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Potassium iodide enhances the photobactericidal effect of methylene blue on *Enterococcus faecalis* as planktonic cells and as biofilm infection in teeth

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Abstract

Objective—To explore the effectiveness, biosafety, photobleaching and mechanism of antimicrobial photodynamic therapy (aPDT) using methylene blue (MB) plus potassium iodide (KI), for root canal infections.

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Conflicts of interest

MRH declares the following potential conflicts of interest. Scientific Advisory Boards: Transdermal Cap Inc, Cleveland, OH; BeWell Global Inc, Wan Chai, Hong Kong; Hologenix Inc. Santa Monica, CA; LumiThera Inc, Poulsbo, WA; Vielight, Toronto, Canada; Bright Photomedicine, Sao Paulo, Brazil; Quantum Dynamics LLC, Cambridge, MA; Global Photon Inc, Bee Cave, TX; Medical Coherence, Boston MA; NeuroThera, Newark DE; JOOVV Inc, Minneapolis-St. Paul MN; AIRx Medical, Pleasanton CA; FIR Industries, Inc. Ramsey, NJ; UVLRx Therapeutics, Oldsmar, FL; Ultralux UV Inc, Lansing MI; Illumiheal & Petthera, Shoreline, WA; MB Lasertherapy, Houston, TX; ARRC LED, San Clemente, CA; Varuna Biomedical Corp. Incline Village, NV; Niraxx Light Therapeutics, Inc, Boston, MA. Consulting; Lexington Int, Boca Raton, FL; USHIO Corp, Japan; Merck KGaA, Darmstadt, Germany; Philips Electronics Nederland B.V. Eindhoven, Netherlands; Johnson & Johnson Inc, Philadelphia, PA; Sanofi-Aventis Deutschland GmbH, Frankfurt am Main, Germany. Stockholdings: Global Photon Inc, Bee Cave, TX; Mitonix, Newark, DE.

Methods—Different combinations and concentrations of MB, KI and 660 nm LED light were used against *E. faecalis* in planktonic and in biofilm states by colony-forming unit (CFU), confocal laser scanning microscopy (CLSM), scanning electron microscopy (SEM). Human gingival fibroblasts (HGF) were used for safety testing by Cell Counting Kit-8 (CCK8) and fluorescence microscopy (FLM). The photobleaching effect and mechanisms were analyzed.

Results—KI could not only enhance MB aPDT on *E. faecalis* in both planktonic and biofilm states even in a hypoxic environment, but also produced a long-lasting bactericidal effect after end of the illumination. KI could accelerate photobleaching to reduce tooth staining by MB, and the mixture was harmless for HGFs. Mechanistic studies showed the generation of hydrogen peroxide and free iodine, and iodine radicals may be formed in hypoxia.

Conclusion—aPDT with MB plus KI could be used for root canal disinfection and clinical studies are worth pursuing.

Keywords

antimicrobial photodynamic inactivation; potassium iodide; methylene blue; *Enterococcus faecalis*; root canal disinfection

1 Introduction

Dental pulp infections caused by bacteria are an important cause of the failure of root canal treatments [1]. *Enterococcus faecalis* is one of the most important bacteria that cause persistent dental pulp infections. It can colonize the surface of gutta-percha tips and survive in anaerobic or nutrient-poor environments[2]. These bacteria are resistant to a variety of antibiotics and disinfectants [3–5] and can penetrate into dentin tubules[6], which makes it difficult to be completely removed or killed by mechanical instruments or antiseptics.

Sodium hypochlorite (NaClO) is a powerful disinfectant used in root canal irrigation[7, 8]. On one hand, commonly used concentrations of NaClO solution can kill (> 3 $\log_{10}$) *E. faecalis in vitro* [9] and some studies have shown that the bactericidal ability of NaClO is more powerful than that of recently developed disinfectants[10, 11]. On the other hand, sodium hypochlorite may be too toxic for normal cells [12–15]. Root tip leakage or dripping of NaClO onto the gingival tissues can cause pain and burns[16–19].

Recently, antibiotic photodynamic therapy (aPDT) has been advocated for root canal treatment [20, 21] although it has not yet received regulatory approval. However there have been several clinical trials of aPDT in endodontics using MB-PDT [22, 23] and also using other PS [24]. aPDT is a method of killing microorganisms using a photosensitizer (PS) activated by a specific wavelength of light. Photoexcited PS produce reactive oxygen species (ROS) in the presence of oxygen [25]. Unlike antibiotics, which act against a single target in bacteria, ROS can damage multiple biomolecules, such as lipids, proteins and nucleic acids. Therefore, aPDT is not susceptible to the development of drug resistance [26, 27]. Studies have shown that aPDT can penetrate as deep as 600 μm into dentin tubules compared to NaClO (only 100 μm) [28].

