactive oxygen species; electron spin resonance spectroscopy; hydrogen peroxide.

Introduction

Bacteria and their products are the major causes of periapical lesions (1); thus, the primary objectives of root canal treatment are elimination of bacteria from the root canal system and subsequent repair of periapical periodontal tissue (2). However, hand and rotary instrumentation techniques are not always adequate for complete shaping and cleaning of the root canal, due to its complexity (3).

Present methods of chemomechanical debridement of root canals sometimes leave detectable remnant

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doi.org/10.2334/josnusd.56.35
DN/JST.JSTAGE/josnusd/56.35

bacteria (4,5). Because residual bacteria may worsen treatment outcomes, supplementary approaches have been developed to improve root canal disinfection. One recommended approach to enhancing disinfection is passive ultrasonic irrigation (PUI), which involves ultrasonic activation of an irrigant. PUI was reported to enhance disinfection, possibly because of ultrasonic cavitation and acoustic streaming (6,7). However, findings of antibacterial studies have been inconclusive (8,9). Therefore, additional research is needed in order to determine the effects of PUI on root canal disinfection.

Studies of the bactericidal effects of H_2O_2 in biological systems have shown growth inhibition and/or inactivation of pathogenic bacteria at concentrations greater than 100 ppm (0.01%), under suitable operative conditions (10). However, H_2O_2 at a concentration of 3-5% has been used as an endodontic irrigant (11), which indicates that the antimicrobial efficacy and tissue-dissolving capacity of H_2O_2 used in root canal treatment are less than those of sodium hypochlorite (11). It has been postulated that an irrigation protocol involving alternating use of sodium hypochlorite and H_2O_2 could improve canal cleanliness and reduce intraradicular bacteria; however, this has not been substantiated (12).

H_2O_2 is a member of the family of reactive oxygen species (ROS), which form due to the presence of strongly oxidant chemicals. ROS are chemically reactive molecules containing oxygen and are generated in biological defense systems as part of the immunological response to invading bacteria (13). In addition, H_2O_2 is converted into the hydroxyl radical ($HO^{\bullet}$) in the presence of ferric compounds (14). $HO^{\bullet}$ is also an ROS and has one unpaired electron in its structure, so it is apt to deprive other substances of an electron, thus oxidizing them (15). This makes $HO^{\bullet}$ reactive and toxic to bacteria because it oxidizes sulfhydryl groups and double bonds in proteins, lipids, and surface membranes (16). Moreover, $HO^{\bullet}$ is formed due to energies generated during cavitation bubble collapse when water is treated with ultrasound (17).

Ultrasound, such as endodontic PUI, in the presence of H_2O_2 thus seems a promising option to increase $HO^{\bullet}$ generation, because ultrasonic irradiation accelerates H_2O_2 dissociation, thereby liberating free radicals. In this *in vitro* experiment, we qualitatively assessed $HO^{\bullet}$ generation from H_2O_2 activated by a dental ultrasonic unit and investigated the bactericidal effect of this $HO^{\bullet}$ generation on *Enterococcus faecalis*, which has been implicated in persistent root canal infections.

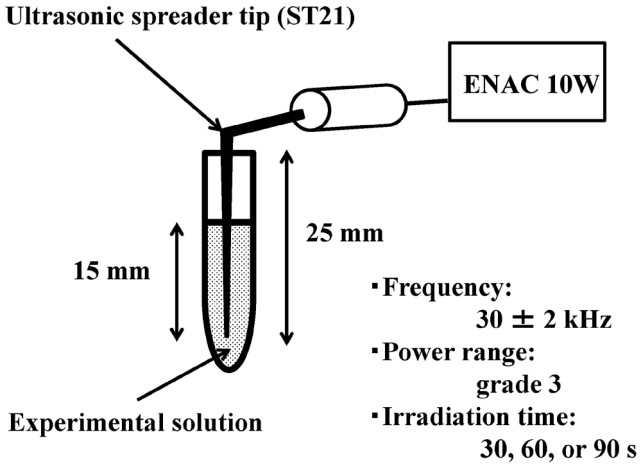

Fig. 1 Schematic illustration of the experimental design.

