Effects of Rose Bengal- and Methylene Blue-Mediated Potassium Iodide-Potentiated Photodynamic Therapy on *Enterococcus faecalis*: A Comparative Study

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Background and Objectives: This study was performed to compare the use of methylene blue (MB) and rose bengal (RB) in antimicrobial photodynamic therapy (PDT) targeting *Enterococcus faecalis* (*E. faecalis*) bacteria in planktonic and biofilm forms with potassium iodide (KI) potentiation.

Study Design/Materials and Methods: *E. faecalis* bacteria in planktonic form were exposed to antimicrobial PDT protocols activating MB and RB, with or without KI potentiation, following laser irradiation with different exposure times, 60 mW/cm^2 laser power, and different photosensitizer agent (PS)/potentiator concentrations to observe relationships among the variables. Two continuous-wave diode lasers were used for irradiation (red light: $\lambda = 660$ nm and green light: $\lambda = 565$ nm). The pre-irradiation time was 10 minutes. The vitality of *E. faecalis* biofilm was assessed by confocal laser scanning microscopy, and the morphology was determined by scanning electron microscopy. The effects on the proliferation of stem cells from the apical papilla (SCAPs) were analyzed by cell counting kit-8 assay. The staining effect of antimicrobial PDT on dentin slices was investigated. Statistical analysis using a one-way analysis of variance was done.

Results: KI-potentiated RB and MB antimicrobial PDT both effectively eradicated *E. faecalis* bacteria in planktonic and biofilm forms. The minimum bactericidal concentrations of PSs (±100 mM KI) were obtained through PDT on planktonic *E. faecalis*, and the optimal light parameters were 60 mW/cm^2, 6 J/cm^2 for 100 seconds. KI-potentiated PDT effectively strengthened the ability to inhibit *E. faecalis* biofilm with $86.50 \pm 5.78\%$ for MB ($P = 0.0015 < 0.01$) and $91.50 \pm 1.75\%$ for RB ($P = 0.0418 < 0.05$) of bactericidal rate, with less toxicity for SCAPs ($P < 0.001$) and less staining. KI could reduce the staining induced by antimicrobial PDT on dentin slices.

Conclusion: A combination of KI and antimicrobial PDT may be a useful alternative to conventional disinfection methods in endodontic treatment. MB and RB antimicrobial PDT at much lower concentrations with KI could hopefully achieve disinfection effects comparable with those of 1.5% NaClO while causing few adverse effects on SCAPs. KI helps to avoid staining problems associated with high concentrations of photosensitizer agents. Lasers Surg. Med. © 2020 Wiley Periodicals, LLC

Key words: antimicrobial photodynamic therapy; potassium iodide; methylene blue; rose bengal; *Enterococcus faecalis*; root canal disinfection

INTRODUCTION

An aseptic environment in endodontic treatment is one of the critical factors for treatment success. Conventional disinfection methods in endodontic therapy consist of mechanical preparation and chemical disinfection. Disinfection in regenerative endodontic therapy mainly relies on irrigation and root canal–sealing drugs because mechanical preparation would result in excessive damage to the dentin wall and the attached stem cells [1]. Pulp necrosis and periapical lesions of young permanent teeth typically occur due to multiple infections of bacteria in planktonic or biofilm forms [2]. The removal of facultative anaerobes is challenging during the disinfection process, and these organisms are more likely to survive than anaerobes [3]. *Enterococcus faecalis*, a Gram-positive, facultatively anaerobic coccus, has been used as a representative bacterium of most resistant and commonly found microorganisms in diseased dental pulp [4]. *E. faecalis* is also the most prevalent microorganism involved in the pathogenicity of refractory endodontic infection in cases of failed treatment [5]. *E. faecalis* is reportedly resistant to sodium hypochlorite (NaClO) [6] and calcium hydroxide [7],

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which are the most effective antibacterial agents in widespread use in endodontic treatment. Thus, there is a need to continually explore new technologies to eradicate microorganisms from the root canal.

One such technique is photodynamic therapy (PDT), which is based on irradiation of a photosensitizer agent (PS) with light at a specific wavelength matched to the peak absorption of the PS in the presence of oxygen; this generates singlet oxygen and reactive-free radicals that cause bacterial cell wall rupture [8]. The therapeutic effects of antibacterial PDT in endodontic treatment have been confirmed in animal experiments [9,10] and clinical studies [11–14]. Phenothiazine dyes, including methylene blue (MB) and rose bengal (RB), are among the most well-characterized PSs. MB has already been applied in the clinical treatment of caries [15,16] and periodontal diseases [17,18], with a human prescription drug code in the United States (0517-0374-05) and a Chinese drug license (H20083164). Another PS, RB, is used as a food coloring in Japan (Food Red No.105 [CI 45440]) and is a US FDA registered drug (FDA application number: 16224). RB PDT has not been applied in dentistry. However, RB in association with a green laser source was proposed as a more efficient approach with regard to the higher rate of inactivation of Gram-positive bacteria than MB [8,19], which may constitute a more efficient method for endodontic disinfection.

However, PDT cannot replace the traditional root canal irrigation strategy because it requires high concentrations of PSs [9,10,12,20]. Commercial MB with concentrations as high as 0.01% (31.2 μM) is generally used. However, such MB products have problems associated with potent cytotoxicity and dentin staining. Indeed, some MB preparations for injection in gel form have been reported to clog dentin tubules [21]. Thus, there remains a need to improve the clinical applicability of PDT in pulp treatment by optimizing PS selection.

Potassium iodide (KI), a nontoxic inorganic salt, has been shown to exhibit potentiating activities on antimicrobial PDT to avoid adverse effects and staining problems [22–25]. Vecchio et al. [25] reported that photodynamic inactivation using MB (10 μM) as a PS and KI (10 mM) as a potentiator could eradicate *Staphylococcus aureus* with an irradiation time of 50 seconds and an irradiance of 100 mW/cm^2. Photodynamic inactivation using KI as a potentiator on *E. faecalis* was first studied by Ghaffaria et al. [26], with the highest photo-killing effect observed at 10 μM toluidine blue O/10 mM KI with an irradiation time of 180 seconds. Yuan et al. [27] reported that KI could enhance the antibacterial efficiency of MB antimicrobial PDT on *E. faecalis* bacteria in planktonic and biofilm forms.