Different photosensitizers have varied affinities to different types of microbial cells. Methylene blue (MB) is used in clinical dental treatment, with an absorption peak at 668 nm [29]. MB is a positively charged hydrophilic PS with a low molecular weight[21]. It can bind to a variety of Gram-positive and Gram-negative oral bacteria[30, 31], and has low toxicity to human gingival fibroblasts and osteoblasts[32]. Researchers have found that many photosensitizers, like Laserphyrin, Photofrin erythrosine B, crystal violet, rose Bengal, new methylene blue, have much higher phototoxicity towards neutrophils than MB. MB can retain the killing effect of neutrophils against pathogens [33]. Therefore, MB has become one of the promising PS used in the field of stomatology. It has received a Chinese drug license (Chinese Drug Approval Number:H20083164).

However, MB has some drawbacks for root canal treatment including a tooth staining effect. At present, MB concentrations have been employed as high as 0.01% (31.3 μM)[34–36] or higher than 100 μM[37]. On one hand, 0.01% MB can stain the teeth [38, 39], and on the other hand, too high MB concentration can reduce the aPDT effect due to self-quenching of the light[40].

The second, *E. faecalis* can colonize the deeper dentin tubules, where lack of enough oxygen can inhibit the PDT bacterial killing. Therefore, the traditional PDT effect in the hypoxic conditions inside root canals may be significantly decreased[41].

Thus, searching for more effective and safer PDT treatments in root canal infection treatment, is a continuing theme. In 2012, Huang et al [42] accidentally discovered that MB combined with sodium azide (NaN_3), which was expected to quench the bactericidal singlet oxygen, unexpectedly enhanced the PDT killing of *Staphylococcus aureus* and *Escherichia coli* without the involvement of oxygen. In order to avoid the toxic effects of NaN_3 on human cells, the team found that potassium iodide (KI) could also enhance the bactericidal effect in PDT. KI has no antimicrobial effect alone when used from 0 mM to 100 mM with or without 5 J/cm^2 660 nm light [43, 44]. Also, oral acute toxicity values (LD50 in mg/kg) of KI are more than 3000 mg/kg in rats [43]. Oral KI is used to treat threshold thyroid radioactive exposures at a dose of 130 mg for adults and is the treatment of choice for cutaneous fungal infections such as sporotrichosis [45]. The only contraindication is hypersensitivity or allergic reactions.

There have been no studies using aPDT mediated by MB combined with KI in dentistry. The present study looks at the sterilization efficiency of photoactivated MB combined with KI on *E. faecalis* in planktonic and biofilm states. MB photobleaching, biosafety for host cells, activity under hypoxic conditions and mechanisms were studied.

2 Material and methods

2.1 Bacterial cultivation

Bacterial strain of *Enterococcus faecalis* ATCC 29212 *(E. faecalis)* was cultivated on brain heart infusion (BHI, Becton, Dickinson and Company, New York, USA) agar plates in an incubator at 37°C. One single colony was chosen to add to 20 mL liquid BHI medium for overnight shaken culture (120 rpm, 37°C). After that, a 1:100 dilution was enriched for 3–5

hours to reach mid-log phase. The concentration of the *E. faecalis* was $10^{(8)}$ CFU/ml (OD_{630}=0.1) and was centrifuged (9000 g, 2 minutes) and resuspended in PBS for subsequent experiments.

2.2 Light source and photosensitizers

A 660 nm deep red LED light source (Fig. 1A M660L4, Thorlabs, New York, USA) was used to deliver light to cover 2×2 wells (spot size 4 cm in diameter) in 48-well plate. A power meter (Thorlabs, New York, USA) was used to measure the light output power which was set at 50 mW/cm^2. Methylene blue (MB), potassium iodide (KI) were purchased from Sigma-Aldrich (St. Louis MO, USA).

