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Bactericidal effect of hydroxyl radicals generated by the sonolysis and photolysis of hydrogen peroxide for endodontic applications

Haruna Ibi [a], Makoto Hayashi [a, *], Fumihiko Yoshino [b], Muneaki Tamura [c], Ayaka Yoshida [b], Yoshimi Kobayash [a], Kohei Shimizu [a], Masaichi-Chang-il Lee [d], Kenichi Imai [c], Bunnai Ogiso [a]

[a] Department of Endodontics, Nihon University School of Dentistry, Tokyo, Japan
[b] Photomedical Dentistry Division, Department of Oral Science, Kanagawa Dental University Graduate School, Kanagawa, Japan
[c] Department of Microbiology, Nihon University School of Dentistry, Tokyo, Japan
[d] Yokosuka-Shonan Disaster Health Emergency Research Center & ESR Laboratories, Graduate School of Dentistry, Kanagawa Dental University, Kanagawa, Japan

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ABSTRACT

The aim of endodontic root canal treatment is the elimination of bacteria and their products from an infected tooth root canal. To effectively disinfect a root canal, an ultrasonic irrigation system, in which hydroxyl radicals (HO˙) generated artificially by sonolysis of H_2O_2, was developed previously for endodontic applications and was demonstrated to have bactericidal efficacy against *Enterococcus faecalis*. To improve this system, we examined the *in vitro* bactericidal effects of HO˙ generated from H_2O_2, activated by simultaneous irradiation with ultrasound for sonolysis and dental LED light for photolysis with a peak wavelength of 405 nm. Regarding the LED irradiation, two methods were used: (i) 'ideal' experimental conditions (irradiation close to the glass tube), and (ii) simulated endodontic conditions (more distant irradiation of a masked glass tube). In these conditions, HO˙ generation from H_2O_2 was detected by electron spin resonance (ESR) spectroscopy, and bactericidal efficacy against *E. faecalis* was assessed by measuring the colony forming units (CFU)/mL. The results indicated that HO˙ generation by ESR measurements and the bactericidal effect on *E. faecalis* by viable count using CFU/mL were enhanced significantly in a time-dependent manner in both conditions. In a comparison of these conditions, bactericidal activity under 'ideal' experimental conditions was similar to that under simulated endodontic conditions. Moreover, the irradiation time for effective killing of *E. faecalis* through the sonolysis and photolysis of H_2O_2 under simulated endodontic conditions was shorter than that with sonolysis alone. These results demonstrate that H_2O_2 activated by ultrasound and LED light may be a safe and effective disinfection technique for endodontic root canal treatment.

1. Introduction

The major cause of periapical periodontitis accompanied by the destruction of peri-radicular tissues is considered to be a persistent bacterial infection in the root canal space of the tooth [1,2]. Thus, the aim of endodontic root canal treatment is the elimination of bacteria and their products from an infected tooth root canal [3]. However, the complex anatomy of the root canal, including the isthmus, fin, and ramifications, makes the complete chemomechanical removal of pathogenic bacteria difficult [4].

The methods presently available for chemomechanical debridement of root canal result in a considerable number of cases with detectable remaining bacteria, leading to persistent inflammation around peri-radicular tissues [5,6]. One approach to enhance disinfection is ultrasonic irrigation of the root canal, which involves ultrasonic activation of an endodontic irrigant such as sodium hypochlorite [7,8]. This technique seems to improve debridement of the root canal, possibly because of ultrasonic cavitation and acoustic streaming. However, additional research is needed to determine the effects of ultrasonic irrigation because the results of previous antibacterial studies have been inconclusive

* Corresponding author. Department of Endodontics, Nihon University School of Dentistry, 1-8-13 Kanda-Surugadai, Chiyoda-ku, Tokyo, 101-8310, Japan.
E-mail address: hayashi.makoto53@nihon-u.ac.jp (M. Hayashi).

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[9,10].

To effectively disinfect a root canal despite its complex anatomy, we previously developed a novel ultrasonic irrigation system for endodontic use in which hydroxyl radicals (HO˙) were generated artificially by the sonolysis of hydrogen peroxide (H_2O_2) [11]. With this system, we demonstrated that the bactericidal effects of HO· generated from H_2O_2 against *Enterococcus faecalis*, which is commonly found in cases of root canal treatment failure [12–14], were increased by ultrasonic irradiation in a time-dependent manner [11]. However, more than 3 min of irradiation time was required for the sonolysis of H_2O_2 in this system.

It has been reported that HO˙ is generated by photolysis of H_2O_2. Some studies have reported that irradiation with ultraviolet light can activate H_2O_2 to form HO˙, which is capable of destroying microbial cells [15,16]. In another study, laser light with a wavelength of 405 nm enhanced HO˙ generation from H_2O_2 and the bactericidal effect [17,18]. These mechanisms of H_2O_2 photolysis are similar to those with sonolysis; the only difference is that the energy required for the generation of HO˙ from H_2O_2 is provided by light as opposed to cavitation bubbles (in the case of ultrasound). However, attention should be paid to patient safety during the use of ultraviolet or laser light in a clinical setting.