Wen et al. [24] reported that RB antimicrobial PDT at a concentration of only 0.1 μM potentiated by 100 mM KI could result in bacterial eradication because Gram-positive bacteria are very susceptible to antimicrobial PDT with anionic PS. In addition, antimicrobial PDT with certain concentrations of RB and irradiation with blue light (380–500 nm) for 240 seconds showed an apparent bactericidal effect without damaging the stem cells, such as human embryonic stem cells, odontoblast-like cells, and undifferentiated dental pulp cells [28]. Therefore, compared with other agents investigated thus far, RB constitutes a better PS with better biocompatibility, sensitivity toward light, and KI photocatalysis.

This study was performed to compare the effects of RB antimicrobial PDT with and without KI potentiation on *E. faecalis* bacteria in planktonic and biofilm forms, with the commonly used PS, MB. We explored the possibility of clinical application of KI-potentiated RB antimicrobial PDT using *E. faecalis* as a representative-resistant endodontic bacterium.

MATERIALS AND METHODS

The study was conducted in the microbiology and molecular biology facilities at Peking University School of Stomatology, Beijing, China. Experiments with human dentin slices and stem cells from the apical papilla (SCAPs) were approved by the Institutional Review Board of Peking University School of Stomatology (PKUSSIRB-201944049).

Preparation of *E. faecalis*

E. faecalis strain ATCC 29212 was used in all microbiological procedures. Single colonies were suspended in 5 ml of brain-heart infusion (BHI) broth and grown overnight in a shaker incubator at 120 rpm under aerobic conditions at 37°C. Aliquots of 1 ml of the overnight suspension were incubated in fresh BHI for 4–6 hours at 37°C until they reached a mid-log phase. Cell concentrations were estimated by measuring the optical density at 630 nm (optical density of $0.1 = 10^8$ colony-forming units/ml). The bacterial suspension was centrifuged, washed, and resuspended in phosphate-buffered saline (PBS; Solarbio, Beijing, China) for the *in vitro* experiments.

Photosensitizers and Lasers

MB and RB (both from Sigma-Aldrich, St. Louis, MO) in powder form were used to prepare stock solutions with concentrations of 1 mM (MB) and 5 mM (RB) in sterile PBS, and were filtered using 0.22 μM syringe filters (Corning, Corning, NY) to eliminate any bacterial contamination. The filtered stock solutions were stored in the dark at 4°C for no more than 2 weeks before use. A 200 mM stock solution of KI (Amresco, Washington, DC) was prepared in sterile PBS and stored at room temperature for no more than 2 weeks before use.

A diode laser system capable of producing wavelengths of 660 and 565 nm (Thorlabs, Newton, NJ) was used in continuous wave mode (Fig. 1) with an output laser power of 60 mW/cm^2 that resulted in the influence of 6 J/cm^2 for an irradiation interval of 100 seconds, as measured by a power meter (Thorlabs). Details and specific parameters regarding each protocol are shown in Table 1. The radiation distance from the tip of the diode laser to the bottom of the multi-well plates was 1 cm, and the beam diameter was 4 cm.

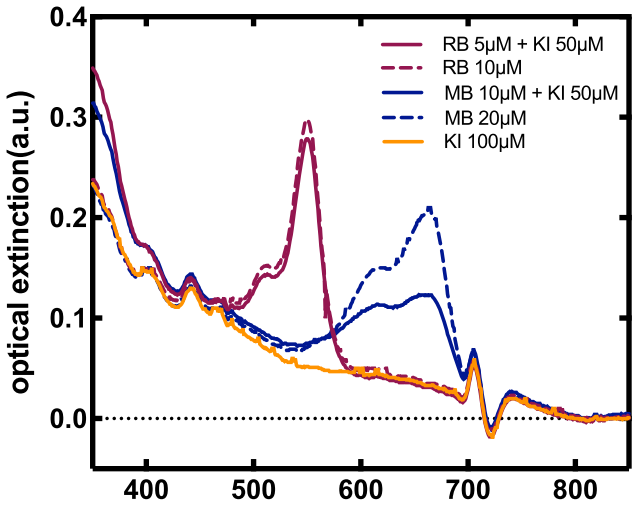

Fig. 1. The absorption spectrum of methylene blue (MB) and rose bengal (RB) with or without potassium iodide (KI) with peak absorptions around 660 and 550 nm, respectively.

PDT Experiments on *E. faecalis* Bacteria in Planktonic Form

Ninety six-well plates (Corning) were used in the experiments because the diameter of the wells matched the radiation beam diameter; this maximized the irradiated surface covered. Bacteria were suspended in sterile PBS (pH 7.2). To verify the relationship between light doses and antibacterial effect, we initially compared the microbial killing with increasing light doses (0–9 J/cm^2) when KI (100 mM) was added to the mixture of *E. faecalis* incubated with the appropriate concentration of MB/RB (0.3 μM MB or 0.05 μM RB) and then illuminated with red/green light. The PS and KI concentration ranges determined in the pilot study [27] were also selected to determine the extents of the PDT effects at different concentrations, yielding the following four concentration ranges: MB, 0.1–20 μM; RB, 0.05–10 μM; KI, 0–100 mM. Our pilot studies showed that using light, KI, or RB/MB PS alone without illumination did not inhibit bacterial growth. The experiments were carried out with the following experimental groups shown as follows for each of the abovementioned concentration ranges.

(1) PS concentration (μM) ranges with the addition of KI (100 mM) (6 J/cm^2):MB concentrations: 0.1 μM, 0.2 μM, 0.3 μM, 0.4 μM;RB concentrations: 0.05 μM, 0.1 μM, 0.15 μM, 0.2 μM.
(2) PS concentration (μM) ranges without the addition of KI (6 J/cm^2):MB concentrations: 5 μM, 10 μM, 15 μM, 20 μM;RB concentrations: 2.5 μM, 5 μM, 7.5 μM, 10 μM.
(3) Fluence (J/cm2) ranges for MB (0.3 μM) and RB (0.1 μM) with the addition of KI (100 mM):Fluences: 0, 6, 12, 18 J/cm^2.
(4) KI concentration (mM) ranges for MB (0.4 μM) and RB (0.2 μM):

KI concentrations: 0 μM, 25 μM, 50 μM, 75 μM, 100 μM.