2.3 Tooth preparation

The study protocol was approved by the Ethics committee of Peking University School of Stomatology (Ethics review sheet number: PKUSSIRB-201631102) with patient informed consent. Biofilm were established on teeth without other debris, or organic matter [46]. Briefly, 40 freshly extracted wisdom teeth were selected and kept in 5.25% sodium hypochlorite (NaClO) overnight, soft tissue, debris, and calculus were removed. The teeth were cut both transversely, others vertically by a diamond Wire Saw (STX-202A, Kejing, China) to form dentin blocks under cutting fluids [47]. 5.25% NaClO, 17% Ethylene Diamine Tetraacetic Acid (EDTA) were used for more than 10 minutes to clean the smear layer, then blocks were washed three times by distilled water and sterilized by autoclaving (121°C, 20 minutes).

2.4 aPDT parameter exploration in planktonic cells

The planktonic experiment was chosen for studying the dose-effect relationship of each parameter. In order to optimize the experimental parameters, two of the three factors (KI, MB, and light dosage) were initially fixed based on existing data, to test the dose-response relationship of the other parameter.

Resuspended planktonic *E. faecalis* cells ($10^{(8)}$/ml, 200 μL) in PBS (Solarbio, Beijing, China), were incubated with different concentrations of MB (100 μL) and KI (100 μL) in a 48 well plate in the dark at room temperature. Blocks of 4 wells (2×2) were covered uniformly by the light spot at a power density of 50 mW/cm^2[48]. The dark group in a separate plate was covered with aluminum foil. The incubation time in the literature has varied from 2–30 minutes [40, 49], but we choose 15 minutes as pre-irradiation time to allow the PS to penetrated into the bacterial cells and into the deeper dentinal tubules. After completion of the illumination each sample was placed in a new 96-well plate for serial 10-fold dilution from 10^1 to 10^6 times in PBS. 10 μL of diluted bacteria were streaked horizontally on square BHI agar plates in triplicate. Each group was repeated at least 3 times. The plates were placed in CO_2 incubator for around 18 hours at 37°C. Colony-forming units were used to calculate the survival fraction of bacteria.

Varying MB concentration—Considering the staining ability of MB, we wanted to use lower MB concentration but more KI to reduce the staining effect, so KI (100 mM) was used to find a more suitable MB dosage compared to that used by other researchers[44]. For light,

we also needed a shorter illumination time with 50mw/cm^2 light, so we chose 6 J/cm^2 [43] (2minutes) to test the sterilization concentration of MB needed, because many researchers have used 5–30 J/cm(2)[50–52] as the light dosage in PDT treatment. Thus, to verify whether the addition of KI could potentiate MB against *E. faecalis* in dark and light, a series of MB concentrations were tested (0, 0.1, 0.2, 0.3, 0.4, 1, 2 μM) with or without KI (100 mM) [48]and with or without 6 J/cm^2 (50 mw/cm^2, 2 minutes) 660 nm LED light[50–52]. (Table 1)

Varying KI concentration—In order to find the dose-effect relationship of KI to potentiate *E. faecalis* sterilization, we tested different combinations of MB (0.1 and 0.4 μM, which was ineffective on its own but was effective with 100 mM KI and 6J/cm^2 light respectively, according to the results above), combined with KI (0, 10, 25, 50, 100 mM) and red light (6 J/cm^2) as shown in Table 2. In previous studies, the addition of KI (10 mM) to MB had been shown to give a good antibacterial effect [44]. In this study we tested a wider range of KI concentrations. (Table 2)

Varying light dosage—To study the dose-effect relationship of light, we tested different combinations of LED energy density. These light dosage parameters were fixed by illumination times (2, 4, 6, 8, 10 minutes) that corresponded to light dosages of 6, 12, 18, 24, 30 J/cm^2 (with 50 mW/cm^2) [43]. In this part, 660 nm LED light (0, 6, 12, 18, 24 J/cm^2) were used to excite MB (0.1~0.4μM) with KI (100mM) as well as MB (0.4 μM) alone. (Table 3)

2.5 Long-lasting effects and effects of hypoxia

Long-lasting killing effect of aPDT—In order to test whether aPDT plus KI could still have a killing effect after stopping illumination, aPDT groups with or without KI were sampled at different times after the end of light delivery. Samples were incubated with with or without KI (100 mM) for 15 minutes, then exposed to 6 J/cm^2 light [44, 53]. Samples took place before light started, and at 0, 5, and 10 minutes after the end of the light (Table 4).

aPDT effect in hypoxia—The deeper dentinal tubules always lack oxygen[54], causing lower efficiency of aPDT. To test whether KI could still enhance the antimicrobial effect of photoexcited MB in the absence of oxygen, MB (0.4 μM), MB (0.4 μM) with KI (100 mM) were incubated with *E. faecalis* for 15 minutes, then exposed to 660 nm LED (6 J/cm^2) either inside or outside an anaerobic incubator (Bacbasic, Shellab Bactron, USA) (Table 5).

