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Antimicrobial photodynamic therapy using a plaque disclosing solution on *Streptococcus mutans*

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ABSTRACT

Objectives: Photodynamic therapy with a bactericidal action is called antimicrobial photodynamic therapy (aPDT),which is a method of staining an object with a photosensitizing dye and then sterilizing by irradiating the dye at it's excitation wavelength. In this study, we aimed to investigate a caries pathogenic bactericidal method in a site difficult to mechanically remove, by examining aPDT effect on *Streptococcus mutans* (*S. mutans*), which is a typical caries pathogenic bacteria by applying the plaque disclosing solution as photosensitizing dye.
Methods: The absorption wavelength spectrum of irradiating plaque staining agent phloxine B (PB) was analyzed using UV–vis. Reactive oxygen species (ROS) generated by photo excitation with blue LED irradiation was measured by electron spin resonance technique. *S. mutans* was cultured according to a conventional method and the effect of aPDT after PB staining was evaluated by a Colony Forming Unit (CFU). In addition, protein carbonyl (PC), an oxidative stress marker, was also measured by western blotting.
Results: Singlet oxygen was generated by PB with blue light. As a result of aPDT treatment on *S. mutans* under this condition, it was recognized that CFU was suppressed dependent on irradiation intensity of blue light. In addition, the expression of PC was enhanced by aPDT.
Conclusions: aPDT is demonstrated by staining *S. mutans* with PB and irradiating blue light used for resin polymerization and tooth bleaching to generate ROS. Therefore, plaque-disclosing solution-based aPDT against *S. mutans* might represent a new method for cleaning pit and fissure grooves.

1. Introduction

Dental caries is the most common disease found in baby teeth and juvenile permanent teeth [1,2]. In dental clinics, pit and fissure sealants are used to prevent dental caries, and strong evidence suggests that dental sealants are effective at preventing dental caries in children at varying degrees of risk [3,4]. In addition, it has been reported that sealants not only directly close fissures, but as fissures can serve as reservoirs for *Streptococcus mutans* (*S. mutans*), the use of permanent first molar sealants can reduce salivary *S. mutans* levels in children without dental caries [5]. In this way, sealants are very useful for dental caries prevention. However, a number of associated clinical problems exist when using sealants. In long-term studies by Jensen et al. and Shapira et al., it was reported that sealant treatment did not necessarily result in a stable long-term prognosis [6,7]. The reasons for this include insufficient cleaning of the tooth surface during the pretreatment procedure, the complexity of the form of the pit fissure groove, abrasion of the sealant material itself, marginal fractures, and accompanying microleakage. Thus, the removal of the *S. mutans* present in biofilms via pit and fissure sealant treatment is very important. However, currently only physical cleaning methods, involving the use of brush cones and rubber cups, are employed for such purposes. It is difficult to completely remove biofilm from all sites, including pit and fissure grooves, via these methods. Therefore, a method for removing microorganisms from sites at which it is not possible to remove microorganisms using current dental techniques is required for preventing secondary caries.

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In recent years, photodynamic therapy (PDT) has been proposed as an alternative method to treating several types of cancer [8] and neurovascular age-related macular degeneration [9]. This treatment does not have severe side effects and can often be repeated [10]. It has also been used for sterilization; i.e., for the photoinactivation of Gram-negative/-positive microorganisms [11], and such treatment is referred to as antimicrobial PDT (aPDT). In dentistry, an increasing number of in vivo and in vitro studies have obtained positive results regarding the utility of aPDT [12]. In the dental field, aPDT has demonstrated great potential as an adjunct treatment for various dental diseases, including caries [13], endodontic infections [14], periodontitis [15] and peri-implantitis [16]. For instance, aPDT is used to eradicate microorganisms from the periodontal pocket. It is necessary to stain the target microorganisms with some kind of photosensitizer and then to irradiate the dye at an appropriate excitation wavelength [17]. In this case, the main target of aPDT is periodontal pathogens, including *Porphyromonas gingivalis* (*P. gingivalis*). Other aPDT has also been used against *S. mutans*, a representative cariogenic pathogen. However, many dyeing agents, such as toluidine and methylene blue, which are effective dyeing reagents in periodontitis, are also used to detect *S. mutans* [18,19]. There have also been some reports about the effects of photoirradiation on *S. mutans* using rose bengal (RB) or erythrosine as a plaque-disclosing solution. However, there are few reports about the detailed bactericidal mechanism of aPDT against *S. mutans* [20–22].

