# Hydroxyl radicals generated from a low concentration hydrogen peroxide with ultrasonic irradiation exhibit bactericidal effect against *Enterococcus faecalis*

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The following two articles are part of this doctoral dissertation:

Yoshimi Kobayashi et al. *Journal of Clinical Biochemistry and Nutrition,* 2014 *(in press)*

Yoshimi Kobayashi et al. *Journal of Oral Science*, 2014 *(in press)*

## Abstract

The primary objectives of root canal treatment are elimination of bacteria from the root canal system and subsequent repair of periapical periodontal tissue. However, the current endodontic techniques are not always adequate for complete shaping and cleaning of the root canal, due to its complexity. Therefore, one recommended approach to enhancing disinfection is passive ultrasonic irrigation (PUI), which involves ultrasonic activation of endodontic irrigants.

Hydrogen peroxide ($H_2O_2$) at a concentration of 3-5% has been used as endodontic irrigants, however, the antimicrobial efficacy and tissue-dissolving capacity of $H_2O_2$ are less than those of sodium hypochlorite. On the other hand, $H_2O_2$ is converted into the hydroxyl radical ($HO^{\cdot}$), which is toxic to bacteria, in the presence of ferric compound, ultraviolet radiation, or ultrasonic irradiation. PUI in the presence of $H_2O_2$ thus seems a promising option in the elimination of bacteria from root canal *via* $HO^{\cdot}$ formation.

The purpose of this study was to assess $HO^{\cdot}$ generation from $H_2O_2$ activated by ultrasonic irradiation and investigate its bactericidal effect against *Enterococcus faecalis*, which have been implicated in persistent root canal infections.

In cahapter 1, $HO^{\cdot}$ generation and its bactericidal effect against *E. faecalis* from a

low concentration $H_2O_2$ with ultrasonic irradiation were examined *in vitro*. An ultrasonic tip was submerged in 0.5 or 1.0 M $H_2O_2$ in a microfuge tube. $H_2O_2$ was irradiated with the ultrasound, the tip of which was maintained centered in the tube to mimic ultrasonic irrigation. $HO^{\cdot}$ generation was assessed by electron spin resonance (ESR) spectroscopy. Subsequently, *E. faecalis* suspension in $H_2O_2$ was prepared and irradiated as described above. Bactericidal effects were assessed by viable counting. ESR measurements showed that $HO^{\cdot}$ generation increased significantly in a time- and dose-dependent manner (two-way ANOVA and Tukey's test, $\alpha = 0.05$). Moreover, the bactericidal effect of $H_2O_2$ against *E. faecalis* were enhanced by ultrasonic irradiation in a time- and dose-dependent manner with more than 4-log reduction of viable counting.

In chapter 2, $HO^{\cdot}$ generation by a dental ultrasonic unit as oscillator for ultrasound was observed and its bactericidal effect was assessed. A dental ultrasonic tip was submerged in 0.45 mol/L (1.5%) $H_2O_2$ in a microfuge tube. $H_2O_2$ was activated by a dental 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 *E. faecalis* suspension in $H_2O_2$ was then prepared and activated as described above. Bactericidal effect was assessed by viable counting (two-way ANOVA and Tukey's test, $\alpha = 0.05$).

$HO^{\cdot}$ 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.

Within the limitation of this study, these results suggest that $HO^{\cdot}$ generated from a low concentration hydrogen peroxide with ultrasonic irradiation exhibit bactericidal effect against *E. faecalis*. This technique might serve as a new disinfection strategy in endodontic treatment.

## Capter 1: Bactericidal effect of hydroxyl radicals generated from a low concentration hydrogen peroxide with ultrasound in endodontic treatment

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## Introduction

Bacteria and their products are considered to be major etiological agents of endodontic infections (1). Thus, the primary objective of root canal treatment is the elimination of these bacteria from the root canal system with subsequent repair of the periodontal tissue (2). However, the complexity of the root canal renders complete shaping and cleaning using various instrumentation techniques difficult (3).

Studies have shown that the methods presently available for chemomechanical debridement of the root canal result in a considerable number of cases with detectable remaining bacteria (4,5). Because residual bacteria may place the treatment outcome at risk, supplementary approaches have been developed to improve root canal disinfection. One approach to enhance disinfection is ultrasonic irrigation, which involves ultrasonic

activation of an endodontic irrigant, such as sodium hypochlorite. Such ultrasonic irrigation has been reported to enhance disinfection in the root canal, possibly because of ultrasonic cavitation and acoustic streaming (6,7). However, the findings of previous antibacterial studies have been inconclusive (8,9). As a result, additional research is needed to determine the effects of ultrasonic irrigation in root canal treatment.

Bactericidal effects of $H_2O_2$ in biological systems have been reported, with growth inhibition and/or inactivation of pathogenic bacteria when $H_2O_2$ is used at an appropriate disinfecting concentration and under suitable operating conditions (10). Concentrations in the range of 3-5% $H_2O_2$ have been used as endodontic irrigants (11). However, the antimicrobial efficacy and tissue-dissolving capacity of $H_2O_2$ used in root canal treatment are lower than those of the commonly used endodontic irrigant, sodium hypochlorite (11).

