Bactericidal Action of Photoirradiated Gallic Acid via Reactive Oxygen Species Formation

Keisuke Nakamura,*,[†] Yasutomo Yamada,[‡] Hiroyo Ikai,[‡] Taro Kanno,[‡] Keiichi Sasaki,[‡] and Yoshimi Niwano[†]

[†]Laboratory for Redox Regulation, Tohoku University Graduate School of Dentistry, 4-1 Seiryo, Aoba-ku, Sendai 980-8575, Japan

[‡]Division of Fixed Prosthodontics, Department of Restorative Dentistry, Tohoku University Graduate School of Dentistry, 4-1 Seiryo, Aoba-ku, Sendai 980-8575, Japan

ABSTRACT: It is known that gallic acid shows antimicrobial activity. In the present study, photoirradiation induced reactive oxygen species formation was investigated for augmentation of the antimicrobial activity of gallic acid. *Staphylococcus aureus* suspended in 4 mmol/L gallic acid was exposed to blue light of a LED at 400 nm. This treatment killed the bacteria, and a >5-log reduction of the viable counts was observed within 15 min. By contrast, neither the LED treatment alone nor the treatment with gallic acid alone showed substantial bactericidal effect. When hydroxyl radical scavengers were added to the suspension, the bactericidal effect of photoirradiated gallic acid was attenuated. Furthermore, electron spin resonance analysis demonstrated that hydroxyl radicals were generated by the photoirradiation of gallic acid. Thus, the present study suggests that the photo-oxidation can enhance the antimicrobial activity of gallic acid via hydroxyl radical formation.

KEYWORDS: gallic acid, bactericidal action, hydroxyl radical, hydrogen peroxide, photo-oxidation

INTRODUCTION

Gallic acid (3,4,5-trihydroxybenzoic acid) is a naturally occurring polyphenolic compound in fruits, nuts, and flowers. Polyphenolic compounds are noteworthy for their antioxidant activity.[1−3] The phenolic hydroxyl group in their structure acts as a hydrogen donor, and they can effectively scavenge free radicals.[4] Some polyphenolic compounds are also antimicrobial and antiviral.[5−9] Taguri et al. investigated the antimicrobial activity of 22 polyphenols, including gallic acid, against 26 bacterial species, and found that the polyphenols with pyrogallol groups had higher activity than those with catechol or resorcinol groups.[10] In addition, Mabe et al. studied the antimicrobial activity of catechins, which are a group of polyphenolic compounds active against *Helicobacter pylori*.[11] Catechins with gallate (gallic acid ester) showed higher antimicrobial activity than those without gallate. These results indicate that the trihydroxyphenyl group of polyphenolic compounds, especially gallate, affects the antimicrobial activity of polyphenolic compounds.

Polyphenolic compounds exert their antimicrobial activity in several ways, such as inhibition of nucleic acid synthesis, dysfunction of cytoplasmic membrane, and disruption of energy metabolism.[8] Regarding the underlying mechanism of such antimicrobial actions, generation of hydrogen peroxide (H_2O_2) is one of the most important contributors. Arakawa et al. demonstrated that H_2O_2 generated in a solution of (−)-epigallocatechin gallate exerted a bactericidal effect.[12] They also demonstrated that addition of catalase to the solution eliminated the bactericidal effect. Akagawa et al. demonstrated that both catechins and gallic acid generate H_2O_2 by autoxidation under neutral-alkaline condition.[13] In addition, exposure to ultraviolet radiation and sunlight causes photo-oxidation of gallic acid.[14] However, there is little or no information available about photo-oxidation generation of H_2O_2 from gallic acid.

The bactericidal activity of H_2O_2 is well recognized and 3% H_2O_2 is used as a disinfectant in medical fields. In an earlier study, we found that the bactericidal activity of H_2O_2 was enhanced by laser irradiation of H_2O_2 at 405 nm, which induced photolysis of H_2O_2.[15] On the basis of these results, we hypothesized that photo-oxidation of gallic acid at around 400 nm would initiate generation of H_2O_2, and then the H_2O_2 would be photolyzed by photoirradiation and generate the hydroxyl radical (·OH). Because ·OH is more reactive and a stronger oxidant than H_2O_2,[16,17] it is expected that photo-irradiation of gallic acid could enhance the antimicrobial activity. Since gallic acid is a food ingredient, it is supposed to be safe for humans, so that it can be used for medical application as a disinfectant. The aim of the present study was to verify this hypothesis by examining the bactericidal effect of photoirradiated gallic acid and the mechanism of bactericidal action in relation to reactive oxygen species (ROS) formation.

MATERIALS AND METHODS

Reagents. Reagents were purchased from the following sources: gallic acid hydrate from Tokyo Chemical Industries (Tokyo, Japan); 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) from Labotec (Tokyo, Japan); catalase, sodium formate (HCOONa), and dimethyl sulfoxide (DMSO) from Wako Pure Chemical Industries (Osaka, Japan); 4-hydroxy-2,2,6,6-tetramethylpiperidine *N*-oxyl (TEMPOL) from Sigma Aldrich (St. Louis, MO); iron(II) sulfate heptahydrate ($FeSO_4$) from Kanto Chemical Co. (Tokyo, Japan); hydrogen peroxide (H_2O_2) from

Santoku Chemical Industries (Tokyo, Japan); and thiobarbituric acid reactive substances (TBARS) assay kit from ZeptoMetrix (Buffalo, NY). All other reagents used were of analytical grade.

Light Source. An experimental device equipped with light emitting diode (LED) with a wavelength of 400 nm (NHH105UV, Lustrous Technology, Shiji, Taiwan) was used. According to a data sheet from the manufacturer, the minimum and the maximum wavelengths of the light are 380 and 420 nm, respectively, and the full width half-maximum (fwhm) is less than 14 nm.[18] The LEDs were chosen based on our previous study in which we demonstrated that the LED radiation could effectively photolyze H_2O_2.[19] A glass tube containing the sample was placed in the experimental device, followed by exposure to the LED radiation. The irradiance of the LED measured by a portable irradiance gauge (Delta OHM, Caselle di Selvazzano, Italy) was 80 mW/cm^2 at a distance of 15 mm from the LED.