Following the addition of the PS, the plates were incubated for 10 minutes at 37°C to allow the uptake of PS into bacterial cells. KI was added immediately before laser irradiation. The same procedure was performed for different irradiation times. After laser treatment, aliquots of 100 μl were taken from each experimental group and serially diluted in PBS, up to a dilution factor of 10^{-6}, and plated using the spot test protocol [29] (10 μl) in triplicate on BHI agar (90 × 90 mm). The plates were incubated at 37°C for 24 hours, followed by enumerations of colony-forming units.

TABLE 1. Details and Specific Lighting Parameters

Laser	Red	Green
Wavelength (nm)	660	565
Irradiance (mW/cm^2)	60	60
Pre-irradiation time	10 minutes	10 minutes
Irradiation time (s)	100	100
Fluence (J/cm^2)	6	6
Photosensitizer	MB	RB

MB, methylene blue; RB, rose bengal.

PDT Experiments on *E. faecalis* Biofilm

Confocal laser scanning microscopic (CLSM) analysis. *E. faecalis* was grown on glass-bottomed plates (Nest Biotechnology, Wuxi, China). 1 ml *E. faecalis* (1×10^8 CFU/ml) was added into the plates and refreshed every day to form biofilms cultured for 3 weeks. For the sake of simplicity, sets with the most active groups with the lowest concentrations of PSs were used. PBS and 1.5% NaClO were added to test as the negative control group (group 1) and the positive control group (group 2) separately. The control and experimental groups were established as follows:

Group 1: PBS (negative control group);
Group 2: 1.5% NaClO (positive control group);
Group 3: MB (0.4 μM)-PDT;
Group 4: MB (0.4 μM)-PDT + KI;
Group 5: MB (20 μM)-PDT;
Group 6: RB (0.2 μM)-PDT;
Group 7: RB (0.2 μM)-PDT + KI;
Group 8: RB (10 μM)-PDT.

The illumination parameters were as follows with considerations of the limitations of the equipment and time of the clinical operation: the PS was incubated for 10 minutes, irradiated with lasers at wavelengths of 660 nm/565 nm for 100 seconds, using an output power of 60 mW/cm^2; the illumination energy was 6 J/cm^2. The biofilm was washed with 0.9% saline solution and stained with a LIVE/DEAD BacLight Bacterial Viability Kit containing SYTO9 and propidium iodide, in accordance with the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA). The samples were visualized by confocal laser scanning microscopy.

Preparation of human dentin slices. Human dentin slices were prepared as previously described [30]. The dentin

slices were cut into cross-sections 1 mm thick with a diamond line saw (STX-402; Shenyang Kejing Auto-Instrument Co., Ltd., Kejing, China). The dentin slices were treated with 17% ethylenediaminetetraacetic acid for 5 minutes, followed by 3% NaClO for 5 minutes. After the slices had been rinsed in sterile water for 10 minutes, they were autoclaved at 121°C for 30 minutes.

Scanning electron microscopy visualization of *E. faecalis* biofilms. Scanning electron microscopy images were taken to observe the destruction of 3-week-old *E. faecalis* biofilms on human dentin slices, following PDT treatments. The negative control group, the positive control group, and the experimental groups in this portion (groups 1–8) were established as CLSM analysis. Besides, a blank control group (group 0, with no contamination) was set up to confirm the biofilm formation.

All teeth were instrumented by the same investigator. The samples were fixed with 2.5% glutaraldehyde (Sigma-Aldrich) and 4% paraformaldehyde, then washed with PBS. The biofilms were dehydrated using increasing concentrations of ethanol (30%, 50%, 70%, 80%, 90%, and 100% for 15 minutes each). Specimens were mounted on aluminum stubs with double-sided adhesive tape, coated by gold sputtering, and examined by field emission scanning electron microscopy (SU8018; Hitachi, Ibaraki, Japan).

Biosafety Assessment in Cell Culture of SCAPs

Isolation and culture of SCAPs. The apical papillae were gently separated, minced, and digested with 3 mg/ml type I collagenase (Sigma-Aldrich) and 4 mg/ml dispase (Sigma-Aldrich) for 1 hour at 37°C. Single-cell suspensions obtained using a 70 μm strainer (Falcon, BD Biosciences, San Jose, CA) were seeded and cultured in alpha minimal essential medium (GIBCO-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (GIBCO-BRL), 100 U/ml penicillin, and 100 μg/ml streptomycin (Hyclone, Logan, UT) in a humidified incubator under an atmosphere of 5% CO_2 at 37°C. SCAPs from passage 3–5 were used for experiments.

Cell proliferation assay. SCAPs were seeded in 96-well culture plates (5×10^3 cells/well) and preincubated in alpha minimal essential medium containing 10% fetal bovine serum and antibiotics for 24 hours. The medium was replaced with PSs in the experimental groups. To evaluate the minimum effect on cell viability, the concentrations were chosen based on the minimum bactericidal concentration in the previous bacterial experiments. The negative control group, the positive control group, and the experimental groups in this portion (groups 1–8) were established as CLSM analysis. Besides, the KI control group (group 9) was set up.

Cells were illuminated as described above and replaced with the standard medium afterward. The concentrations were chosen based on the minimum bactericidal concentration in the previous bacterial experiments to minimize the unfavorable effect on cell viability. After 0, 1, 3, and 5 days, cell proliferation was evaluated using a cell counting kit-8 (CCK-8; Dojindo, Kumamoto, Japan), in accordance with the manufacturer's instructions, with three replicates.

Dentin Staining Test

Dentin disks were placed on 24-well plates, followed by the addition of a total volume of 400 μl of PS and KI in the wells. The final concentrations of the different wells are shown as follows: MB (20 μM)-PDT; MB (20 μM)-PDT + KI; MB (0.4 μM)-PDT + KI; RB (10 μM)-PDT; RB (10 μM)-PDT + KI; RB (0.2 μM)-PDT + KI. Plates containing the PSs, with or without KI, were incubated for 10 minutes at 37°C, followed by illumination (0, 6, 12, 18 J/cm^2). Images were taken before and after illumination.