$H_2O_2$ is generally considered to be a reactive oxygen species (ROS). ROS are chemically reactive molecules containing oxygen that are generated in biological defense systems as part of the immunological response to invading bacteria (12). Additionally, $H_2O_2$ can be converted into $HO^{\cdot}$ by the Fenton (13) and Haber-Weiss (14) reactions. $HO^{\cdot}$ is also a ROS and has one unpaired electron in its structure, so it is apt to

remove an electron from other substances, thereby oxidizing them (15). This makes $HO^{\cdot}$ reactive and toxic to bacteria because it oxidizes sulfhydryl groups and double bonds in proteins, lipids, and membrane surfaces (16). Moreover, $HO^{\cdot}$ is formed due to the energy involved during cavitation bubble collapse when water is treated with ultrasound (17).

Thus, the use of ultrasound in the presence of $H_2O_2$ seems a promising option to increase the generation of $HO^{\cdot}$, which can be used for root canal disinfection in endodontic treatment. The purpose of this study was to qualitatively assess $HO^{\cdot}$ generation from $H_2O_2$ activated by an ultrasonic unit *in vitro* and the bactericidal effect of such $HO^{\cdot}$ generation against *Eenterococcus faecalis (E. faecalis)*, which has been implicated in persistent root canal infections.

## Materials and Methods

Reagents and ultrasonic unit

5-(2,2-dimethyl-1,3-propoxycyclophosphoryl)-5-methyl-1-pyrroline *N*-oxide (CYPMPO) was purchased from Radical Research (Tokyo, Japan). Dimethyl sulfoxide (DMSO) and $H_2O_2$ were purchased from Wako Pure Chemical Industries, Ltd. (Osaka,

Japan). The 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 or 20 W.

Experimental design

Experimental solutions (360 μL) consisted of 0.5 or 1.0 mol/L $H_2O_2$ diluted with 0.025 mol/L Tris-HCl buffer (pH 7.0); 0.025 mol/L Tris-HCl buffer alone was used as a control. The ultrasonic tip was inserted into the experimental solution in a 600-μL microfuge tube. The soaking length was fixed at 20 mm of the ultrasonic tip. Then, the experimental solution was activated with ultrasonic irradiation (UI) for 1, 2, or 3 min on ice to avoid temperature change, during which the ultrasonic tip was maintained centered in the microfuge tube to mimic endodontic ultrasonic irrigation. Four experimental conditions were tested: 1) Tris-HCl buffer without UI, 2) $H_2O_2$ without UI, 3) Tris-HCl buffer with UI, and 4) $H_2O_2$ with UI.

$HO^{\cdot}$ generation from $H_2O_2$ with UI

$HO^{\cdot}$ generation under the four experimental conditions was analyzed quantitatively using an electron spin resonance (ESR) spin-trapping technique. This analysis was

conducted using a ROS-generating system containing CYPMPO. Briefly, CYPMPO (40 μL) was added to each solution, to yield a final $H_2O_2$ concentration of 0.45 or 0.90 mol/L (1.5 or 3.0%). $HO^{\cdot}$ generation was assessed using the experimental conditions described above. The ESR observations were performed with a JES-RE1X (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 sec. The results are expressed as the signal intensity (peak height).

Viable counting for bactericidal activity

*E. faecalis* JCM5803 stock culture (Japan Collection of Microorganisms, RIKEN BioResource Center, Tsukuba, Japan) was 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 re-suspended in the same buffer. The cell density of suspensions was adjusted to ~$2.0 \times 10^7$ cells/mL. In a 600-μL microfuge 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 $1.0 \times 10^7$ cells/mL and 0.45 or 0.90 mol/L (1.5 or 3.0%) $H_2O_2$, as for the 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 was spread on BHI agar (Becton Dickinson Labware). Plates were cultured at 37°C for 18 hrs under the conditions described above, and then numbers of colony-forming unit (CFU)/mL were determined.

Statistical analysis

All tests were performed in six sets ($n$ = 6). To assess the statistical significance of differences among groups, two-way analysis of variance and Tukey's test were used ($\alpha$ = 0.05).

## Results

$HO^{\cdot}$ generation from $H_2O_2$ with UI

$HO^{\cdot}$ generation from $H_2O_2$ with UI was investigated using the ESR spin-trapping technique with CYPMPO. $HO^{\cdot}$ generated with 10 or 20 W irradiation in the presence of CYPMPO led to the formation of a characteristic CYPMPO-OH spin adduct spectrum

(hyperfine coupling constants: $A_N$ = 1.37 mT, $A_H$ = 1.37 mT, and $A_P$ = 4.88 mT), with hyperfine splitting, giving rise to 14 resolved peaks (Figs. 1A and 1B). CYPMPO-OH spin adduct formation increased significantly in a time- and dose-dependent manner ($p < 0.05$; Fig. 2). Moreover, the presence of $HO^{\cdot}$ was confirmed because the intensity of CYPMPO-OH spin adduct was decreased by the addition of 10.0 mol/L DMSO, a scavenger of $HO^{\cdot}$ (Fig. 3). In the experimental solutions without UI, little CYPMPO-OH signal intensity was observed, regardless of the experimental duration (data not shown).