Bactericidal Assay. *Staphylococcus aureus* JCM 2413 purchased from the Japan Collection of Microorganisms, RIKEN BioResource Center (Wako, Japan) was used. A bacterial suspension was prepared in sterile physiological saline from a culture grown on brain heart infusion (BHI) agar (Becton Dickinson Labware, Franklin Lakes, NJ) aerobically at 37 °C for 12 h. A 5 mmol/L stock solution of gallic acid was prepared in pure water and then sterilized by filtration. The pH of the stock solution was at 3.3 ± 0.1 measured using a pH meter (twin pH meter, Horiba, Kyoto, Japan). Gallic acid was used in the experiments without further pH adjustment. In a glass tube, 800 μL of gallic acid or pure water was mixed with 200 μL of the bacterial suspension to reach final concentrations of 4 mmol/L for gallic acid and 4×10^7 colony forming units (CFU)/mL for the bacteria. Then, the sample was exposed to the LED radiation for 0, 5, 10, or 15 min. After irradiation, 50 μL of the sample was mixed with an equal volume of sterile catalase solution (5000 U/mL) to terminate the bactericidal effect of H_2O_2 generated by photo-oxidation of gallic acid. A 10-fold serial dilution of the mixture was then prepared using sterile physiological saline, and 10 μL of the diluted solution was seeded onto BHI agar to evaluate the number of viable microorganisms in the suspension. The agar medium was aerobically cultured at 37 °C for 24 h and the number of CFU/mL was determined. A bacterial suspension that was kept in a dark box instead of being irradiated was also subjected to the same procedure.

To examine if the bactericidal effect of photoirradiated gallic acid could be attributed to ·OH, DMSO and HCOONa, which are well-known ·OH scavengers,[17] were added to the reaction mixture. DMSO and HCOONa were used at final concentrations of 1.4 mol/L and 100 mmol/L, respectively. Then, the sample was exposed to the LED radiation for 8 min. The CFU was determined after each treatment as described above.

To investigate the influence of dissolved oxygen on the bactericidal action, dissolved oxygen in the samples was replaced with argon gas. One milliliter of a sample containing 4 mmol/L gallic acid and the bacterial suspension (4×10^7 CFU/mL) in a glass tube was bubbled with argon gas for 5 min. The glass tube was then sealed with an airtight stopper and exposed to the LED radiation for 8 min. The CFU was determined as described above. Quantitative analysis of dissolved oxygen in 4 mmol/L gallic acid with or without the argon gas bubbling was also conducted using an oxygen sensor (Microx TX3, PreSens, Regensberg, Germany). All tests were performed in quintuplicate.

TBARS Assay. If the ROS play a critical role in the bactericidal action of photoirradiated gallic acid, the level of lipid peroxidation of the bacterial cells should increase. Thus, lipid peroxidation was analyzed by the TBARS assay. *S. aureus* was suspended in 4 mmol/L gallic acid to a level of 10^{9-10} CFU/mL, which was determined by the viable count method with seeding 100 μL of a 10^7-fold dilution of the suspension on BHI agar. One milliliter of the suspension in a glass tube was exposed to the LED radiation for 15 min. An aliquot of the suspension was kept in the dark as a control. After the treatment, the cells were collected by centrifugation at 5000*g* for 15 min, and then resuspended in 50 μL of saline. The suspension was mixed with 50 μL of sodium dodecyl sulfate in a vial, and 1.25 mL of thiobarbituric acid buffer reagent was added. The vials containing the reaction mixture were then incubated in a block incubator at 95 °C for 60 min. After

incubation, the vials were cooled to room temperature in an ice bath for 10 min. To remove any solid material, the samples were centrifuged at 5000*g* for 15 min. Fluorescence analysis of the supernatant was performed with excitation at 530 nm and emission at 550 nm using a spectrofluorometer (FP-8200, JASCO, Tokyo, Japan). The fluorescence intensity was converted to malondialdehyde (MDA) equivalents per 10^{10} cells using a MDA standard curve. All tests were performed in quintuplicate.

Electron Spin Resonance (ESR) Analysis of Oxygen Radicals. Qualitative and quantitative analyses of oxygen radicals generated by photoirradiation of gallic acid were performed using an ESR-spin trapping technique as described in our earlier study.[20] Gallic acid was mixed with DMPO in a glass tube to reach final concentrations of 4 mmol/L for gallic acid and 300 mmol/L for DMPO. Then, the sample was exposed to the LED radiation for 15 min. After irradiation, the sample was transferred to a quartz cell for ESR spectrometry, and the ESR spectrum was recorded on a X-band ESR spectrometer (JES-FA-100; JEOL, Tokyo, Japan). The measurement conditions for ESR were as follows: field sweep, 331.41−341.41 mT; field modulation frequency, 100 kHz; field modulation width, 0.1 mT; amplitude, 200; sweep time, 2 min; time constant, 0.03 s; microwave frequency, 9.420 GHz; and microwave power, 4 mW. TEMPOL (10 μmol/L) was used as a standard to calculate the concentration of spin-trapped radicals, and the ESR spectrum of manganese (Mn^{2+}) held in the ESR cavity was used as an internal standard. The concentration of ·OH was determined using Digital Data Processing (JEOL).

DMPO−OH, a spin adduct of ·OH, can be formed even in the absence of ·OH under certain conditions.[21] Consequently, additional ESR analysis was conducted to confirm if the DMPO−OH was derived from the reaction between ·OH and DMPO. If ·OH are generated, the intensity of DMPO−OH signal decreases and a signal for a spin adduct of the methyl radical (DMPO−CH$_3$) appears when a ·OH scavenger containing a methyl group is added to the reaction system.[22] DMSO was added to the reaction mixture to reach final concentrations of 1.4 mol/L for DMSO, 4 mmol/L for gallic acid, and 300 mmol/L for DMPO. Then, the sample was exposed to the LED radiationfor 15 min. ESR analysis was performed as described above.