Statistical Analysis

Colony-forming unit counts were log-transformed for statistical analysis. Statistical significance was analyzed by a one-way analysis of variance. In all analyses, differences with $P < 0.05$ were considered to indicate statistical significance ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

RESULTS

E. faecalis Minimum Bacteriostatic Concentration of PSs in KI-Potentiated MB/RB Antimicrobial PDT

The survival fraction curves shown in Figure 2a verified the light-dependent photochemical process involved in the bactericidal effects. The survival fraction curves obtained with different concentrations of KI (Fig. 2b) and of MB or RB concentrations, with and without the addition of 100 mM KI, showed that the addition of KI produced a 4–6 log increase in bacterial killing effect against *E. faecalis*. KI showed the most exceptional enhancement effect at 100 mM. The minimum bactericidal PS concentrations for suspended *E. faecalis* in the MB-PDT (Fig. 2d), MB-PDT + KI (Fig. 2c), RB-PDT (Fig. 2d), and RB-PDT + KI groups (Fig. 2c) were 20, 0.4, 10, and 0.2 μM, respectively.

KI-Potentiated MB/RB-Antimicrobial PDT Disrupts *E. faecalis* Biofilm

Reduction of *E. faecalis* bacteria in biofilm form, observed by confocal laser scanning microscopy. As shown in Figure 3 and Table 2, SYTO9/PI staining showed disruption of the *E. faecalis* biofilm in the MB (20 μM)-PDT group (group 5) ($P = 0.4014$), RB (10 μM)-PDT group (group 8) ($P = 0.1663$) with no significant difference between these two groups ($P = 0.9565$). MB (0.4 μM)-PDT + KI group (group 4) ($P = 0.0015 < 0.01$) and RB (0.2 μM)-PDT + KI group (group 7) ($P = 0.0418 < 0.05$) showed lower bactericidal rates than the positive control (NaClO) with no inter-group difference ($P = 0.3766$). PBS was used as a negative control for comparison with the other experimental groups; it showed no effect on the bacterial growth.

Disruption of *E. faecalis* biofilm morphology, determined by scanning electron microscopy.

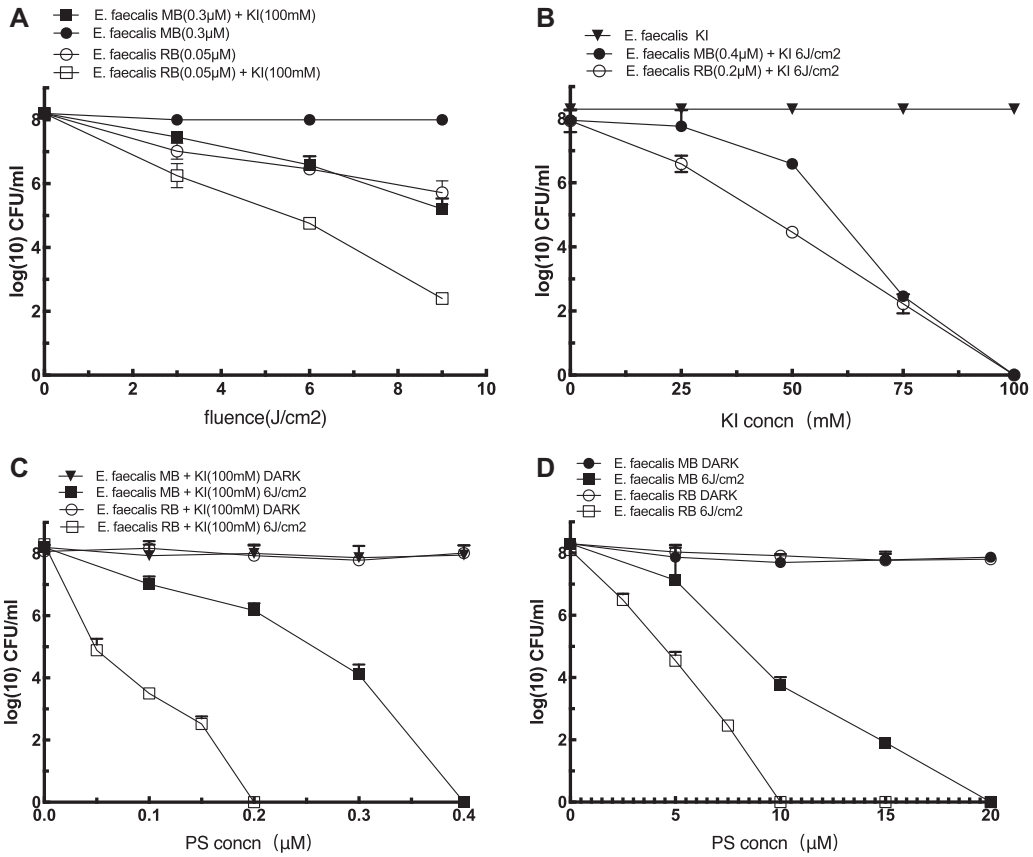

Fig. 2. Survival fraction curves against *Enterococcus faecalis* incubated for 10 minutes with low concentrations of 0.3 μM MB or 0.05 μM RB, with and without the addition of 100 mM KI, and increasing doses of light (0–9 J/cm^2) (**A**); with different concentrations of KI (0–100 mM) (**B**); and with a range of concentrations of MB or RB, with and without the addition of 100 mM KI (**C** and **D**). MB, methylene blue; RB, rose bengal; KI, potassium iodide.