Viable counting for bactericidal activity

The bactericidal effect against *E. faecalis* in $H_2O_2$ with UI was examined by viable counting. *E. faecalis* were effectively killed, with a 4-log reduction under the conditions of 1.0 mol/L $H_2O_2$ with 10 W irradiation in 3 min (Fig. 4A). On the other hand, no bactericidal effects were observed in both 0.5 mol/L $H_2O_2$ and Tris-HCl with UI regardless experimental duration. Figure 4B shows the bactericidal effect in $H_2O_2$ with 20 W irradiation. The number of CFU/mL dramatically decreased in 1.0 mol/L $H_2O_2$, and an approximately 4-$\log_{10}$ reduction was obtained within 2 min. Moreover, the

bacteria were killed effectively in 0.5 mol/L $H_2O_2$ with UI in 3 min (~4-log reduction). The condition with Tris-HCl alone killed almost no bacteria, even after 3 min UI.

## Discussion

Ultrasonic irrigation for root canal treatment relies on the transmission of acoustic energy from an oscillating ultrasonic instrument to an irrigant in the root canal. The energy is transmitted by means of ultrasonic waves and can induce acoustic streaming and cavitation of the irrigant (7,11). $H_2O_2$ has been reported to act as a source of $HO^{\cdot}$ in the dissociation process (13) and $HO^{\cdot}$ is formed due to the energy available generated during cavitation bubble collapse (17). $HO^{\cdot}$ generation from $H_2O_2$ with UI using this system was confirmed owing to the identical hyperfine coupling constants to previously reported constants of $HO^{\cdot}$ by Kamibayashi *et al*. (18) and spectrum elimination with DMSO in a same way by Mukohda *et al*. (19). Thus, the mechanism of $HO^{\cdot}$ generation may be explained by both acceleration of $H_2O_2$ dissociation by UI and ultrasonic cavitation activity. On the other hand, the small amount of CYPMPO-OH spin adduct spectrum was observed during UI of Tris-HCl (control solution). This phenomenon may be caused of $HO^{\cdot}$ formation due to ultrasonic cavitation activity in aqueous solutions.

The ESR spin-trapping technique is used for the quantitative assessment of ROS, such as $HO^{\cdot}$. This technique involves compounds that readily react with free radicals to produce a relatively long-lived free radical product (spin adduct), which can then be identified by its ESR spectrum (20,21). An ESR-based technique was developed to detect free radical reactions induced by ROS in biological systems *in vitro* and *in vivo* (20,21). Among *in vitro* ESR applications, the spin-trapping technique is well known for its abilities to detect ROS and quantify spin adduct concentration in experimental systems (20,21). Although the half-life of $HO^{\cdot}$ is extremely short (22), total $HO^{\cdot}$ generation throughout the experimental period can be directly and specifically assessed using the ESR technique. In the present study, the amounts of $HO^{\cdot}$ generated from $H_2O_2$ with UI at 10 and 20 W increased significantly in a time- and dose-dependent manner. These results would suggest that UI continuously formed $HO^{\cdot}$ throughout the ultrasonic exposure period. Moreover, it appears that the amount of $HO^{\cdot}$ generation increased in proportion to output power in this system. Therefore, further researches are needed to assess $HO^{\cdot}$ generation in several output power strengths such as more than 30 W.

The bactericidal effect of $H_2O_2$ with UI on *E. faecalis* was also examined. *E. faecalis*, a Gram-positive anaerobic facultative coccus, has been recovered from

several oral sites (23). This bacterium was used because it exhibits a high level of resistance to a wide range of medications (24), and is commonly found in cases of root canal treatment failure associated with persistent apical periodontitis (25,26). The present results indicated that the bactericidal effects were time- and dose-dependently enhanced by UI. The number of CFU/mL after 10 and 20 W irradiation in 1.0 mol/L $H_2O_2$ markedly decreased in 3 and 2 min irradiation time, respectively. In addition, *E. faecalis* was effectively killed with the conditions of 20 W irradiation for 3 min in 0.5 mol/L $H_2O_2$. These findings indicated that even lower concentration of $H_2O_2$ could be effective against *E. faecalis* in this system. This may depend upon the amount of $HO^{\cdot}$ from $H_2O_2$ because the ESR measurements showed more $HO^{\cdot}$ with longer irradiation times and higher $H_2O_2$ concentration. In biological system, $HO^{\cdot}$ causes radical chain reactions and leads to generation of many types of ROS including alkoxyl- and alkylperoxyl-radicals. These reactions in lipids of cell membrane refer to lipid peroxidation, which might be toxic to bacteria or cells (27). Therefore, bactericidal effect of this system may be due to not only $HO^{\cdot}$ but also other types of ROS. Thus, present results suggest that appropriate $H_2O_2$ concentration and irradiation time are important factors in the achievement of an optimal bactericidal effect in this system.

The conventional $H_2O_2$ concentration for endodontic application is approximately 3-5% (11), whereas 1.5 and 3.0% $H_2O_2$ were used in this study. Chemical disinfectants, such as the commonly used endodontic irrigant, sodium hypochlorite, can cause problems, such as tissue damage or accidental injury caused by leakage (28,29). 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 may be clinically desirable from patient-safety viewpoint.

ROS cause oxidative damage to tissues or cells if not controlled (30). Although no detrimental effect on the oral mucosa or healing of wounded skin in $HO^{\cdot}$ generation from $H_2O_2$ by laser irradiation was reported (31), it is difficult to compare UI with laser irradiation. Further studies are required to assess the safety for clinical use of present method.