The influence of dissolved oxygen on the generation of ·OH was also examined to see if the radicals were derived from the dissolved oxygen as described in the bactericidal assay. Briefly, 1 mL of a mixture of 4 mmol/L gallic acid and 300 mmol/L DMPO in a glass tube was bubbled with argon gas for 5 min to replace the dissolved oxygen. The glass tube was then sealed with an airtight stopper and exposed to the LED radiation for 15 min. ESR analysis was performed as described above. All tests were performed in triplicate.

ESR Analysis of H_2O_2 Coupled with a Fenton Reaction. The yield of H_2O_2 was determined utilizing an ESR-spin trapping technique coupled with a Fenton reaction, in which H_2O_2 reacts with ferrous ions and produces ·OH. A standard curve was constructed using H_2O_2 solutions that were prepared by dilution with 4 mmol/L gallic acid or pure water. Each standard solution (160 μL) was mixed with 20 μL of DMPO (final concentration: 300 mmol/L), and 20 μL of FeSO$_4$ was added to initiate the Fenton reaction. The final concentrations of FeSO$_4$ were 50 μmol/L in the solution of H_2O_2 diluted with gallic acid, and 10 μmol/L in the solution of H_2O_2 diluted with pure water. These concentrations were chosen to optimize the analytical sensitivity for each experiment. ESR analysis was performed 60 s after the addition of FeSO$_4$, using the same conditions as described above. The yield of DMPO−OH in the reaction system was converted to the yield of H_2O_2 using the standard curves. The yields of H_2O_2 generated by LED-irradiation of 4 mmol/L gallic acid or pure water were compared with the yields of H_2O_2 in the mixtures kept in the dark.

Gallic acid after replacement of the dissolved oxygen with argon was exposed to LED radiation and the yield of H_2O_2 was examined. An aliquot (1 mL) of 4 mmol/L gallic acid was bubbled with argon gas for 5 min. Then, the glass tube was sealed and exposed to the LED radiation for 15 min. Quantitative analysis of H_2O_2 in the sample was performed as described above. All tests were performed in triplicate.

Statistical Analyses. Statistical significance ($p < 0.05$) in the level of lipid peroxidation with or without the LED irradiation and in the yield of DMPO−OH and H_2O_2 under each condition was assessed by Student's t-test for pairwise comparisons and Tukey-Kramer test for multiple comparisons.

■ RESULTS

Bactericidal Assay. LED irradiation of a *S. aureus* suspension containing 4 mmol/L gallic acid exerted a bactericidal effect that was dependent on the irradiation time (Figure 1). Photoirradiated gallic acid killed the bacteria, and a
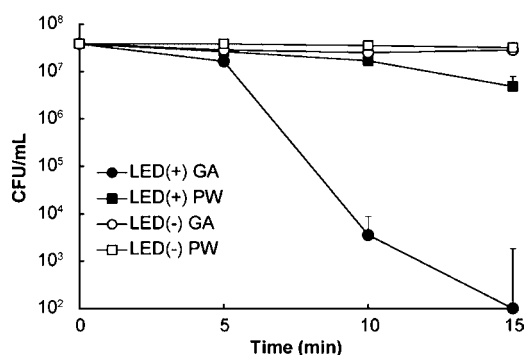
Figure 1. Number of viable *S. aureus* in the suspension after each treatment. Each value is the mean of quintuplicate measurements with the standard deviation. GA: gallic acid, PW: pure water.

>5-log reduction was observed within 15 min on average. When pure water was used instead of gallic acid, the number of bacteria also decreased as the LED irradiation time increased. However, the reduction in the CFU was <1-log in 15 min. Without the LED irradiation, the change in the CFU was absent or minimal with both pure water and 4 mmol/L gallic acid (Figure 1).

When DMSO and HCOONa were added to the reaction mixture, the bactericidal effect of photoirradiated gallic acid was attenuated (Figure 2). Replacement of the dissolved oxygen in the sample with argon also reduced the bactericidal effect of
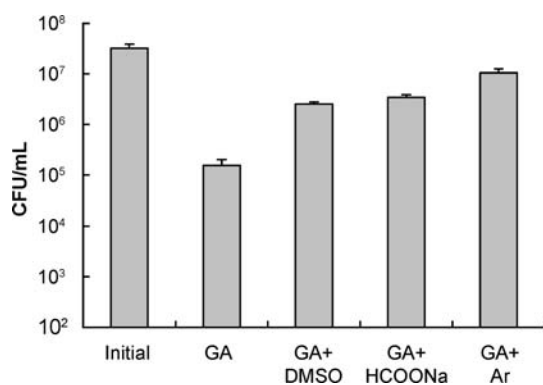

Figure 2. Influence of ·OH scavengers and argon replacement of dissolved oxygen on the bactericidal effect of photoirradiated gallic acid. The LED irradiation was performed for 8 min. When 1.4 mol/L DMSO (GA + DMSO) and 100 mmol/L HCOONa (GA + HCOONa) presented in the reaction mixture, the bactericidal effect of photoirradiated 4 mmol/L gallic acid was attenuated. Replacement of the dissolved oxygen in the sample with argon (GA + Ar) also reduced the bactericidal effect of photoirradiated gallic acid. Each value is the mean of quintuplicate measurements with the standard deviation.

photoirradiated gallic acid (Figure 2). After only 5 min of bubbling with argon gas, the concentration of dissolved oxygen in 4 mmol/L gallic acid was reduced from 8 mg/L to less than 0.5 mg/L. When the glass tube was sealed with an airtight stopper after argon gas replacement, the level of dissolved oxygen was kept for 15 min.