As shown in Figure 4, in the blank control group (group 0), dentinal tubule openings were visible without a smear layer. In the infected control group (group 1), after 3 weeks of *E. faecalis* biofilm formation, the surface of the dentin slice was overlaid by a thick biofilm. Bacterial clusters bound to the canal surface, with bacterial cells extending into dentinal tubules. In the positive control group (1.5% NaClO, group 2), the surface of intertubular dentin was clean without bacterial colonization; however, residual bacteria remained in dentinal tubules. In antimicrobial PDT with MB (20 μM) (group 3) (Fig. 4f) and RB (10 μM) (group 6) (Fig. 4h), bacterial membranes were damaged with some residual bacteria in the dentinal tubules, similar to the positive control group. Surprisingly, in antimicrobial PDT with RB (10 μM) (group 8) (Fig. 4i), although the surface of intertubular dentin was less clean than in the positive control group, a significant reduction was observed in terms of bacteria in dentinal tubules, with the lowest amount in dentinal tubules within the treated samples.

Effect of KI-potentiated MB/RB antimicrobial PDT on the proliferation of SCAPs

As shown in Figure 5, antimicrobial PDT with MB (20 μM) and RB (10 μM) alone or MB (0.4 μM) plus KI (100 mM) and RB (0.2 μM) plus KI (100 mM) killed a subset of the cells, but the SCAPs remained viable. In contrast, very few live cells remained after treatment with 1.5% NaClO.

Dentin Staining Test

Blue staining of dentin could be observed in disks treated with MB-PDT at 20 μM. More severe pink staining was observed on disks treated with RB-PDT at 10 μM. The degree of staining was reduced when dentin was treated with RB PDT at 10 μM potentiated with KI (100 mM); however, some orange staining remained. There was no visible staining of dentin treated with MB (20 μM)-PDT + KI, MB (0.4 μM)-PDT + KI, or RB (0.2 μM)-PDT + KI (Fig. 6).

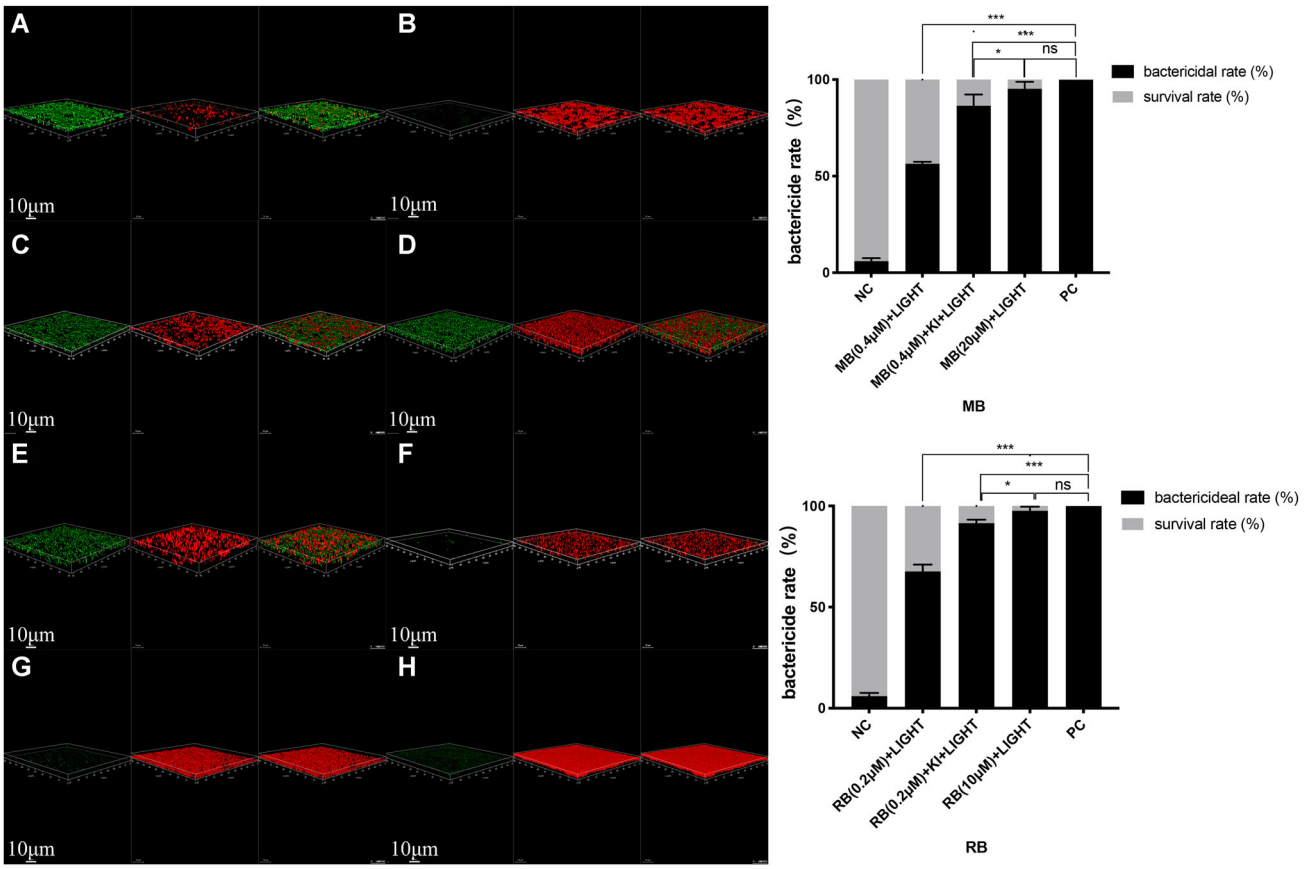

Fig. 3. *Enterococcus faecalis* biofilms were treated with (**A**) negative control (PBS) (group 1), (**B**) positive control (NaClO) (group 2), (**C**) MB (0.4 μM)-PDT (group 3), (**D**) RB (0.2 μM)-PDT (group 6), (**E**) MB (0.4 μM)-PDT + KI (group 4), (**F**) RB (0.2 μM)-PDT + KI (group 7), (**G**) MB (20 μM)-PDT (group 5), and (**H**) RB (10 μM)-PDT (group 8). *E. faecalis* biofilms were stained with a LIVE/DEAD staining kit. The *E. faecaliss* biofilms were visualized by confocal laser scanning microscopy: Green (SYTO9; live cells); Red (propidium iodide; dead cells). ANOVA, analysis of variance; KI, 100 mM potassium iodide; MB, methylene blue; RB, rose bengal. The areas of bacterial aggregates were analyzed using ImageJ software. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, Ns, not significant. The results shown are representative of three independent experiments. Statistical comparisons were carried out by one-way ANOVA.