The aim of this study is to evaluate the effects of aPDT, involving blue light irradiation of a plaque-disclosing solution (which was also used as a photosensitizer), against *S. mutans* and the bactericidal mechanism underlying these effects.

2. Materials and methods

2.1. Reagents

4-Hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxide (TEMPOL) and 2,2,6,6-tetramethyl-4-piperidinol (4-OH-TEMP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phloxine B (PB) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Phosphate-buffered saline (PBS, pH 7.2) was purchased from the Invitrogen Corporation (Carlsbad, CA, USA), and L-histidine was acquired from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other reagents were of analytical grade.

2.2. Light source and conditions

The setting of the light irradiation condition was conducted with a minor modification to the method described in Yoshida et al [23]. The spectrum of the G180T microscope illuminator (RBG) (SCOPELED; Richmond, CA, USA) in blue light mode was obtained with an Exemplar charge-coupled device (CCD) spectrometer (BRC115P, B&W TEK, Newark, DE, USA) and the BWSpec 4 software (B&W TEK, Newark, DE, USA). In each experiment, the light-emitting diode (LED) power output level was set to 370 mW/cm^2 and the peak wavelength was set to 475 nm using an optical power meter (8230E, ADC Corporation, Tokyo, Japan). The *S. mutans* suspensions in each well were irradiated with the tip of the light source from the top of the plate. The 96-well plates had a depth of 10.9 mm (clear-bottomed black plates; Corning Incorporated, Corning, NY, USA), whereas the 24-well plates had a depth of 14.6 mm (clear-bottomed black plates; Eppendorf AG, Hamburg, Germany). Therefore, in the reactive oxygen species (ROS) measurement, cell viability analysis, and oxidative stress analysis the light irradiation distance was set at 13.0, 10.9, and 14.6 mm, respectively (the optimal values for each experiment). Also, in each experiment the beam diameter was set at 10.0 mm, and the amount of light energy was set at a level that would not affect the parameter being examined (see each section).

2.3. Spectroscopic analysis

The fluorescence spectra of blue light-irradiated 0.01% PB were obtained with an Exemplar CCD spectrometer and the BWSpec 4 software.

2.4. In vitro electron spin resonance (ESR) measurement

The singlet oxygen ($^1O_2$) generated upon the blue light irradiation of PB was analyzed quantitatively using ESR spectroscopy [24,25]. It was detected by adding an $^1O_2$-trapping agent (20 mM 4-OH-TEMP) to PB solutions that had been irradiated with blue light (475 nm, 370 mW/cm^2). The concentration of the PB solution was 0, 0.001, or 0.01%, and the blue light irradiation dose was 0, 1, or 10 J. We assessed the inhibitory effect of 5 mM L-histidine on ROS production in blue light-irradiated PB-containing solutions [26]. We compared the double integrals of the experimental spectra of 4-OH-TEMP with those of a 10 μM TEMPOL standard, both of which were measured under identical conditions, to estimate the $^1O_2$ adduct concentration [27]. The ESR measurements were performed using a JES-RE1X (JEOL, Tokyo, Japan) connected to a WIN-RAD ESR data analyzer (Radical Research, Tokyo, Japan) at the following instrument settings: microwave power, 8.00 mW; magnetic field, 335.8 ± 5.0 mT; field modulation width, 0.1 mT; sweep time, 1 min; and time constant, 0.03 s. All experiments were repeated three times.