From the results of this study, ultrasonic irrigation in the presence of $H_2O_2$ could provide the capacity for disinfection by not only ultrasonic cavitation and acoustic streaming, but also $HO^{\cdot}$ generation. In conclusions, the ESR spin-trapping technique and a bactericidal assay in the present study showed that $HO^{\cdot}$ was generated from a low

concentration of $H_2O_2$ activated by ultrasound, and that it exerted a bactericidal effect against *E. faecalis*. Thus, this method may be usefully applied in root canal disinfection.

## Capter 2: 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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## Introduction

From the results of chapter 1, the ESR spin-trapping technique and a bactericidal assay showed that $HO^{\cdot}$ was generated from a low concentration of $H_2O_2$ activated by ultrasound, and that it exerted a bactericidal effect against *E. faecalis*. Before the clinical application of this technique as PUI for endodontic treatment, the assessment of the effect of $HO^{\cdot}$ generation by a dental ultrasonic unit as oscillator for ultrasound is required. Therefore, the aim of chapter 2 was to evaluate $HO^{\cdot}$ generation by a dental ultrasonic unit and its bactericidal effect against *E. faecalis*.

## Materials and Methods

Reagents and dental ultrasonic unit

The sources of reagents used in this study were obtained from the same industry as

those of chapter 1. Osada 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. 5. 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 sec, 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.

$HO^{\cdot}$ generation from $H_2O_2$ with PUI and its bactericidal activity assay

$HO^{\cdot}$ generation in the four experimental conditions and its bactericidal assay were analyzed according to the methods described in chapter 1.

Statistical analysis

All tests were performed according to the method described in chapter 1.

## Results

$HO^{\cdot}$ generation from $H_2O_2$ with or without PUI

The results of $HO^{\cdot}$ generation from $H_2O_2$ with or without PUI investigated using the ESR spin-trapping technique with CYPMPO are shown in Fig. 6. $HO^{\cdot}$ generated from $H_2O_2$ with PUI increased significantly in a time-dependent manner. Moreover, the presence of $HO^{\cdot}$ was confirmed, because $HO^{\cdot}$ generation was abolished by the addition of L-ascorbic acid (0.1 mol/L), a major antioxidant of $HO^{\cdot}$ (data not shown). $HO^{\cdot}$ generation from $H_2O_2$ without PUI was significantly lower than with PUI, irrespective of irradiation time (Fig. 6). Levels of $HO^{\cdot}$ from Tris-HCl buffer were lower regardless of PUI use.

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 to $H_2O_2$ with PUI for 90 sec was significantly lower than after exposure for 30 or 60 sec (Fig. 7). Moreover, after ultrasound treatment for 90 sec, 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. 7).

## 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 $HO^{\cdot}$ generation from $H_2O_2$ in the present study. $H_2O_2$ serves as a source of $HO^{\cdot}$ in the dissociation process, (13) and $HO^{\cdot}$ is

formed from energies generated during cavitation bubble collapse (17). Using present PUI system, enhanced $HO^{\cdot}$ generation from $H_2O_2$ with ultrasonic waves was observed. Thus, $HO^{\cdot}$ generation might be caused by both acceleration of $H_2O_2$ dissociation due to PUI and ultrasonic cavitation activity.

In the present study, the amount of $HO^{\cdot}$ generated from $H_2O_2$ with PUI significantly time-dependently increased. This suggests that PUI stimulated continuous formation of $HO^{\cdot}$ throughout the ultrasonic irradiation time. $HO^{\cdot}$ generation from $H_2O_2$ without PUI was significantly lower than with PUI, perhaps due to spontaneous $H_2O_2$ decomposition, which liberates $HO^{\cdot}$. The specificity of $H_2O_2$ for $HO^{\cdot}$ generation was confirmed because little $HO^{\cdot}$ was detected in Tris-HCl buffer with or without PUI.

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) was also examined. 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 sec was significantly lower than after exposure for 30 and 60 sec. These findings indicate that the total viable count of *E. faecalis* depends on the amount of $HO^{\cdot}$ generated and exposure time, because ESR measurements showed that $HO^{\cdot}$ 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 sec. The differences in these effects may be due to $HO^{\cdot}$ 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-sec ultrasound group and the other groups. This may explain the amount of $HO^{\cdot}$ generated due to PUI. Future research should determine how to improve $HO^{\cdot}$ generation in this system by using more-powerful ultrasound.

From the results of chapter 2, the ESR spin-trapping technique and viable counting showed that $HO^{\cdot}$ 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.

# Conclusions

Within the limitation of the present *in vitro* study, the following conclusions were drawn:

1. $HO^{\cdot}$ generation by ultrasonic irradiation and passive ultrasonic irrigation showed bactericidal effect against *E. faecalis*.
2. ESR measurements showed that $HO^{\cdot}$ generation from a low concentration of $H_2O_2$ significantly increased by ultrasonic irradiation in a time- and dose-dependent manner. Moreover, the bactericidal effects of $H_2O_2$ against *E. faecalis* were enhanced by ultrasonic irradiation in a time- and dose-dependent manner with more than 4-log reduction of viable counting.
3. $HO^{\cdot}$ was generated from a low concentration of $H_2O_2$ activated by passive ultrasonic irrigation and the number of CFUs of *E. faecalis* was reduced in a time- and dose-dependent manner.