Materials and Methods

Reagents and dental ultrasonic unit

The names and sources of reagents used in this study were 5-(2,2-dimethyl-1,3-propoxycyclophosphoryl)-5-methyl-1-pyrroline-*N*-oxide (CYPMPO) from Radical Research (Tokyo, Japan), 4-hydroxy-2,2,6,6-tetramethyl-piperidinoxyl (tempol) from Sigma Aldrich (St. Louis, MO, USA), and 30% H_2O_2 from Wako Chemical Industries (Tokyo, Japan).

An ENAC 10W device (Osada Electric Co., Inc., Tokyo, Japan) with an ultrasonic spreader tip (ST-21, Osada Electric Co., Inc.) was used as the dental ultrasonic unit and was operated at a fixed driving frequency of 30 ± 2 kHz. In accordance with the manufacturer's instructions for endodontic use, the ENAC 10W was operated at power range 3 without water flow.

Experimental design

The design of the study is shown in Fig. 1. Briefly, the experimental solutions (360 μL) consisted of either 0.5 mol/L H_2O_2 diluted with 0.025 mol/L Tris-HCl buffer (pH 7.0) or 0.025 mol/L Tris-HCl buffer alone. The ultrasonic tip was inserted into the experimental solution in a 600-μL microfuge tube. Soaking length was fixed at 15 mm of the ultrasonic tip (total length: 25 mm). Then, the experimental solution was passively activated, using the dental ultrasonic unit, for 30, 60, or 90 s, during which the ultrasonic tip was kept centered in the microfuge tube, to mimic PUI conditions. Four experimental conditions were tested: 1) Tris-HCl buffer without PUI, 2) H_2O_2 without PUI, 3) Tris-HCl buffer with PUI, and 4) H_2O_2 with PUI.

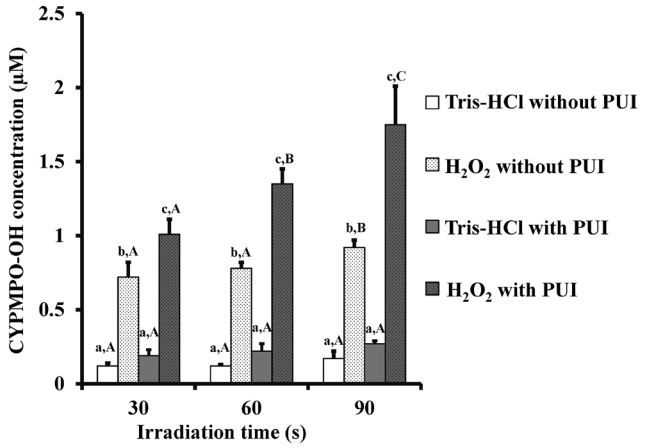

Fig. 2 Determination of CYPMPO-OH concentration as a measure of HO• generation ($n = 6$). Statistically significant differences were assessed by two-way ANOVA and Tukey's test. Within experimental solutions, means sharing the same upper-case letter are not significantly different ($P > 0.05$). Between experimental solutions at the same irradiation time, means sharing the same lower-case letter are not significantly different ($P > 0.05$).

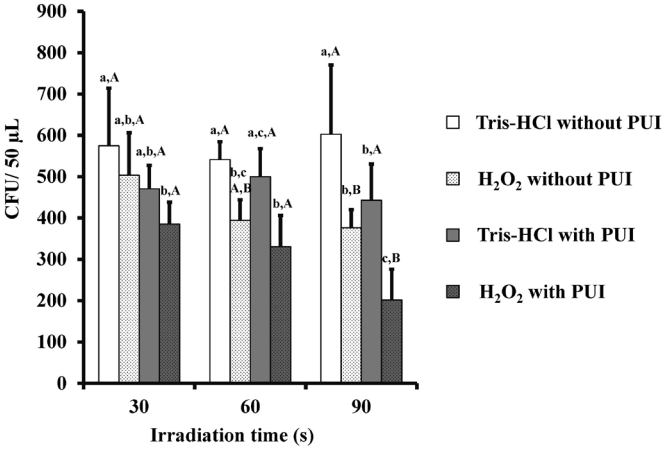

Fig. 3 Viable counting of *E. faecalis* ($n = 6$). Statistically significant differences were assessed by two-way ANOVA and Tukey's test. Within experimental solutions, means sharing the same upper-case letter are not significantly different ($P > 0.05$). Between experimental solutions at the same irradiation time, means sharing the same lower-case letter are not significantly different ($P > 0.05$).