2.5. Bacterial strain and cultivation conditions

The bacterial strain used in this study was *S. mutans* Ingbritt. The *S. mutans* was grown in brain heart infusion broth (BHI broth; Difco Laboratories, Detroit, MI, USA) at 37 °C for 18 h under anaerobic conditions (85% N_2, 10% H_2, and 5% CO_2). The *S. mutans* was washed twice with PBS before each experiment to avoid the influence of pigments in the culture medium.

2.6. Survival of phloxine B-dyed S. mutans after blue light irradiation

The survival of *S. mutans* after blue light irradiation was determined according to Yoshida and co-workers [23] with a minor modification as follows: The washed *S. mutans* suspensions were adjusted to an optical density of 0.6 at 550 nm. In order to minimize the effects of PB photoexcitation due to indoor light, the concentration of PB was set at 0.001%, and the light energy was set at a level at which irradiation would not result in an increase in the temperature of the target. The washed suspensions were supplied with or without 0.001% PB and left for 1 min. The samples were added to a 96-well plate at 200 μL per well, and then were irradiated with 0 or 10 J. The survival of the *S. mutans* cells was estimated by counting the number of colony-forming units (CFU) after 18 h culturing on BHI agar media under anaerobic conditions. The data are shown as the logarithm of the number of CFU per milliliter.

2.7. Oxidative stress assessment

The washed *S. mutans* was adjusted to an optical density of 1.0 at 550 nm. Even in this experiment, the PB concentration was set at 0.001% in order to reduce the influence of ROS generation due to indoor light, and the light energy was set at a level at which irradiation would not result in an increase in the temperature of the target. Then, the *S. mutans* was suspended in PBS with 0.001% PB or distilled water and plated in a 24-well plate at 1.8 mL per well, before being irradiated with 0 or 100 J of blue light. The precipitates obtained from the collected samples after 2 min centrifugation at 12,000 G were used for protein extraction with CelLytic B, according to the manufacturer's instructions (Sigma-Aldrich Co. LLC., MO, USA). Bacterial protein carbonylation was determined using a modified procedure based on 2,4-

dinitrophenyl hydrazine (DNPH)-derivatization [28–31]. An equal volume of 10 mM DNPH in 10% trifluoroacetic acid was added and incubated at room temperature for 15 min. The reaction was neutralized and normalized to the protein concentration. In order to detect carbonylation, samples were prepared as described in the Bolt bis-tris plus mini gel manual (Thermo Fisher Scientific Inc., MA, USA), run on the gels (4–12% gradient gels), and electroblotted onto polyvinylidene difluoride (PVDF) membranes. According to the instructions in the iBind flex western system manual (Thermo Fisher Scientific Inc., MA, USA), the PVDF membranes were treated with rabbit anti-dinitrophenyl (DNP) primary antibodies (SHIMA Laboratories Co., Ltd., Tokyo, Japan) followed by goat F(ab')2 anti-rabbit IgG (H + L) and mouse/human ads-horseradish peroxidase secondary antibodies (SouthernBiotech, AL, USA). The DNP-derivatized protein carbonyls (PC) were visualized using a chemiluminescent reagent (Amersham ECL prime Western blotting detection reagent, GE Healthcare, IL, USA) using the LAS-3000 FUJIFILM luminescent image analyzer (FUJI FILM Corporation, Tokyo, Japan). The protein bands on the PVDF membranes were visualized by washing the blots extensively in PBS containing 0.3% Tween 20 and then staining them with India ink (PILOT CORPORATION, Tokyo, Japan).

2.8. Statistical analyses

Quantitative data are represented as the mean ± standard deviation (SD) of a minimum of three separate experiments. All statistical analyses were performed with the unpaired *t*-test or Tukey's multiple comparisons test using the GraphPad Prism 6 software (GraphPad Software Inc., La Jolla, CA, USA). P-values of < 0.05 were considered statistically significant.

3. Results

3.1. Blue LED light spectra and absorbance spectra of PB

The spectrum of the blue LED light and the absorbance spectrum of PB are shown in Fig. 1. The blue LED light spectrum peaked at 471.5 nm, and the absorbance of PB peaked at 540 nm.