TBARS Assay. The TBARS assay kit coupled with the fluorometric analysis could provide high sensitivity to detect very low TBARS levels. The detection limit under the conditions used in the present study was 0.1 nmol/L MDA. Bacteria at 10^{9-10} CFU were used to obtain fluorescence values above the detection limit. When the bacteria suspended in 4 mmol/L gallic acid were irradiated with the LED-light for 15 min, the TBARS level (MDA equivalents per 10^{10} cells) significantly increased ($p < 0.01$) compared to that of the suspension without the LED radiation (Figure 3).
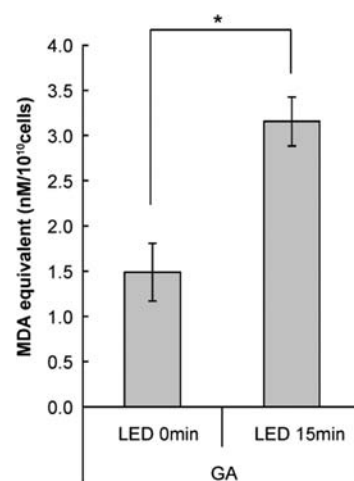

Figure 3. Lipid peroxidation with or without LED irradiation of a bacterial suspension in 4 mmol/L gallic acid (GA). Lipid peroxidation was determined by the TBARS assay and reported as MDA equivalents per 10^{10} cells. Each value is the mean of quintuplicate measurements with the standard deviation. *$p < 0.01$.

ESR Analysis of Oxygen Radicals. When gallic acid and pure water solutions of DMPO (300 mmol/L) were exposed to the LED radiation, the ESR signal of the ·OH spin adduct (DMPO−OH) was detected (Figure 4A). The presence of the spin adduct was confirmed by hyper fine coupling constants of $a_N = a_H = 1.49$ mT for DMPO−OH.[23] Both gallic acid and pure water samples without LED irradiation showed small signals for DMPO−OH, and LED irradiation of the samples significantly increased the yield of DMPO−OH (Figure 4B). The average yields of DMPO−OH after LED irradiation of gallic acid and pure water for 15 min were 0.7 and 0.3 μmol/L, respectively. ESR signals other than that of DMPO−OH, such as DMPO−OOH, were not observed under the conditions used in the present study.

Addition of DMSO to the reaction mixture significantly decreased the signal of DMPO−OH (Figure 5), and a signal for DMPO−CH_3 was observed (data not shown), which was identified by its hyper fine coupling constants of $a_N = 1.64$ mT and $a_H = 2.35$ mT.[23] This suggests that the DMPO−OH was generated by the reaction between ·OH and DMPO. The yield of DMPO−OH also significantly decreased when the dissolved oxygen in the sample was replaced with argon (Figure 5).
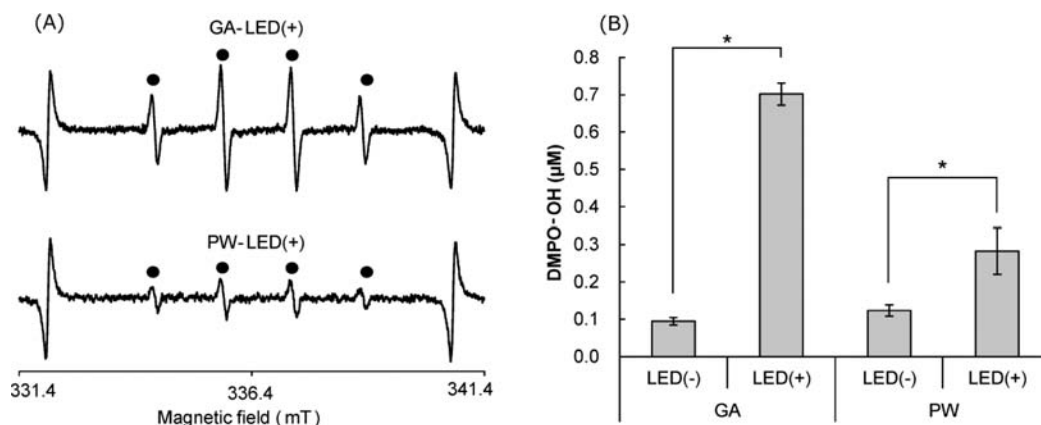
Figure 4. DMPO−OH formation with or without LED irradiation for 15 min. (A) Representative ESR spectra of DMPO−OH (solid circle) generated by the LED irradiation of 4 mmol/L gallic acid (GA) and pure water (PW). (B) The LED irradiation of 4 mmol/L GA and PW significantly increased the yield of DMPO−OH. Each value is the mean of triplicate measurements with the standard deviation. *$p < 0.01$.
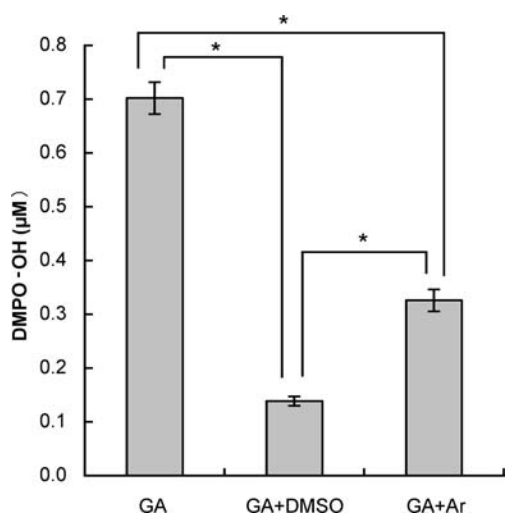

Figure 5. Influence of DMSO and argon replacement of dissolved oxygen on the generation of DMPO−OH in photoirradiated gallic acid. The yield of DMOP−OH was significantly attenuated by addition of 1.4 mol/L DMSO (GA + DMSO) and argon replacement (GA + Ar). Each value is the mean of triplicate measurements with the standard deviation. *$p < 0.01$.