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# Figures

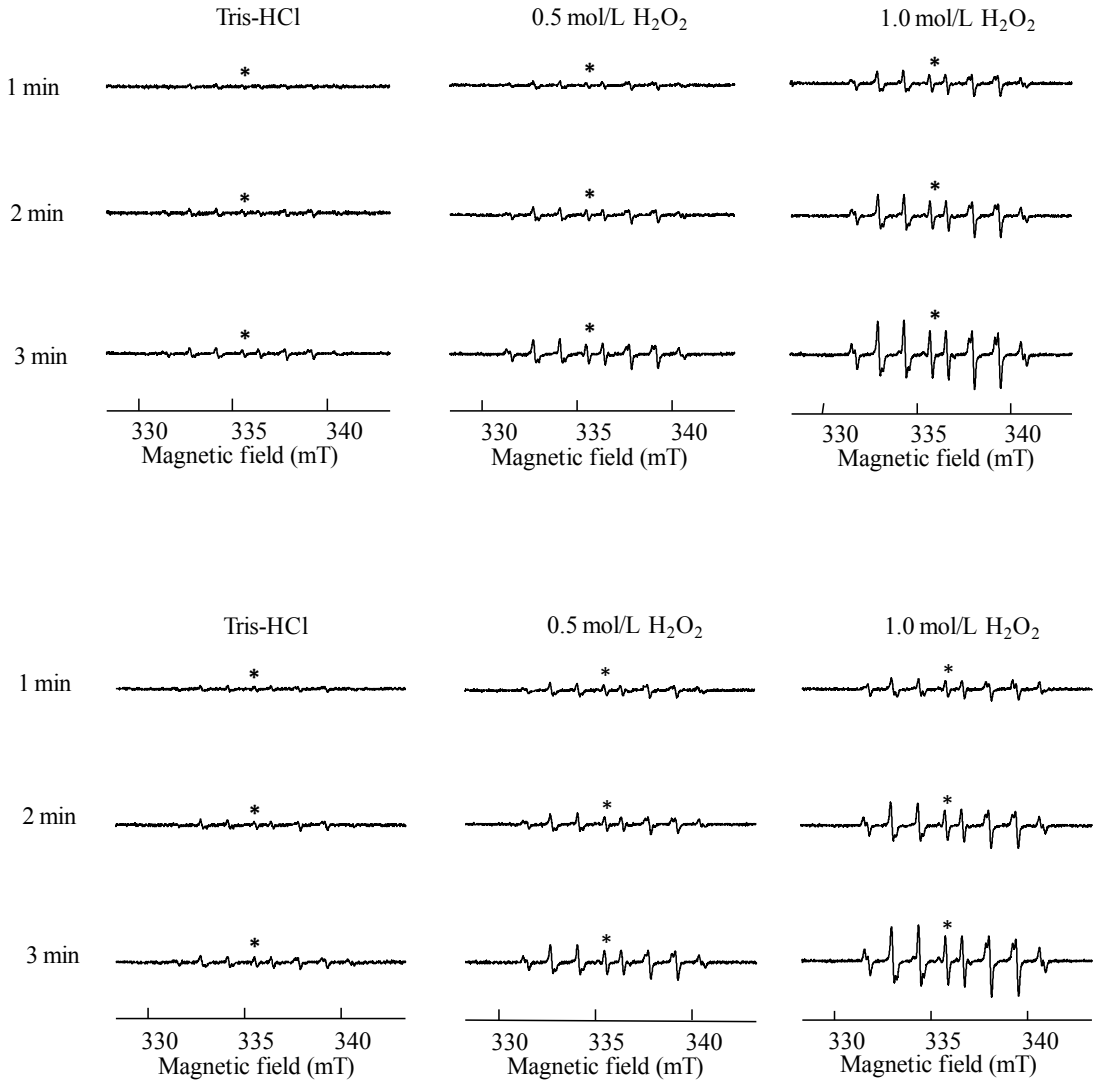


Fig. 1 $HO^{\cdot}$ generation from $H_2O_2$ by ultrasonic irradiation. A and B: ESR spin trapping measurement of $HO^{\cdot}$ generated from $H_2O_2$ by 10 W (A) and 20 W (B) ultrasonic irradiation with CYPMPO as the spin trap. The asterisk (*) indicates the signal intensity used for the analysis of $HO^{\cdot}$ generation.