3.2. ROS production induced by blue light-irradiated PB

We investigated the ROS generated by PB subjected to blue light-induced excitation. In this study, the characteristic ESR spectral pattern included three intense lines derived from the 2,2,6,6-tetramethyl-4-hydroxyl-piperidinyloxy (4-OH-TEMPO) radical, indicating that $^1O_2$ generation occurred when blue light was used to excite PB in the presence of 4-OH-TEMP. Even at high concentrations of PB, hardly any $^1O_2$ generation was observed in the absence of blue light irradiation.

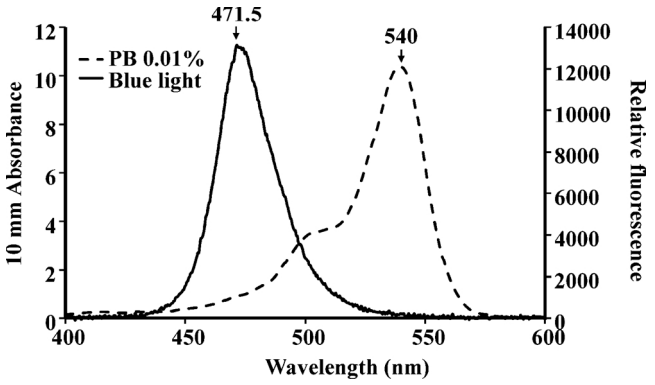

Fig. 1. Relative spectral emission curve of blue LED light and relative spectral absorbance of PB.

Similarly, little $^1O_2$ was generated by high-joule irradiation in the absence of PB. The spin concentration was about 33 μM after the blue light irradiation of 0.1% PB with 100 J (Fig. 2A). The spin concentration of the products of 0.1% PB excited with 100 J blue light was reduced by the addition of L-histidine (Fig. 2B).

3.3. Viability of S. mutans after blue light irradiation

We investigated the effect of PDT on *S. mutans* by subjecting bacteria in liquid medium containing PB to blue light irradiation. As shown in Fig. 3, the viability of the *S. mutans* (according to the logarithm of the number of CFU per mL) was significantly suppressed when 0.001% PB was added to the bacterial solution and the mixture was subjected to 10 J blue light irradiation. On the other hand, when 0.001% PB was added to the bacterial solution and no irradiation was delivered or the bacterial solution was irradiated, but not stained with PB, no differences in survival were observed between the test and control groups (which were not subjected to PB staining or irradiation).

3.4. Oxidative stress induced in S. mutans by blue light-irradiated PB

To investigate the effects of oxidative stress on PB-stained *S. mutans* subjected to blue light irradiation, we evaluated DNPH-derivatized PC formation using immunoblotting. Marked bands were detected in the DNPH-derivatized samples from the bacteria that were stained with 0.001% PB, before being subjected to 100 J blue light irradiation, which indicated that the bacterial proteins were highly carbonylated. In the other DNPH-derivatized samples, almost no bands were detected in the control (which was not subjected to PB staining or irradiation), whereas faint bands were detected in the irradiation alone or staining alone samples (Fig. 4A). When India ink staining of the PVDF membranes was performed to visualize all proteins, similar bands were observed in all samples, and it was confirmed that equivalent amounts of protein were transferred to the PVDF membranes (Fig. 4B). In addition, no bands were detected during chemiluminescent immunoblotting of the samples that were not subjected to DNPH derivatization (data not shown).

4. Discussion

In PDT, it is important that the photosensitizer is transported to the target tissues/bacterial cells. Generally, in clinical PDT-based cancer therapy the photosensitizer is transported to the cancer tissue via the circulatory system. One of the problems limiting the use of many photosensitizers is the difficulty in preparing pharmaceutical formulations that enable their parenteral administration. Due to their low water solubility, hydrophobic photosensitizers cannot be injected intravenously [32]. On the other hand, the oral cavity can be directly viewed, and so it is a very suitable environment for both PDT and aPDT. In addition, aPDT has a promising future in the face of the unrelenting increase in antibiotic resistance [33]. Therefore, in this study attempts were made to eradicate *S. mutans*, a representative cariogenic bacterium, by irradiating a dye found in a dental plaque-disclosing solution, which is widely used as a photosensitizer. Blue light, which is used for resin polymerization and tooth bleaching in dentistry, was employed in order to allow the rapid clinical application of this technique.