DISCUSSION

Antimicrobial PDT has a number of unique advantages in dental pulp treatment. First, PDT shows better bactericidal effects on deeply embedded bacteria [31], especially with regard to biofilm destruction [14,32,33]. In addition, within a specific concentration range, the cytotoxicity of PDT is weaker than that of NaClO, which is the most commonly used root canal disinfectant [34]. This study provided

TABLE 2. Bactericidal Rates and Survival Rates of Bacteria in Biofilm Form in Various Groups

Group (No.)	Bactericidal rate (%)	Survival rate (%)
Negative control (PBS) (1)	6.02	93.98
Positive control (NaClO) (2)	99.99	0.01
MB (0.4 μM)-PDT (3)	56.43	43.57
MB (0.4 μM)-PDT + KI (4)	86.50	13.50
MB (20 μM)-PDT (5)	95.27	4.733
RB (0.2 μM)-PDT (6)	67.66	32.34
RB (0.2 μM)-PDT + KI (7)	91.50	9.50
RB (10 μM)-PDT (8)	97.58	2.42

MB, methylene blue; PBS, phosphate-buffered saline; PDT, photodynamic therapy; RB, rose bengal.

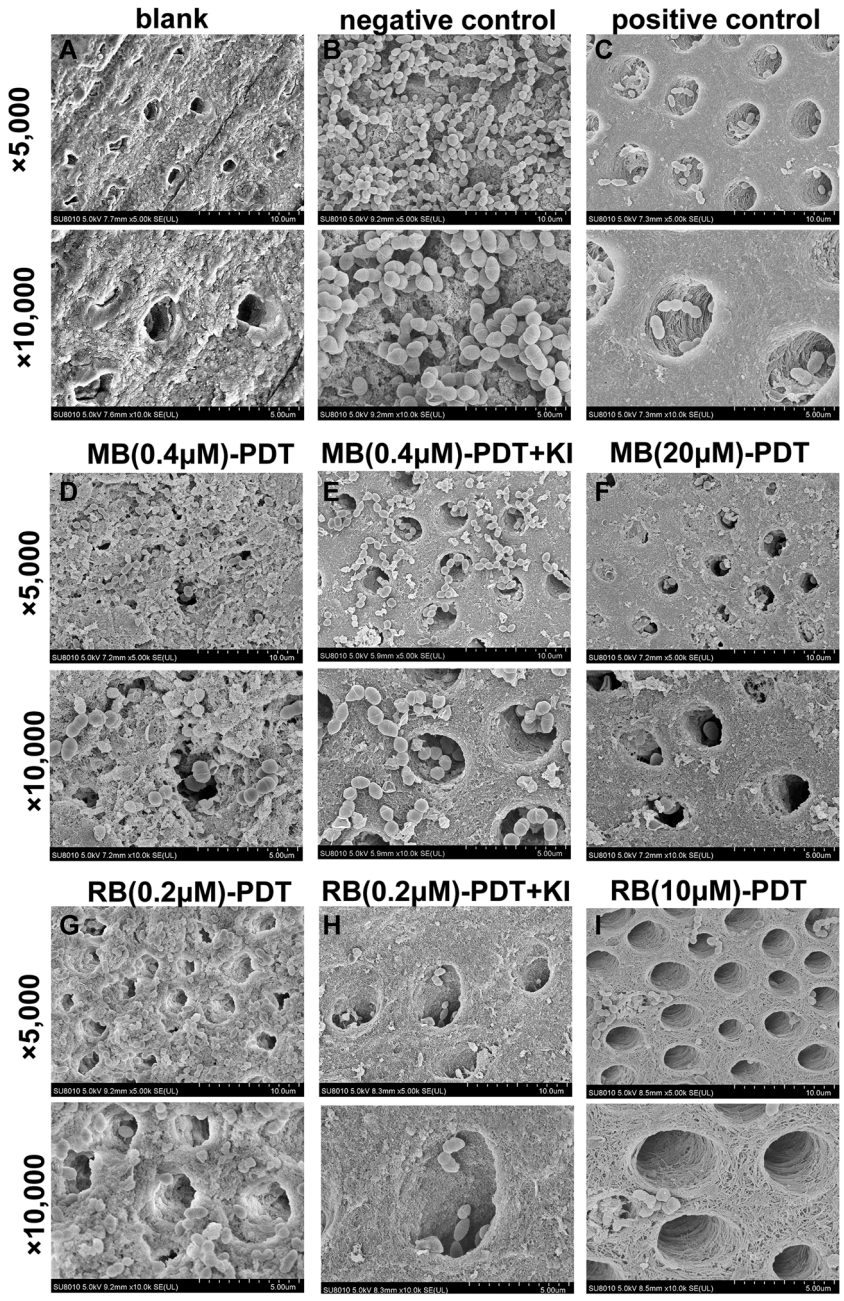

Fig. 4. Scanning electron microscopy images of *Enterococcus faecalis* biofilms after treatment, KI, 100 mM potassium iodide; MB, methylene blue; RB, rose bengal: (**A**) blank control (no biofilm) (group 0), (**B**) negative control (PBS) (group 1), (**C**) positive control (1.5% NaClO) (group 2), (**D**) MB (0.4 μM)-PDT (group 3), (**E**) MB (0.4 μM)-PDT + KI (group 4), (**F**) MB (20 μM)-PDT (group 5), (**G**) RB (0.2 μM)-PDT (group 6), (**H**) RB (0.2 μM)-PDT + KI (group 7), and (**I**) RB (10 μM)-PDT (group 8). PBS, phosphate-buffered saline; PDT, photodynamic therapy.

additional therapeutic options by comparing the antimicrobial PDT bactericidal effects of two PSs, with and without potentiation by KI.