HO• generation from H_2O_2 with or without PUI

HO• generation in the four experimental conditions was analyzed quantitatively by an electron spin resonance (ESR) spin-trapping technique. This was conducted using a ROS-generating system containing CYPMPO. Briefly, CYPMPO (40 μL) was added to each solution, to produce a final H_2O_2 concentration of 0.45 mol/L (1.5%). HO• generation was assessed using the experimental conditions described above. The ESR observations were performed with a JES-RE1X device (JEOL, Tokyo, Japan) connected to a WIN-RAD ESR Data Analyzer (Radical Research, Tokyo, Japan) with the following instrument settings: microwave power, 8.00 mW; magnetic field, 335.6 ± 7.5 mT; field modulation width, 0.079 mT; sweep time, 1 min; and time constant, 0.03 s.

For HO• quantification, tempol (100 μmol/L) was used as the standard to calculate CYPMPO-OH concentration. HO• concentration was determined using digital data processing (JEOL) and is expressed as the concentration of CYPMPO-OH. All experiments were performed in six sets ($n = 6$).

Bactericidal activity assay

The *E. faecalis* JCM5803 stock culture was obtained from the Japan Collection of Microorganisms (RIKEN BioResource Center, Tsukuba, Japan). Bacteria were cultured aerobically in brain-heart infusion (BHI) broth (Becton Dickinson Labware, Franklin Lakes, NJ, USA) at 37°C and, after harvesting by centrifugation, were washed once in 0.025 mol/L Tris-HCl buffer (pH 7.0) and resuspended in the same buffer. The cell density of suspensions was adjusted to approximately 2.0×10^7 cells/mL. In a 600-μL microcentrifuge tube, 200 μL of the suspension was mixed with 200 μL of 0.9 mol/L H_2O_2 diluted with Tris-HCl buffer to yield a final concentration of 1.0×10^7 cells/mL and 0.45 mol/L (1.5%) H_2O_2, for ESR measurement. Immediately after mixing, the suspension was exposed to ultrasound as described above. A 100-fold serial dilution of the mixture was then prepared using Tris-HCl buffer, and 50 μL were spread on BHI agar (Becton Dickinson Labware). Plates were cultured at 37°C for 18 h under the conditions described above, and then numbers of colony forming units (CFUs)/50 μL were determined.

Statistical analysis

All tests were performed in six sets ($n = 6$). Two-way analysis of variance (ANOVA) and Tukey's test were used to assess the statistical significance of differences among groups ($P < 0.05$).

Results

HO• generation from H_2O_2 with or without PUI

HO• generation from H_2O_2 with or without PUI was investigated using the ESR spin-trapping technique with CYPMPO. HO• generated from H_2O_2 with PUI increased significantly in a time-dependent manner (Fig. 2). Moreover, the presence of HO• was confirmed, because HO• generation was abolished by the addition of L-ascorbic acid (0.1 mol/L), a major antioxidant of HO• (data not

shown). HO• generation from H_2O_2 without PUI was significantly lower than with PUI, irrespective of experimental duration (Fig. 2). Levels of HO• from Tris-HCl buffer were lower regardless of PUI use (Fig. 2).

Bactericidal activity assay

The bactericidal effect against *E. faecalis* was examined by viable counting. Viable counting of *E. faecalis* was decreased by PUI in a time-dependent manner. The number of CFUs after exposure of bacteria to H_2O_2 with PUI for 90 s was significantly lower than after exposure for 30 or 60 s (Fig. 3). Moreover, after ultrasound treatment for 90 s, the number of CFUs significantly differed after exposure of bacteria to H_2O_2 with PUI versus exposure to H_2O_2 without PUI (Fig. 3).

Discussion

Two types of ultrasonic irrigation for root canal treatment have been reported. One combines irrigation with simultaneous ultrasonic instrumentation. The other (ie, PUI) does not use simultaneous instrumentation (6,11). PUI relies on transmission of acoustic energy from an oscillating ultrasonic instrument to an irrigant in the root canal. Energy is transmitted by ultrasonic waves, which can induce acoustic streaming and cavitation of the irrigant (6,11). This induction enhanced HO• generation from H_2O_2 in the present study. H_2O_2 serves as a source of HO• in the dissociation process, (14) and HO• is formed from energies generated during cavitation bubble collapse (17). Using our PUI system, we observed enhanced HO• generation from H_2O_2 with ultrasonic waves. Thus, HO• generation might be caused by both acceleration of H_2O_2 dissociation due to PUI and ultrasonic cavitation activity.