First, we investigated whether the absorption wavelength of PB, which is representative of red dental plaque dyes, falls within the spectrum of blue light. It has been reported that this dye has photodynamic effects when it is irradiated with white light [34]. In the current study, it was shown that the absorption wavelength of PB was near to the output wavelength of the blue light source (Fig. 1). Originally, it was predicted that in PDT ROS would be most efficiently generated at the maximum absorption wavelength of the dye. Specifically, the combination of a drug, usually referred to as a photosensitizer; the optimal light wavelength for exciting the photosensitizer;

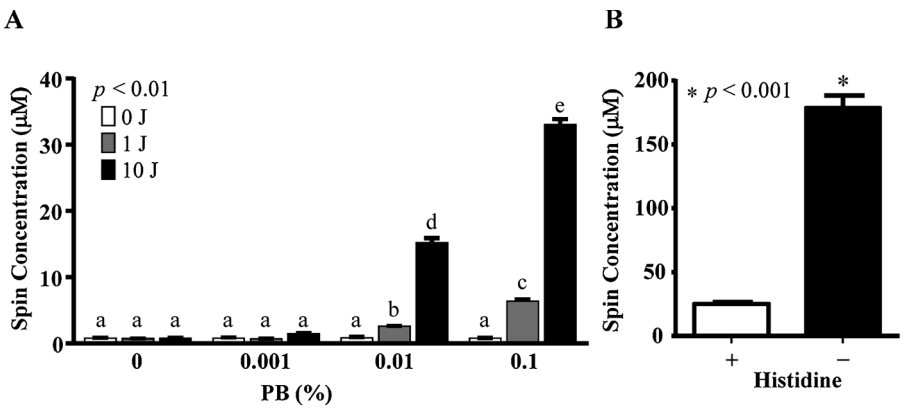

Fig. 2. Singlet oxygen ($^1O_2$) generation induced by PB upon blue light irradiation.
(A) The concentration of $^1O_2$.
(B) The generation of $^1O_2$ in the presence and absence of L-histidine under 100 J of irradiation.
The data are expressed as mean ± SD values (n = 3). Different characters indicate a significant difference at $p < 0.01$, and asterisks (*) indicate significant differences at $p < 0.001$.

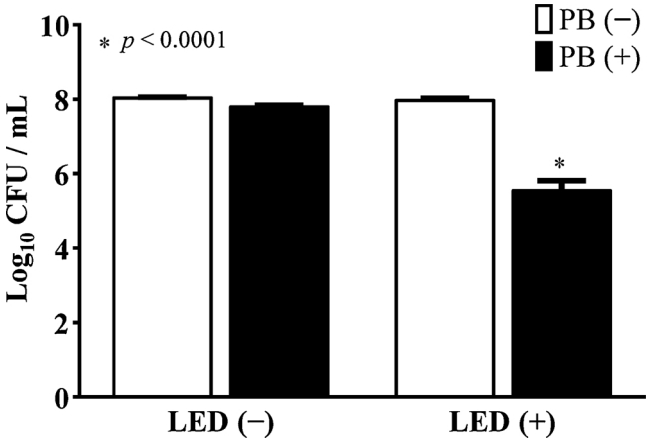

Fig. 3. Effect of blue light irradiation on the viability of *S. mutans* in PB-containing solution.
The data are expressed as mean ± SD values (n = 3). Asterisks (*) indicate significant differences at $p < 0.0001$.