ESR Analysis of H_2O_2 Generation Coupled with a Fenton Reaction. The concentrations of H_2O_2 in the gallic acid and pure water samples were determined by an ESR-spin trapping technique coupled with the Fenton reaction. The concentration of H_2O_2 was highly correlated to the yield of DMPO−OH generated in the Fenton reaction (Figure 6A). The detection limits of H_2O_2 dissolved in 4 mmol/L gallic acid and pure water were 1.25 and 0.25 μmol/L, respectively. Standard curves were used to analyze H_2O_2 generation caused by LED irradiation of gallic acid or pure water. When 4 mmol/L gallic acid was exposed to the LED radiation, H_2O_2 was generated (Figure 6B). By contrast, H_2O_2 was not detected in gallic acid without the irradiation. Similarly, H_2O_2 was not detected in the pure water irrespective of whether it was irradiated or not (data not shown). The average yield of H_2O_2 generated in gallic acid with LED irradiation for 15 min was 7.1 μmol/L. When the dissolved oxygen in the sample was replaced with argon, the yield of H_2O_2 significantly decreased to 2.0 μmol/L (Figure 6B).

DISCUSSION

The present study demonstrated that by LED irradiation at 400 nm, gallic acid exerted a bactericidal effect and reduced the CFU by >5-log within 15 min. Because ·OH was detected by the ESR analysis and the bactericidal effect was attenuated by ·OH scavengers, these results suggest that the major contributor to the bactericidal effect of photoirradiated gallic acid is ·OH.

Gallic acid has antimicrobial activity.[10,24,25] Akiyama et al. reported that the minimum inhibitory concentration against *S. aureus* was 8000 mg/L (47 mmol/L).[26] In the present study, gallic acid was used at a concentration of less than one-tenth of the minimum inhibitory concentration (4 mmol/L), and the bacteria were exposed to gallic acid for only 15 min at most. Thus, the gallic acid treatment was not solely responsible for the reduction in the CFU of *S. aureus*. With LED irradiation, a minimal reduction in bacteria suspended in pure water was observed after 15 min. This finding agrees with an earlier study, which showed that irradiation with ultraviolet or blue light could exert bactericidal action depending on the level of irradiation.[27] Although the LED irradiation performed in the present study could kill the bacteria, the bactericidal effect was relatively weak as shown by the <1-log reduction of the CFU in 15 min. Even though treatment with 4 mmol/L gallic acid or LED irradiation alone was not effective, LED irradiation of the suspension in 4 mmol/L gallic acid could kill the bacteria effectively.

The bactericidal activity of (−)-epicatechin gallate is attributed to the generation of H_2O_2, which increases at higher pH values, and especially over pH 6.[12] Other studies have reported H_2O_2 generation in solutions of catechins and other polyphenolic compounds under neutral or alkaline conditions.[13,28−30] Mochizuki et al. proposed a mechanism for H_2O_2 generation in a catechin solution, with initiation of the reaction by oxidation of the polyphenolic hydroxyl group by dissolved oxygen (autoxidation) and generation of semiquinone radicals.[31] Then, superoxide anion radicals ($O_2^-\cdot$) would be generated because of a reaction between the semiquinones and dissolved oxygen. Finally, the $O_2^-\cdot$ could react with the polyphenolic hydroxyl groups generating H_2O_2. In the present study, gallic acid was dissolved in pure water and used at pH 3.3 without further pH adjustment. Thus, it was thought that autoxidation of gallic acid would not be induced. As expected, the yields of H_2O_2 and ·OH in 4 mmol/L gallic acid without
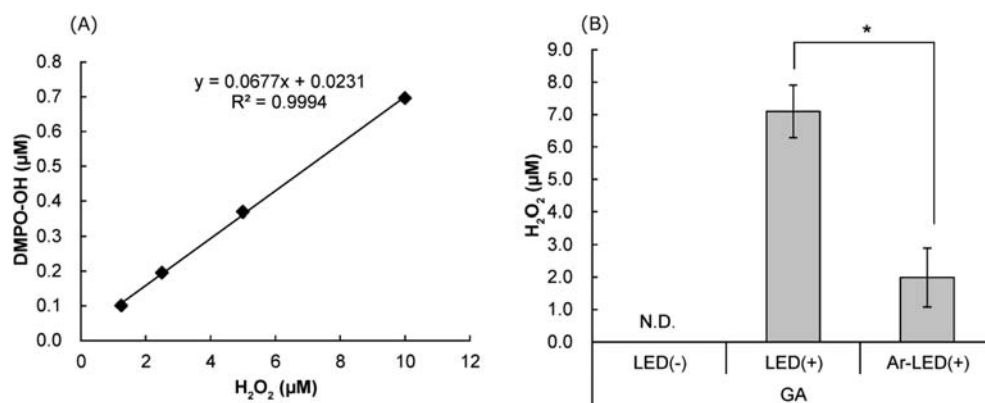
Figure 6. Quantitative analysis of H_2O_2 generated by LED irradiation of samples. The yield of H_2O_2 was determined by ESR-spin trapping technique coupled with a Fenton reaction. (A) Standard curve for H_2O_2 diluted with 4 mmol/L gallic acid (GA). (B) The yield of H_2O_2 generated in 4 mmol/L GA with or without LED irradiation (LED(+), LED(−)) and the yield of H_2O_2 in GA with argon replacement of dissolved oxygen (Ar-LED(+)). H_2O_2 was not detected for LED(−), while the LED irradiation of GA generated H_2O_2. The yield of H_2O_2 was significantly decreased by argon replacement of dissolved oxygen. Each value is the mean of triplicate measurements with the standard deviation. $*p < 0.01$. N.D.: not detected (<1.25 μmol/L).

LED irradiation were at trace levels or not detectable. By contrast, LED irradiation of 4 mmol/L gallic acid generated H_2O_2 and ·OH, which suggests that 400 nm light can initiate oxidation of gallic acid. In addition, generation of both ROS was significantly reduced by replacement of dissolved oxygen by argon, which indicates that the ROS were derived from a reaction involving dissolved oxygen. The bactericidal effect of photoirradiated gallic acid was also attenuated by argon replacement. Therefore, the presence of dissolved oxygen is a key factor in the bactericidal action of photoirradiated gallic acid.