Here, we considered that a light-emitting diode (LED) with a peak wavelength of 405 nm, as used in conventional tooth whitening treatments, might improve the bactericidal efficacy of H_2O_2 sonolysis. Thus, the purpose of the present study was to assess qualitatively HO˙ generation from H_2O_2 activated by ultrasound and a conventional dental LED light *in vitro* and the bactericidal effect of HO˙ generation against *E. faecalis*.

2. Materials and methods

2.1. Reagents

5-(2,2-dimethyl-1,3-propoxycyclophosphoryl)-5-methyl-1-pyrroline-*N*-oxide (CYPMPO) was obtained from Mikuni Pharmaceutical Industrial Co., Ltd. (Osaka, Japan). Dimethylsulfoxide (DMSO) and H_2O_2 were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.2. Ultrasonic unit and LED irradiator

A Handy Sonic UR-20P (Tomy Seiko Co., Ltd., Tokyo, Japan) with an active ultrasonic tip (φ 2.5-mm) was used as the ultrasonic unit, operated at a fixed driving frequency of 28 kHz with an output power of 10 W. A dental LED light, a Pencure 2000 (J Morita Mfg. Corp., Kyoto, Japan), with a peak wavelength of 405 nm (**a range of 400 to 410 nm and** 300 mW/cm^2) was used as the light source.

2.3. Ultrasound irradiation

Ultrasound irradiation of H_2O_2 was performed according to a previous report [11] with modifications. Briefly, experimental solutions (500 μL) consisted of 0.5 or 1.0 M H_2O_2 diluted with 0.025 M Tris-HCl buffer (pH 7.0); 0.025 M Tris-HCl buffer alone was used as a control. The ultrasonic tip was inserted into the experimental solution in a glass tube (diameter, 11 mm; height, 20 mm). The soaking length was fixed at 7 mm of the ultrasonic tip. Then, the experimental solution was activated with ultrasound for 1, 2, or 3 min on ice (to avoid temperature changes), during which the ultrasonic tip was maintained in the centre of the glass tube to mimic clinical endodontic ultrasonic irrigation.

2.4. Simultaneous ultrasound and LED light irradiation

LED light irradiation was performed in the sonolysis system described above under two conditions: (i) LED irradiation under 'ideal' experimental conditions for the photolysis of H_2O_2 (irradiation close to the glass tube); the head of LED irradiator was placed at the lateral surface of the glass tube to irradiate the centre of the experimental solution with LED light (the distance between the head of LED irradiator and the glass tube was kept at 1 mm) (Fig. 1A), and (ii) LED irradiation under simulated endodontic conditions (more distant irradiation to a masked glass tube). The lateral surface of the glass tube was masked with black tape to avoid transmitting LED light, then the head of the LED irradiator was placed at the orifice of the glass tube to mimic clinical endodontic conditions. The distance between the head of the LED irradiator and the top surface of experimental solution was kept at 10 mm (Fig. 1B). Also, irradiation with the LED light alone was performed to confirm the specific effect of LED light on HO˙ generation.

2.5. Measurement of HO˙ generation from H_2O_2

HO˙ generation was analyzed quantitatively using an electron spin resonance (ESR) spin-trapping technique, as described previously [11]. This analysis was conducted using a reactive oxygen species-generating system containing CYPMPO as a spin trap agent. Briefly, 50 μL of CYPMPO was added to each solution, to yield a final H_2O_2 concentration of 0.5 or 1.0 M. Then, HO˙ generation was assessed under the irradiation conditions described above. The ESR observations were performed with a JES-RE1X (JEOL, Tokyo, Japan) connected to a WIN-RAD ESR data analyzer (Shidai System, Saitama, 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. This method produces a relatively long-lived free radical product (spin adduct), and it can be identified by its ESR spectrum with signal specific to HO˙. Therefore, HO˙ generation was expressed as the signal intensity of spin adduct of CYPMPO-OH (peak height).

2.6. Bactericidal activity assay

The bactericidal effect of HO˙ against *E. faecalis* was evaluated using the numbers of colony forming units (CFUs), as described previously [11]. Briefly, an *E. faecalis* JCM5803 stock culture was obtained from the Japan Collection of Microorganisms (RIKEN BioResource Center, Tsukuba, Japan). The cells were cultured aerobically in brain–heart infusion (BHI) broth (Becton Dickinson Labware, Franklin Lakes, NJ, USA) at 37 °C for 18 h and, after harvesting by centrifugation, were washed once in 0.025 M Tris-HCl buffer (pH 7.0) and resuspended in the same buffer. The cell density of the suspensions was adjusted to ~2.0×10^7 cells/mL. In the glass tube, 250 μL of the suspension was mixed with 250 μL of 0.9 M H_2O_2 and then diluted with Tris-HCl buffer to yield a final concentration of 1.0×10^7 cells/mL and 0.5 or 1.0 M H_2O_2, as for ESR measurement. Immediately after mixing, the suspension was exposed to ultrasound and/or LED light, 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). The Plates were cultured at 37 °C for 18 h under the conditions described above, and the CFU/mL were determined.

2.7. Statistical analysis

All experiments were performed in six sets ($n = 6$). To assess the statistical significance of differences among groups in terms of