and molecular oxygen leads to the production of ROS via electron or energy transfer (type I or type II reactions, respectively) from the triplet excited-state of the photosensitizer to molecular oxygen [35]. Therefore, it is desirable to use an appropriate excitation wavelength for PDT. However, in the current study even though PB was irradiated with a blue light source, which are commonly used in dental offices, it resulted in ROS formation via photoexcitation reactions. Specifically, the blue light irradiation of the photosensitizer caused the formation of $^1O_2$ (a representative ROS) (Fig. 2A). This result was supported by the experiment performed in the presence of the $^1O_2$-specific quencher L-histidine (Fig. 2B) [36]. $^1O_2$ generation increased with the radiation intensity and dye concentration. Similar results were obtained for red pigments (RB and erythrosine) that are found in dental plaque staining-solutions other than PB, but the greatest $^1O_2$ formation was detected after the irradiation of PB with blue light (data not shown). $^1O_2$ is an ROS, which is known to exert very strong oxidizing power towards cell membranes and DNA [37]. During PDT, photodestructive oxidative bursts occur in close proximity to the photosensitizer [38]. It has also been reported that $^1O_2$ has cytotoxic and antimicrobial effects against microorganisms, including *Escherichia coli* [39,40]. On the other hand, the hydrogen peroxide produced in response to aPDT also has bactericidal properties. Unfortunately, when hydrogen peroxide is produced, catalase expression might be promoted in the surviving bacteria. Therefore, catalase expression might help bacteria to survive photooxidative stress induced by aPDT [41]. For this reason, it is considered that it is desirable to produce $^1O_2$ during aPDT. In addition, we have reported that when *P. gingivalis*, a typical periodontitis-related pathogen, was irradiated with blue light the porphyrin dye in the mitochondria was excited, which resulted in antimicrobial effects due to the generation of $^1O_2$ [23]. However, the photoinactivation of cells by photosensitizers mainly results in the oxidation of biomolecules, such as lipids, proteins, and nucleic acids, and the production of $^1O_2$ via type II reactions, resulting in cell death [42–47]. Therefore, applying plaque-staining dye to *S. mutans* and inducing $^1O_2$ generation via irradiation with blue light (via a different mechanism to that seen in *P. gingivalis*, which is a Gram-negative bacterium) might result in sufficiently effective aPDT.

In order to demonstrate the effects of aPDT based on the blue light

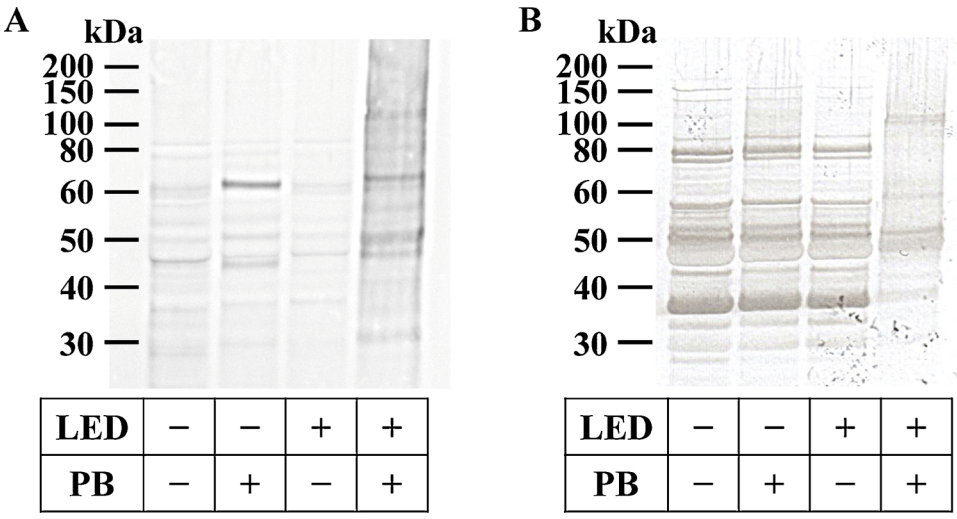

Fig. 4. PB plus blue light irradiation-induced formation of protein carbonyls in *S. mutans*.
S. mutans was treated with 0 or 0.0001% PB stain and irradiated with 0 or 100 J of blue light. Then, the protein carbonylation of bacterial proteins was examined using a Western blotting assay, involving carbonyl derivatization with DNPH, as described in Section 2.
(A) A representative Western blot of the protein carbonylation pattern.
(B) The corresponding India ink protein staining.