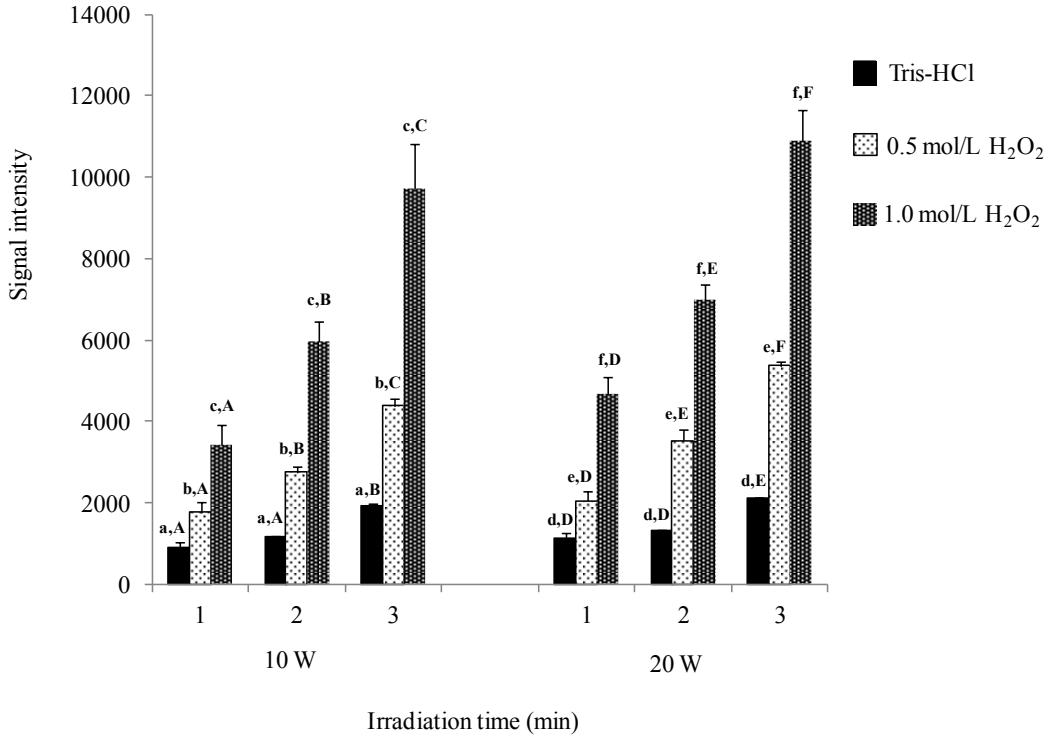


Fig. 2 Signal intensities of the ESR spectrum of CYPMPO-OH by 10 W and 20 W ultrasonic irradiation. Within experimental solutions, means sharing the same upper-case letter are not significantly different ($p > 0.05$). Between experimental solutions at the same irradiation time, means sharing the same lower-case letter are not significantly different ($p > 0.05$).

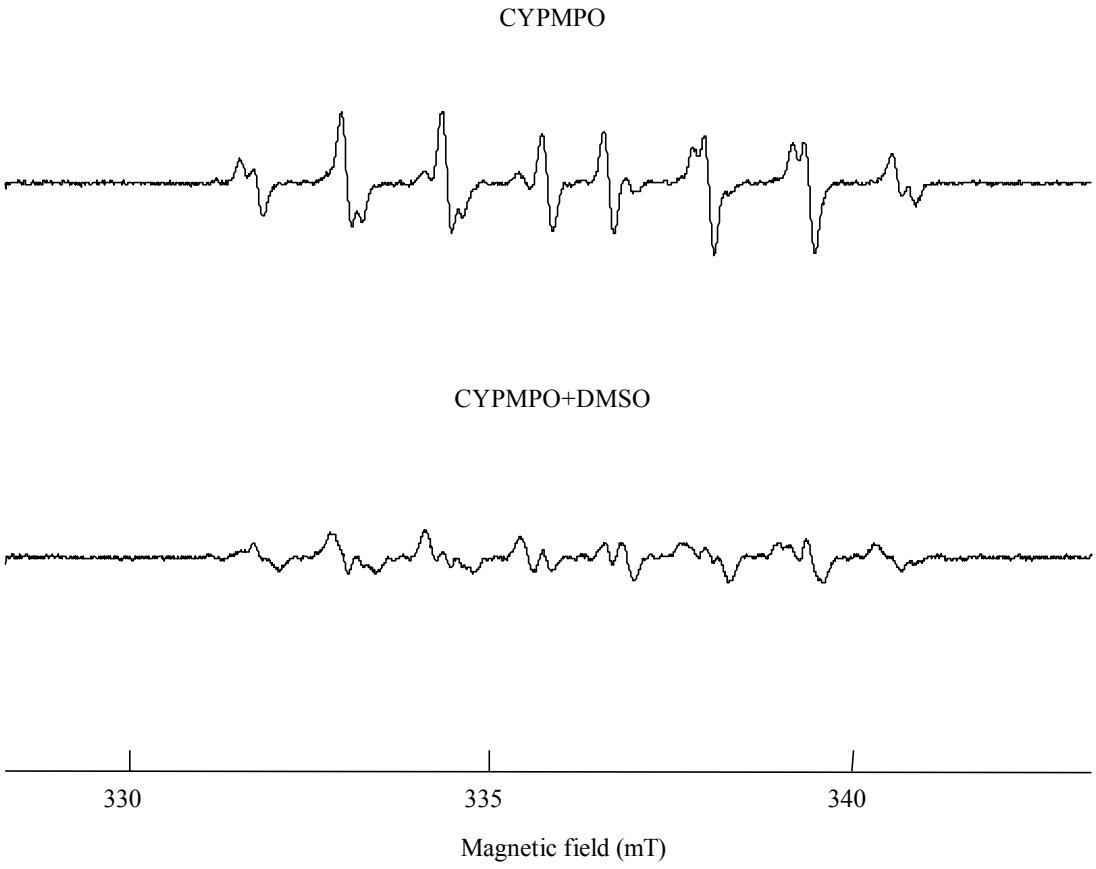


Fig. 3 Influence of DMSO on ESR spectra of $HO^{\cdot}$ generated from $H_2O_2$ by 20 W ultrasonic irradiation with CYPMPO as the spin trap.