In the present study, neither O_2^-· nor semiquinone radicals derived from gallic acid were detected. If photo-oxidation of gallic acid generates ROS as is the same way proposed in the autoxidation, both radicals are involved in the reaction. The reason why O_2^-· could not be detected is probably due to the low yield and low rate constant between DMPO and O_2^-·.[32] Concerning the semiquinone radicals, it is considered that the yield was below the detection limit. In the case of the semiquinone radicals, unlike the spin adducts of DMPO, they are assumed to be transformed immediately to quinone structure.[33] Thus, only H_2O_2 and DMPO−OH which can accumulate in a time dependent manner could be detected even though the generation rates were low. The possible involvements of these radicals should be further studied in the future. Regarding the DMPO−OH detected in the present study, DMSO inhibited formation of DMPO−OH and induced formation of DMPO−CH_3, which suggests that the DMPO−OH was derived from ·OH and DMPO. Moreover, the bactericidal effect was reduced by addition of DMSO and HCOONa, which are known ·OH scavengers.

Involvement of ·OH in the bactericidal action of photoirradiated gallic acid was also confirmed by the TBARS assay. Nonenzymatic lipid peroxidation could be initiated by free radicals, such as ·OH, alkoxyl radicals and peroxyl radicals, but not H_2O_2 or O_2^-·.[34] There are a few reports of lipid peroxidation of bacteria caused by ·OH with analysis by the TBARS assay. Maness et al. demonstrated that the TBARS level of *Escherichia coli* was increased by ·OH generated by photocatalytic reaction of TiO_2.[35] Hong et al. demonstrated that the TBARS level of *E. coli* increased when the bacteria was in contact with copper or copper alloy.[36] They discussed that

lipid peroxidation would probably be caused by ·OH generated via a Fenton-like reaction. In the present study, photoirradiation peaked at 400 nm and a fwhm of <14 nm of the bacterial suspension in 4 mmol/L gallic acid significantly increased the TBARS level of *S. aureus*. This suggests that ·OH functioned as an initiator of lipid peroxidation resulting in oxidative damage to the bacteria.

According to the ESR analysis, the amounts of both ROS generated by LED irradiation of gallic acid were very small. The average yields of H_2O_2 and ·OH were 7.1 and 0.7 μmol/L, respectively, when 4 mmol/L gallic acid was irradiated with the LED-light for 15 min. Our earlier results suggested that 200−300 μmol/L ·OH would be needed to produce a >5-log reduction in *S. aureus* within a short time as 3 min.[15] In addition to the difference in the exposure time, one of the reasons for this discrepancy might be because of the affinity of gallic acid to the bacterial cell membrane. Catechins with gallate, such as epicatechin gallate and epigallocatechin gallate, possess higher affinity to cell membranes than those without gallate.[37,38] Ikigai et al. demonstrated that the antimicrobial activity of epigallocatechin gallate was higher than that of epicatechin, and showed that epigallocatechin gallate but not epicatechin damaged the lipid bilayers of liposomes.[39] Thus, it is thought that gallic acid would also have high affinity to the cell membrane. Consequently, the ·OH generated around the phospholipid membrane of the bacteria will affect the membrane structure and function even though the yield of the radicals is relatively low. It is reasonable to think that H_2O_2 would probably act as a source of ·OH in the photolysis reaction, rather than be a major contributor to the bactericidal action in a short time (15 min). This is because the reactivity and the oxidative power of ·OH are much higher than those of H_2O_2.[17] Indeed, in our previous study, H_2O_2 at a high concentration as 500 mM could kill *S. aureus* only with 1-log reduction.[40] In the present study, H_2O_2 at the concentration of less than 10 μM was generated, indicating that H_2O_2 cannot be a contributor of the bactericidal activity even though it would be generated around the phospholipid membrane of the bacteria as discussed above. These results strongly suggest that ·OH could be the main contributor to the bactericidal effect of the photoirradiated gallic acid.

A possible scheme for the bactericidal action via ·OH formation is illustrated in Figure 7. Gallic acid would interact
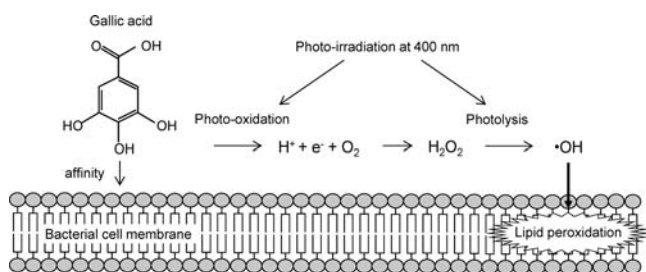

Figure 7. Proposed mechanism of the bactericidal action in photoirradiation of gallic acid. Photo-oxidation of gallic acid is induced by the LED irradiation. Through the photo-oxidation, a proton coupled electron transfer to dissolved oxygen results in H_2O_2 generation, followed by photolysis of H_2O_2. Consequently, the ·OH generated by photolysis causes lethal oxidative damage including lipid peroxidation of the bacterial cells.

with the bacterial cell membrane because of its high affinity to the membrane as discussed above for gallate-containing catechins.[37–39] Then, the polyphenolic hydroxyl group of gallic acid would be oxidized by the photoradiation peaked at 400 nm and a fwhm of <14 nm. Because of the oxidation, a proton coupled electron transfer to dissolved oxygen would result in H_2O_2 generation,[12] followed by photolysis of H_2O_2.[15,19,41] Consequently, the ·OH generated by photolysis would cause lethal oxidative damage including lipid peroxidation of the bacterial cells. In this series of reactions, photoirradiation would probably function in two different ways, that is, photo-oxidation of gallic acid and photolysis of H_2O_2.

The present study suggests that photoirradiated gallic acid could be used for disinfection. Gallic acid is regarded as relatively safe, and is ingested by humans daily as an ingredient of various fruits, vegetables and functional foods. Furthermore, gallic acid can function as an antioxidant to terminate excessive oxidative damage after the treatment.[2,3] The ·OH is only generated in gallic acid during photoirradiation, which means that its generation is controllable through termination of the light irradiation. Thus, the residual toxicity would probably be negligible, and this disinfection technique could be used in various situations.