irradiation of PB, the inhibitory effects of such treatment on the growth of *S. mutans* were examined. No change in the number of CFU was observed in the *S. mutans* subjected to blue light irradiation alone or PB staining alone. Conversely, a significant reduction in the number of CFU was observed after blue light irradiation + PB staining (Fig. 3). This result suggests that aPDT involving blue light irradiation + PB has adverse effects on *S. mutans*. In order to examine the detailed mechanism responsible for the abovementioned effects on *S. mutans*, protein carbonylation was assessed. Protein carbonylation is a type of protein oxidation, which can be promoted by $^1O_2$ and/or other ROS, and it is widely used as a marker of oxidative stress [48]. A significant increase in *S. mutans* protein carbonylation was observed after the aPDT (Fig. 4). The usage of protein carbonylation as a biomarker of oxidative stress has some advantages in comparison with the measurement of other oxidation products because of the relative early formation and relative stability of PC. Most assays for detecting protein carbonylation involve the derivatization of carbonyl groups with DNPH, which leads to the formation of stable dinitrophenyl hydrazone products. At present, the measurement of protein carbonylation after the derivatization of proteins with DNPH is the most widely utilized measure of protein oxidation [49]. Therefore, the abovementioned results suggest that aPDT induced the generation of $^1O_2$ around and/or inside *S. mutans*, which caused oxidative damage and exerted bactericidal activity against *S. mutans*. In addition, it has been reported that Gram-positive bacteria, which are typified by *Staphylococcus aureus* (*S. aureus*), are more susceptible to aPDT than Gram-negative bacteria, as they have less complex cell walls [50,51]. On the other hand, the outer membranes of Gram-negative bacteria are composed of a lipid bilayer surrounding a thin multilayered peptidoglycan layer. Furthermore, since the outer membranes of Gram-negative bacteria contain large amounts of lipopolysaccharides, they do not readily allow the permeation of dye into their interiors [52]. Moreover, although protein carbonylation and the chemistry of the reactions that give rise to carbonyl groups are well characterized, the biology of oxidative protein modifications remains complex and incompletely defined [53]. Kato et al. reported that xanthene dyes, such as PB and RB, are taken up into the cytoplasmic membranes of the Gram-positive bacterium *S. aureus* [54]. When these dyes are used to generate $^1O_2$ via PDT, the biomolecules present in cytoplasmic membranes are damaged. Therefore, while it is unclear exactly where $^1O_2$ oxidative damage occurred in the *S. mutans* cells in the current study, it is suggested that the mechanism of action aPDT might involve oxidative damage to cell membranes. If the localization of the dye and the expression of oxidative stress markers could be clarified, more detail about the site of action of aPDT might be uncovered. Further studies are required to assess the effects of PB-based aPDT on other bacteria and biofilms in the future.

5. Conclusion

Our ultimate goal with regard to aPDT is to inactivate the *S. mutans* present in deep pits and fissure grooves, which are difficult to remove using sealant-based dental caries-preventative treatments. In previous studies, it was found that chemical cleaning using sodium hypochlorite only dissolved the organic biofilm matter at the entrances of pit and fissure grooves when a normal chair time was employed, so it is not possible to clean deep pit and fissure grooves using this method [55]. In addition, even if ultrasonic waves are used in combination with sodium hypochlorite, ultrasonic vibrations are not transmitted to deep pit and fissure grooves, and so complete cleaning remains impossible [56]. However, the PB molecule is smaller than the width of deep grooves, and so PB can stain biofilms in deep pit and fissure grooves. Furthermore, unlike ultrasonic waves, it is conceivable that sufficient light penetrates into deep pit and fissure grooves to allow aPDT to be performed. Therefore, our results indicate that performing aPDT with PB has the potential to eradicate the pathogenic caries-causing bacteria present in plaque at sites such as deep pits and fissures, which can be stained with plaque-disclosing solution, but cannot be removed.

Conflicts of interest

The authors declare that they have no competing interests.

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