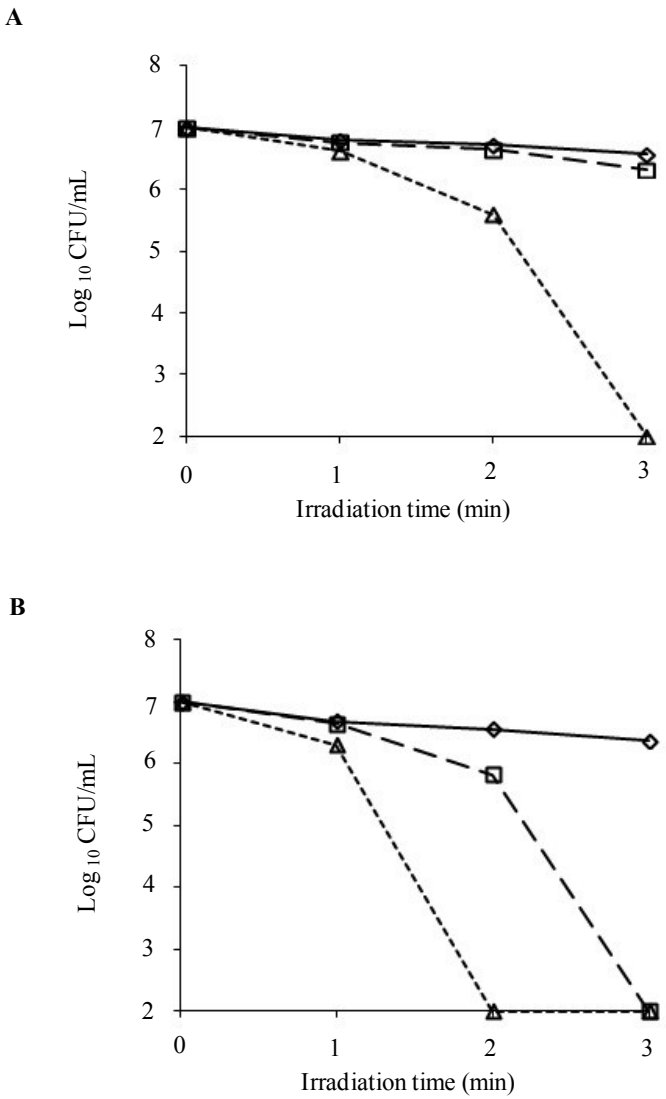


Fig. 4 Viable counting bactericidal activity in the suspension after ultrasonic irradiation. The data points indicate the mean values (n = 6). A: Number of CFU/mL of 10 W irradiation in each experimental solution. B: Number of CFU/mL of 20 W irradiation in each experimental condition.

—◇— : Tris-HCl —□— : 0.5 mol/L $H_2O_2$ - -△- - : 1.0 mol/L $H_2O_2$

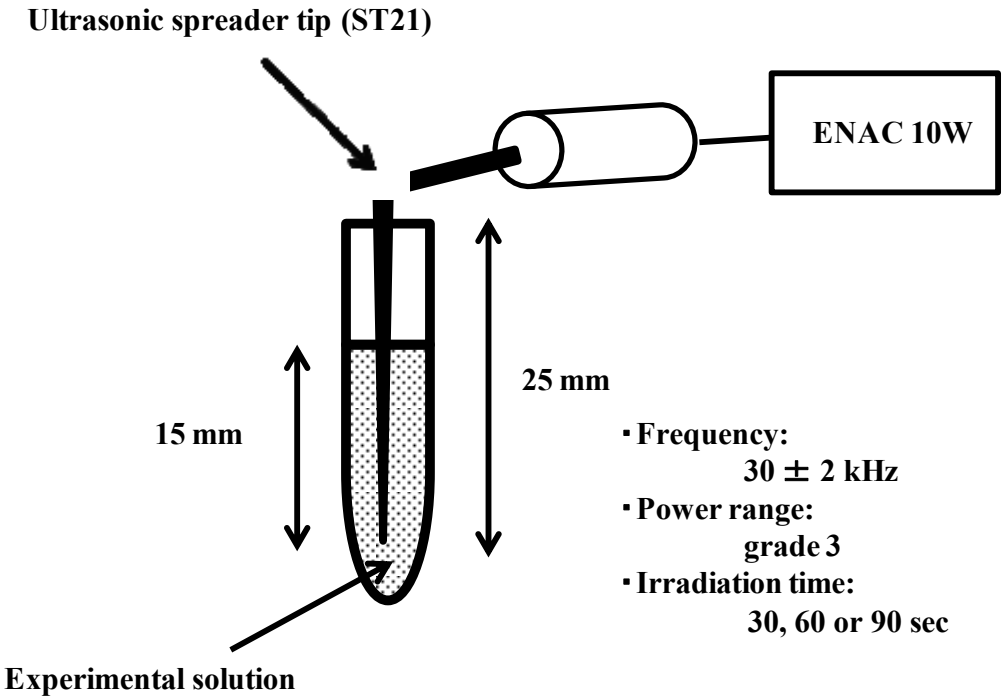


Fig. 5 Schematic illustration of the experimental design.