KI is a biocompatible compound that can be easily acquired, and is both safe and effective, as reported by the US FDA [35]. The results of an in vitro study demonstrated that 10–100 mM KI is not toxic to microbial cells [36] or human fibroblasts [25,27] after 30 minutes of incubation. Hamblin et al. [37] reported that the concentration of KI was critical for determining the exact mechanism underlying the bactericidal effect—at low concentrations up to 10 mM KI, the effect is mainly due to iodine radicals; the antimicrobial effect is mediated mainly by free iodine at higher KI concentrations up to

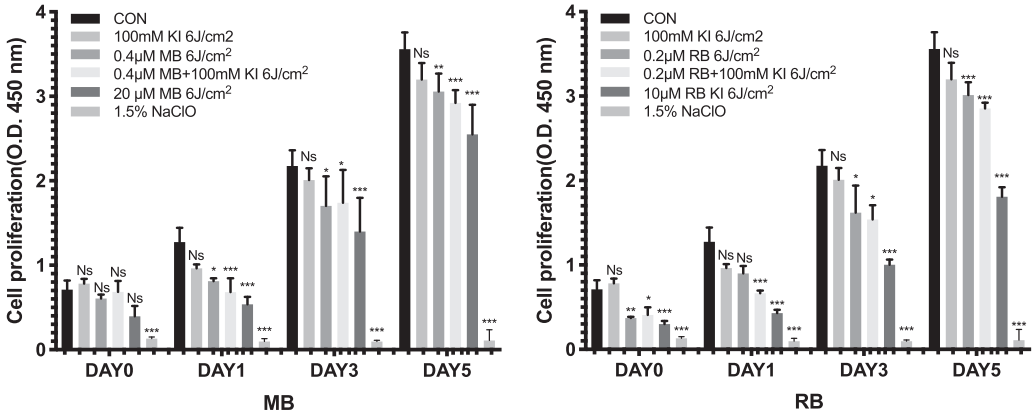

Fig. 5. Cell proliferation was evaluated by CCK-8 assay. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, Ns, not significant (the experimental groups and the positive control group were compared with the negative control group). Error bars: SD. Statistical comparisons were carried out by two-way ANOVA. ANOVA, analysis of variance; CCK-8, cell counting kit-8; KI, 100 mM potassium iodide; MB, methylene blue; OD, optical density; RB, rose bengal; SCAP, stem cells from the apical papilla.

100 mM (or even up to 400 mM). If the bacterial cells were added to the photoactivated photosensitizer plus KI solution, with some stable free iodine as I_2 and/or HOI produced, no bacterial killing was observed [25]. On the contrary, short-lived iodine radicals and peroxyiodide have been reported to play a major role in bactericidal effects [37]. Therefore, we chose 100 mM KI because it could provide the most substantial iodine radical levels. We explored the lowest effective bactericidal concentration of PSs under conditions of potentiation with 100 mM KI, to reduce both the cytotoxicity of PSs and the tooth discoloration effect.

The PDT results against planktonic *E. faecalis* bacteria in the present study would be useful for explorations of the minimum bactericidal concentrations of PSs; they also provided insights into the influences of light parameters and KI concentrations on the effectiveness of PDT (Fig. 2). The enhancing effect of KI observed in the present study corresponded to the effects reported by Vecchio et al. [25] and Wen et al. [24]. At the chosen light dose (6 J/cm^2), based on clinical and mechanical considerations, MB (0.4 μM) or RB (0.2 μM) plus KI (100 mM) and light eradicated *E. faecalis*. Furthermore, the same bactericidal effect was observed with 20 μM MB or 10 μM RB, when KI was omitted.

In general, *in vitro* experiments can be used to select the appropriate antimicrobial PDT parameters with respect to planktonic bacteria. However, it is difficult to kill pathogenic bacteria organized in a biofilm within the root canal system [38]. The biofilm formed by *E. faecalis* is closely associated with primary and secondary endodontic infections [39]. In previous studies, the incubation times of *E. faecalis* bacteria in biofilm form have differed widely, ranging from 24 hours [40] to 48 hours [41], 72 hours [42], 5 days [43], 7 days [44], 21 days [45,46], or 30 days [47,48] on dentin slices or *ex vivo* root canal models. In the present study, the dentin slices were inoculated with *E. faecalis* for 21 days to allow structurally mature biofilm formation on dentin, in agreement with the previous research [45,46]. A period of 21 days was sufficient to promote biofilm formation and adhesion with a sufficient biofilm density. When the biofilm is formed, antimicrobial PDT is less likely to be capable of completely killing the bacteria [14,33,49]. KI-potentiated PDT has been shown to significantly prevent bacterial adhesion and biofilm formation, compared with normal PDT at the same or higher PS concentrations (Fig. 3).

A notable difference between the present study and similar previous studies reported in the literature is that we utilized standardized dentin slices and glass-bottomed dishes to cultivate biofilm bacteria, rather than an *ex vivo* model. Some biofilm-related studies applying *ex vivo* models have suggested that it is difficult for PDT to achieve an effect similar to that of conventional root canal disinfection [49,50]. In such instances, the antibacterial effect can be influenced by the lighting depth and ability of the lighting device to adapt to the root canal structure. Irradiation with different MB concentrations and irradiances for 0.5–1.5 minutes was shown to eradicate up to approximately 98–99% of *E. faecalis* when saline was used as an irrigation agent [42,43]. In the present study, we used a high-power diode laser at 60 mW/cm^2 for 100 s in continuous mode as PDT, which resulted in a significant reduction of *E. faecalis* bacteria in biofilm form. Our study showed that PDT activated by MB and RB with relatively low concentrations, or with KI potentiation, can achieve an effect similar to that of conventional root canal disinfection (Figs. 3 and 4). Therefore, it is necessary to optimize the light equipment for pulpal treatment based on the findings in this study. However, this in vitro study only used a single bacterial biofilm, whereas the biofilm encountered in human patients is composed of a diverse range of resident bacteria. Nonetheless, our results would be beneficial for clinicians to gain additional insights into new disinfection procedures for use in endodontics.