■ AUTHOR INFORMATION

Corresponding Author
*Phone: +81-22-717-8299. Fax: +81-22-717-8299. E-mail: keisuke@m.tohoku.ac.jp.

Notes
The authors declare no competing financial interest.

■ ABBREVIATIONS USED

·OH, hydroxyl radical; ROS, reactive oxygen species; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; DMSO, dimethyl sulfoxide; TEMPOL, 4-hydroxy-2,2,6,6-tetramethylpiperidine *N*-oxyl; TBARS, tiobarbituric acid reactive substances; LED, light emitting diode; CFU, colony forming unit; ESR, electron spin resonance; MDA, malondialdehyde

■ REFERENCES

(1) Kondo, K.; Kurihara, M.; Miyata, N.; Suzuki, T.; Toyoda, M. Scavenging mechanisms of (-)-epigallocatechin gallate and (-)-epicatechin gallate on peroxyl radicals and formation of superoxide during the inhibitory action. *Free Radical Biol. Med.* **1999**, *27*, 855−863.

(2) Liu, Z.; Ma, L.; Zhou, B.; Yang, L.; Liu, Z. Antioxidative effects of green tea polyphenols on free radical initiated and photosensitized peroxidation of human low density lipoprotein. *Chem. Phys. Lipids* **2000**, *106*, 53−63.

(3) Yilmaz, Y.; Toledo, R. T. Major flavonoids in grape seeds and skins: Antioxidant capacity of catechin, epicatechin, and gallic acid. *J. Agric. Food Chem.* **2004**, *52*, 255−260.

(4) Heim, K.; Tagliaferro, A.; Bobilya, D. Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. *J. Nutr. Biochem.* **2002**, *13*, 572−584.

(5) Hatano, T.; Kusuda, M.; Inada, K.; Ogawa, T. O.; Shiota, S.; Tsuchiya, T.; Yoshida, T. Effects of tannins and related polyphenols on methicillin-resistant *Staphylococcus aureus*. *Phytochemistry* **2005**, *66*, 2047−2055.

(6) Kusuda, M.; Inada, K.; Ogawa, T. O.; Yoshida, T.; Shiota, S.; Tsuchiya, T.; Hatano, T. Polyphenolic constituent structures of *Zanthoxylum piperitum* fruit and the antibacterial effects of its polymeric procyanidin on methicillin-resistant S*taphylococcus aureus*. *Biosci., Biotechnol., Biochem.* **2006**, *70*, 1423−1431.

(7) Xu, L.; Liu, R.; Li, D.; Tu, S.; Chen, J. An in vitro study on the dental caries preventing effect of oligomeric procyanidins in sorghum episperm. *Food Chem.* **2011**, *126*, 911−916.

(8) Cushnie, T. P.; Lamb, A. J. Antimicrobial activity of flavonoids. *Int. J. Antimicrob. Agents* **2005**, *26*, 343−356.

(9) Iwasawa, A.; Niwano, Y.; Mokudai, T.; Kohno, M. Antiviral activity of proanthocyanidin against feline calicivirus used as a surrogate for noroviruses, and coxsackievirus used as a representative enteric virus. *Biocontrol Sci.* **2009**, *14*, 107−111.

(10) Taguri, T.; Tanaka, T.; Kouno, I. Antibacterial spectrum of plant polyphenols and extracts depending upon hydroxyphenyl structure. *Biol. Pharm. Bull.* **2006**, *29*, 2226−2235.

(11) Mabe, K.; Yamada, M.; Oguni, I.; Takahashi, T. In vitro and in vivo activities of tea catechins against *Helicobacter pylori*. *Antimicrob. Agents Chemother.* **1999**, *43*, 1788−1791.

(12) Arakawa, H.; Maeda, M.; Okubo, S.; Shimamura, T. Role of hydrogen peroxide in bactericidal action of catechin. *Biol. Pharm. Bull.* **2004**, *27*, 277−281.

(13) Akagawa, M.; Shigemitsu, T.; Suyama, K. Production of hydrogen peroxide by polyphenols and polyphenol-rich beverages under quasi-physiological conditions. *Biosci., Biotechnol., Biochem.* **2003**, *67*, 2632−2640.

(14) Rudrappa, T.; Choi, Y. S.; Levia, D. F.; Legates, D. R.; Lee, K. H.; Bais, H. P. Phragmites australis root secreted phytotoxin undergoes photo-degradation to execute severe phytotoxicity. *Plant Signaling Behav.* **2009**, *4*, 506−513.

(15) Ikai, H.; Nakamura, K.; Shirato, M.; Kanno, T.; Iwasawa, A.; Sasaki, K.; Niwano, Y.; Kohno, M. Photolysis of hydrogen peroxide, an effective disinfection system via hydroxyl radical formation. *Antimicrob. Agents Chemother.* **2010**, *54*, 5086−5091.

(16) Redmond, R. W.; Kochevar, I. E. Spatially resolved cellular responses to singlet oxygen. *Photochem. Photobiol.* **2006**, *82*, 1178−1186.

(17) Halliwell, B.; Gutteridge, J. M. The chemistry of free radicals and related ralactive species. In *Free Radicals in Biology and Medicine*, 4th ed.; Oxford University Press: Oxford, 2007; Chapter 2, pp 30−78.

(18) Lustrous Technology. Technical Datasheet LS04. High power solid-state LED light source. COLOR V. http://www.lustrous.com.tw/download/ffile/LS04-COLOR%20V%20%202010-01-07.pdf (September 13, 2012),

(19) Shirato, M.; Ikai, H.; Nakamura, K.; Hayashi, E.; Kanno, T.; Sasaki, K.; Kohno, M.; Niwano, Y. Synergistic effect of thermal energy on bactericidal action of photolysis of H_2O_2 in relation to acceleration of hydroxyl radical generation. *Antimicrob. Agents Chemother.* **2012**, *56*, 295−301.