The ESR spin-trapping technique is used to quantitatively assess ROS such as HO•. This technique uses compounds that readily react with free radicals to produce a relatively long-lived free-radical product (spin adduct), which can be identified by its ESR spectrum (18,19). We developed an ESR-based technique to detect free-radical reactions induced by ROS in biological systems *in vitro* and *in vivo* (18,19). Regarding *in vitro* ESR applications, the spin-trapping technique is a well known method of using ESR spin adduct to detect ROS by quantifying spin concentration in experimental systems (18,19). Although the half-life of HO• is extremely short, approximately 10^{-9} s (20), total HO• generation throughout the experimental period can be directly and precisely assessed using the ESR technique. In the present study, the amount of HO• generated from H_2O_2 with PUI significantly time-dependently increased. This suggests that PUI stimulated continuous formation of HO• throughout the ultrasonic irradiation time. HO• generation from H_2O_2 without PUI was significantly lower than with PUI, perhaps due to spontaneous H_2O_2 decomposition, which liberates HO•. The specificity of H_2O_2 for HO• generation was confirmed because little HO• was detected in Tris-HCl buffer with or without PUI.

An H_2O_2 concentration of 0.9-1.5 mol/L corresponds to approximately 3-5% H_2O_2, which is the concentration used in conventional endodontic irrigants (11). A lower H_2O_2 concentration was used in this study because chemical disinfectants can cause problems such as tissue damage and accidental injury due to leakage (21,22). Additionally, a subcommittee of the US Food and Drug Administration (FDA, 2003) concluded that H_2O_2 was safe at concentrations of up to 3%. Accordingly, a low-concentration endodontic irrigant with bactericidal effects might be safer for patients. Thus, for reasons of clinical safety the H_2O_2 concentration used in this study was fixed at 0.45 mol/L (1.5%), which is about half the concentration of a conventional endodontic irrigant.

We also examined the bactericidal effect of H_2O_2 with PUI on *E. faecalis*, a gram-positive anaerobic facultative coccus that has been recovered from several oral sites (23). *E. faecalis* was used in the present study because it has a high level of resistance to a wide range of medications, such as calcium hydroxide (24), and is commonly found in cases of root canal treatment failure associated with persistent apical periodontitis (25,26). Viable counting of *E. faecalis* was time-dependently decreased by PUI. The number of CFUs after exposure to H_2O_2 with PUI for 90 s was significantly lower than after exposure for 30 and 60 s. These findings indicate that the total viable count of *E. faecalis* depends on the amount of HO• generated and exposure time, because ESR measurements showed that HO• generated from H_2O_2 with PUI time-dependently increased. Moreover, the number of CFUs after exposure to H_2O_2 with PUI was significantly lower than after exposure to either H_2O_2 without PUI or Tris-HCl buffer with PUI for 90 s. The differences in these effects may be due to HO• generation by PUI. However, the bactericidal effect on this system was not entirely satisfactory as a root canal disinfection method, due to small differences (less than 1 $\log_{10}$) in CFU values between PUI in the 90-s ultrasound group and the other groups. This may explain the amount of HO• generated due to PUI. Future research should determine how to improve HO• generation in this system by using more-powerful ultrasound.

Uncontrolled ROS cause oxidative damage to tissues and cells (27). A previous study of HO• generation by photolysis of 1 M H_2O_2 found no detrimental effect on either oral mucosa or the healing of wounded skin (28).

Moreover, since HO• generation by photolysis of H_2O_2 increases linearly with H_2O_2 concentration, HO• generation with PUI in 0.45 mol/L (1.5%) H_2O_2 would likely have no adverse effect on periodontal tissues.

In conclusion, the ESR spin-trapping technique and viable counting showed that HO• was generated from a low concentration of H_2O_2 activated by PUI and that the number of CFUs of *E. faecalis* was reduced. These results indicate that PUI in the presence of a low concentration of H_2O_2 is a promising new disinfection strategy.

Acknowledgments

This study was supported by Research Grants from the Sato Fund for 2012 and by grants in 2012 and 2013 from the Dental Research Center of the Nihon University School of Dentistry.

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