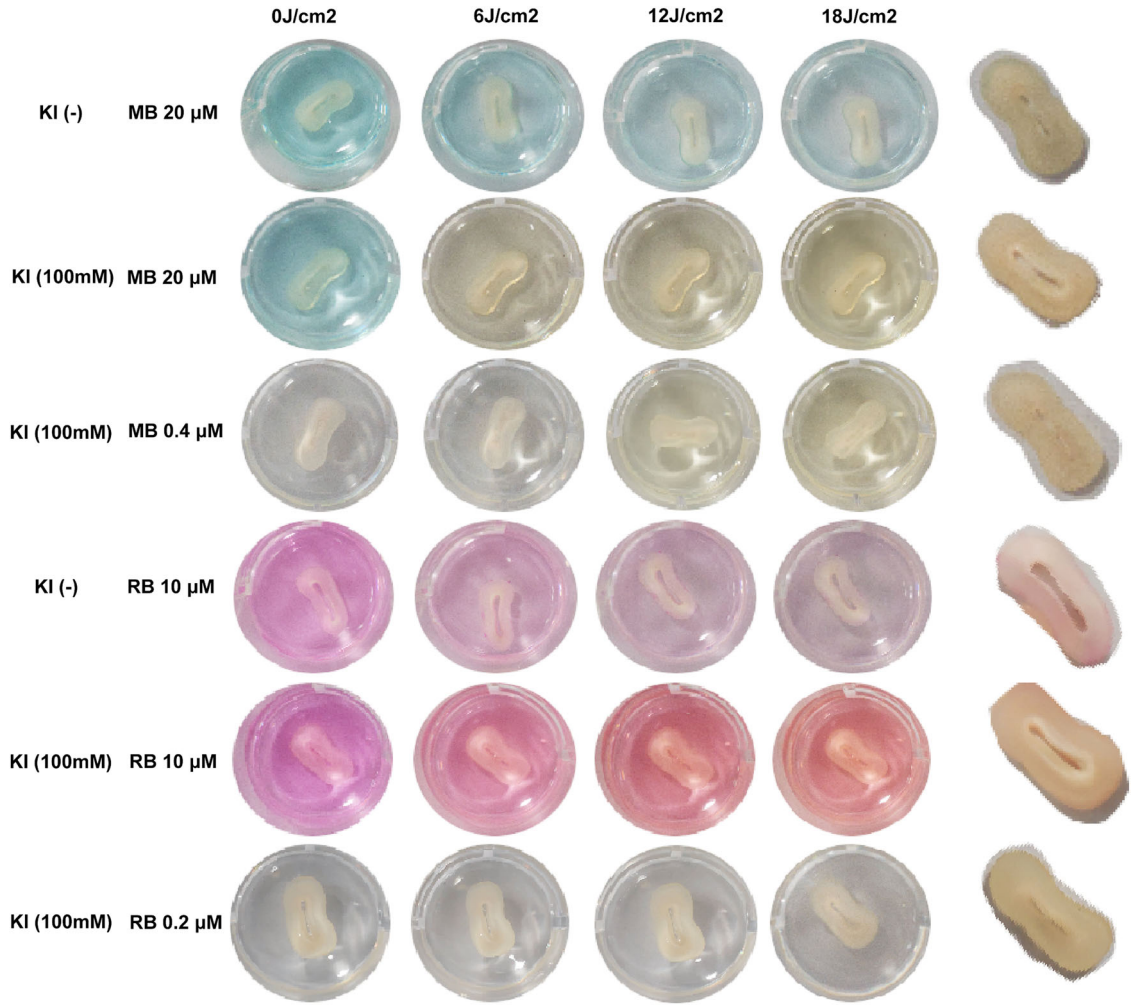

Fig. 6. Dentin conditioning with PSs caused concentration-dependent dentin staining. The colors of the solution were light at the MB/RB concentrations used in the experimental groups in this study. Photographs show representative dentin disks conditioned with MB and RB, with or without KI, before and after irradiation at a dose of 0, 6, 12, 18 J/cm^2. KI, 100 mM potassium iodide; MB, methylene blue; PS, photosensitizer agent; RB, rose bengal.

The main objective of endodontic treatment in affected teeth is to eradicate microorganisms in the root canal and create an internal microenvironment conducive to the proliferation and differentiation of stem cells [51]. The disinfection protocol should be SCAP-friendly, as these cells with osteoblast differentiation ability are important for root development [52]. The positive control group treated with 1.5% NaClO showed eradication of *E. faecalis* in the planktonic form; however, this treatment adversely affected the proliferative capacity of SCAPs. Therefore, the maintenance of periapical health after sterilization of infected root canal systems cannot be achieved with the current irrigation protocol [53]. After PDT, although some cells were inactivated, the remaining cells retained good proliferative capacity (Fig. 5).

The key to the clinical applicability of PDT in endodontic treatment is ensuring resolution of the staining problem and maintenance of the bactericidal effect. Yuan et al. [27] reported that KI accelerated the photobleaching of MB. In the present study, the potentiation of KI lightened the color of PSs at the concentrations described in the text, especially for RB (10 μM) antimicrobial PDT. Even with PSs at higher concentrations, the addition of KI could further provide the possibility of antimicrobial PDT application in endodontic treatment.

This study explored a new therapeutic approach for endodontic disinfection using KI-potentiated PDT for enhanced bacterial killing. RB antimicrobial PDT seemed to be more responsive to KI potentiation than MB antimicrobial PDT on *E. faecalis* bacteria in planktonic and biofilm forms. There is a therapeutic balance regarding the elimination of biofilm for root canal disinfection and maintenance of the viability of stem cells required for pulp regeneration. Wang et al. [54] reported that green (540 nm) wavelengths had positive effects on osteoblastic differentiation of human adipose-derived stem cells.

Therefore, green light-activated PDT may have better biocompatibility with fewer adverse impacts on stem cells. Further research on biological safety is needed.

CONCLUSION

MB and RB antimicrobial PDT at different concentrations with or without KI could achieve sterilization effects comparable with those of 1.5% NaClO while causing few adverse effects on SCAPs. An additive effect of KI on antimicrobial PDT activated by MB and RB was observed in the eradication of *E. faecalis*. KI helps to avoid staining problems associated with high concentrations of PSs. A combination of KI and antimicrobial PDT may be a useful alternative to conventional disinfection methods in endodontic treatment. Further studies are needed with a focus on the optimization of the laser parameters. The efficacy of KI-potentiated antimicrobial PDT should be further validated in animal models and clinical studies.

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