(20) Nakamura, K.; Kanno, T.; Ikai, H.; Sato, E.; Mokudai, T.; Niwano, Y.; Ozawa, T.; Kohno, M. Reevaluation of quantitative ESR spin trapping analysis of hydroxyl radical by applying sonolysis of water as a model system. *Bull. Chem. Soc. Jpn.* **2010**, *83*, 1037−1046.

(21) Britigan, B. E.; Rosen, G. M.; Chai, Y.; Cohen, M. S. Do human neutrophils make hydroxyl radical? Determination of free radicals generated by human neutrophils activated with a soluble or particulate stimulus using electron paramagnetic resonance spectrometry. *J. Biol. Chem.* **1986**, *261*, 4426−4431.

(22) Sato, E.; Mokudai, T.; Niwano, Y.; Kohno, M. Kinetic analysis of reactive oxygen species generated by the in vitro reconstituted NADPH oxidase and xanthine oxidase systems. *J. Biochem.* **2011**, *150*, 173−181.

(23) Buettner, G. R. Spin trapping: ESR parameters of spin adducts. *Free Radical Biol. Med.* **1987**, *3*, 259−303.

(24) Chanwitheesuk, A.; Teerawutgulrag, A.; Kilburn, J. D.; Rakariyatham, N. Antimicrobial gallic acid from *Caesalpinia mimosoides* Lamk. *Food Chem.* **2007**, *100*, 1044−1048.

(25) Li, A.; Chen, J.; Zhu, W.; Jiang, T.; Zhang, X.; Gu, Q. Antibacterial activity of gallic acid from the flowers of *Rosa chinensis* Jacq. against fish pathogens. *Aquacult. Res.* **2007**, *38*, 1110−1112.

(26) Akiyama, H.; Fujii, K.; Yamasaki, O.; Oono, T.; Iwatsuki, K. Antibacterial action of several tannins against *Staphylococcus aureus*. *J. Antimicrob. Chemother.* **2001**, *48*, 487−491.

(27) Vermeulen, N.; Keeler, W. J.; Nandakumar, K.; Leung, K. T. The bactericidal effect of ultraviolet and visible light on *Escherichia coli*. *Biotechnol. Bioeng.* **2008**, *99*, 550−556.

(28) Miura, Y. H.; Tomita, I.; Watanabe, T.; Hirayama, T.; Fukui, S. Active oxygens generation by flavonoids. *Biol. Pharm. Bull.* **1998**, *21*, 93−96.

(29) Nakayama, T.; Enoki, Y.; Hashimoto, K. Hydrogen peroxide formation during catechin oxidation is inhibited by superoxide dismutase. *Food Sci. Technol. Int.* **1995**, *1*, 65−69.

(30) Long, L. H.; Clement, M. V.; Halliwell, B. Artifacts in cell culture: rapid generation of hydrogen peroxide on addition of (-)-epigallocatechin, (-)-epigallocatechin gallate, (+)-catechin, and quercetin to commonly used cell culture media. *Biochem. Biophys. Res. Commun.* **2000**, *273*, 50−53.

(31) Mochizuki, M.; Yamazaki, S.; Kano, K.; Ikeda, T. Kinetic analysis and mechanistic aspects of autoxidation of catechins. *Biochim. Biophys. Acta* **2002**, *1569*, 35−44.

(32) Finkelstein, E.; Rosen, G. M.; Rauckman, E. J. Spin trapping of superoxide and hydroxyl radical: practical aspects. *Arch. Biochem. Biophys.* **1980**, *200*, 1−16.

(33) Tatsumi, H.; Nakase, H.; Kano, K.; Ikeda, T. Mechanistic study of the autoxidation of reduced flavin and quinone compounds. *J. Electroanal. Chem.* **1998**, *443*, 236−242.

(34) Catala, A. Lipid peroxidation. In *Principles of Free Radical Biomedicine. Vol. 1*; Pantopoulos, K., Schipper, H., Eds.; Nova Science Publishers: NY, 2012; Chapter 7, pp 137−159.

(35) Maness, P. C.; Smolinski, S.; Blake, D. M.; Huang, Z.; Wolfrum, E. J.; Jacoby, W. A. Bactericidal activity of photocatalytic TiO_2 reaction: toward an understanding of its killing mechanism. *Appl. Environ. Microbiol.* **1999**, *65*, 4094−4098.

(36) Hong, R.; Kang, T. Y.; Michels, C. A.; Gadura, N. Membrane lipid peroxidation in copper alloy-mediated contact killing of *Escherichia coli*. *Appl. Environ. Microbiol.* **2012**, *78*, 1776−1784.

(37) Kajiya, K.; Kumazawa, S.; Nakayama, T. Effects of external factors on the interaction of tea catechins with lipid bilayers. *Biosci., Biotechnol., Biochem.* **2002**, *66*, 2330−2335.

(38) Kajiya, K.; Kumazawa, S.; Nakayama, T. Steric effects on interaction of tea catechins with lipid bilayers. *Biosci., Biotechnol., Biochem.* **2001**, *65*, 2638−2643.

(39) Ikigai, H.; Nakae, T.; Hara, Y.; Shimamura, T. Bactericidal catechins damage the lipid bilayer. *Biochim. Biophys. Acta* **1993**, *1147*, 132−136.

(40) Kanno, T.; Nakamura, K.; Ikai, H.; Hayashi, E.; Shirato, M.; Mokudai, T.; Iwasawa, A.; Niwano, Y.; Kohno, M.; Sasaki, K. Novel denture cleaning system based on hydroxyl radical disinfection. *Int. J. Prosthodont.* **2012**, *25*, 376−380.

(41) Nakamura, K.; Kanno, T.; Mokudai, T.; Iwasawa, A.; Niwano, Y.; Kohno, M. Microbial resistance in relation to catalase activity to oxidative stress induced by photolysis of hydrogen peroxide. *Microbiol. Immunol.* **2012**, *56*, 48−55.