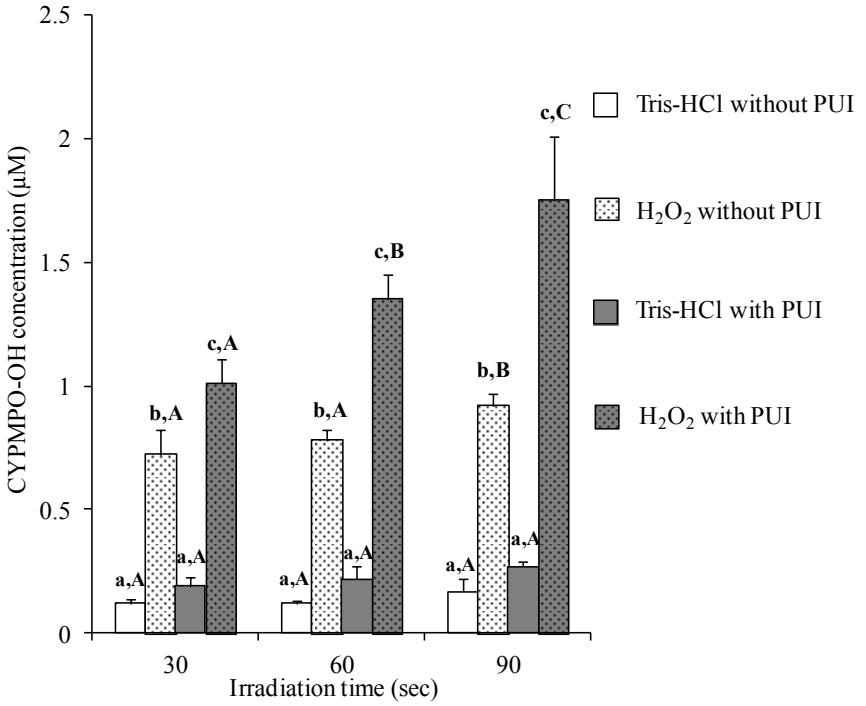


Fig. 6 Determination of CYPMPO-OH concentration as a measure of $HO^{\cdot}$ generation (n = 6). Statistically significant differences were assessed by two-way ANOVA and Tukey's test. Within experimental solutions, means sharing the same upper-case letter are not significantly different ($p > 0.05$).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

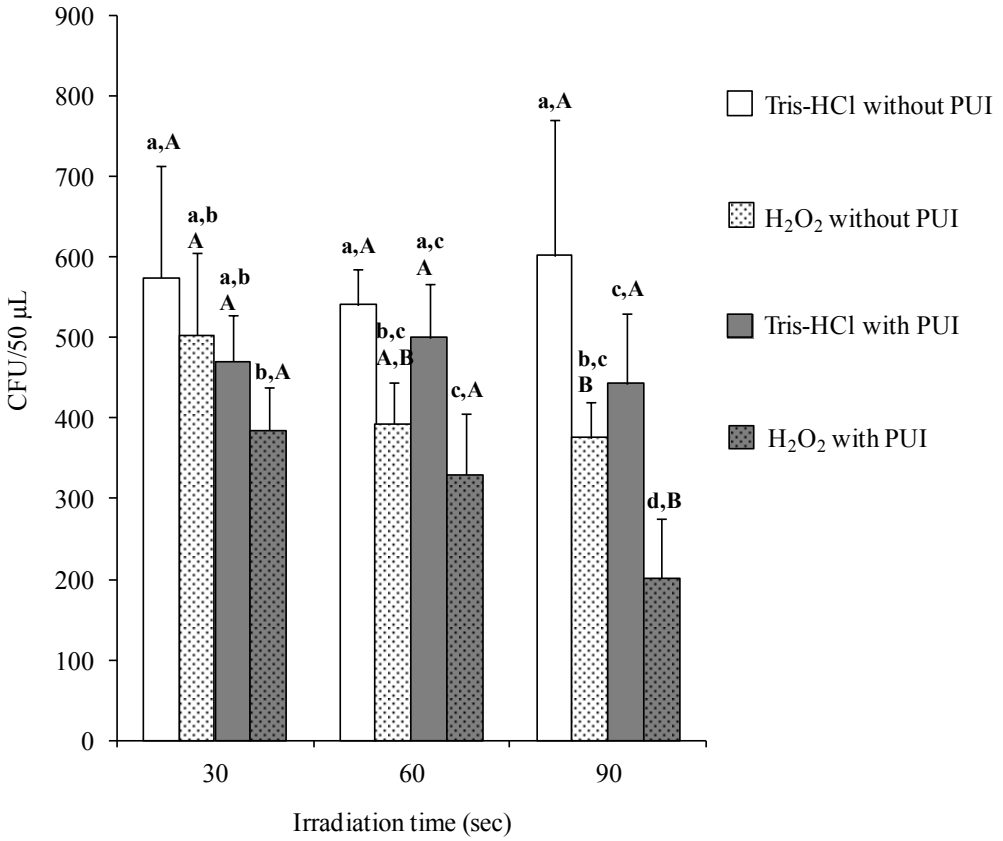


Fig. 7 Viable counting of *E. faecalis* (n = 6). Statistically significant differences were assessed by two-way ANOVA and Tukey's test. Within experimental solutions, means sharing the same upper-case letter are not significantly different ($p > 0.05$). Between experimental solutions at the same irradiation time, means sharing the same lower-case letter are not significantly different ($p > 0.05$).