2.6 Biofilm sterilization study

Bacterial in the biofilm stage can resist the sterilization effect more than planktonic bacteria [55]. With the 50 mW/cm^2 light, we used a total 30 J/cm^2[43] fluence to sterilize the *E. faecalis* biofilm. Also, we used a higher MB (10 μM)[44] concentration group to enhance the killing effect for biofilms.

To find suitable concentrations of MB + KI + light to kill *E. faecalis* biofilm, 1 mL *E. faecalis* (10(8) CFU/ml) was added into a 6-well-plate and refreshed every 2 days to form

biofilms. After 7 days, biofilm was established in each well and was treated with MB in PBS (0, 0.4, 10 μM), or MB (0.4, 10 μM) plus KI (100 mM) followed by 30 J/cm^2 red light. After light delivery, scrapers were used to collect the biofilm into PBS with ultrasound vibration to disperse the cells before measuring CFU/mL (Table 6)

Dentin block infection and treatment—Dentin blocks were placed into 5 mL EP tubes with *E. faecalis* ($10^{(8)}$ CFU/ml) in 2 mL BHI medium. All samples were incubated in a CO_2 incubator for 21 days and the BHI was refreshed every 2 days.

Before the PDT treatments, dentin blocks were washed lightly with PBS. Each sample was incubated with different concentrations of MB (100 μL) and KI (100 μL) in a 12 well plate for 15 minutes in the dark at room temperature. Then the samples were exposed to 660 nm LED as the group demanded. The experimental group details are shown in Table 7.

Confocal laser scanning microscopy (CLSM) observation—CLSM was used to check the live and dead bacteria immediately after PDT using special stains. Bacteria with unbroken cell membranes are stained fluorescent green by SYTO 9, whereas bacteria with damaged membranes are stained fluorescent red by PI. The dentin blocks were grouped as shown in Table 7. Double distilled water was used to clean the dentin blocks. Each group was immersed in Live/Dead BacLight kit L7012 (Invitrogen, Carlsbad, CA) (PI: 535/617, SYTO 9: 485/498) and incubated for 15 minutes in darkness. Fluorescent images were obtained by confocal scanning fluorescence microscope (Zeiss LSM710, Germany).

Scanning Electron Microscopy Analysis (SEM)—The dentin blocks in the same groups as in the CLSM experiment, were slowly washed by 2.5% glutaraldehyde for fixation at 4°C overnight. After that, each simple was immersed in 30%, 50%, 70%, 80%, 90% alcohol for dehydration over 10 minutes. 100% alcohol dehydration 2 times again. After that, in vacuo metal spraying on each sample as shown in Table 7.

2.7 Photobleaching

In our study, we found that MB (100, 10, 0.4 μM) combined with or without KI (100 mM) displayed pronounced color changes before and after 6 J/cm^2 (Table 8).

2.8 Biosafety evaluation

Cell culture—Human gingival fibroblasts (HGF) were a generous gift from Central Laboratory (Peking University School and Hospital of Stomatology, Beijing, China). HGF were maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibico, Thermofisher, USA) with 10% Fetal Bovine Serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Grand Island, NY, USA), streptomycin (100 μg/ml), penicillin (100 U/ml) at 37°C in 5% CO_2 incubator. Then the cells (5×10^4/well,) passage 3–5 were used for the experiment in 96 well plate (Costar; Corning Inc., NY, USA).

Cytotoxicity test—When the cell confluence was about 80%, MB (2 μM), MB (0.4 μM) plus KI (100 mM) were prepared in DMEM for 3*3 wells in a 96 well plate respectively. The doses for these groups were chosen to correspond to the doses that produced bacterial

eradication in the planktonic studies in 2.4. 2*3 wells were prepared for untreated group and 3 wells for blank groups (no cells). Cells were irradiated at 660 nm wavelength with 6 J/cm^2 dose[44, 53]. After treatment, the cells were washed with PBS and replaced with fresh culture medium. Cell viability in each group was measured by Cell Counting Kit-8 (CCK8, Dojindo, JAPAN) before light. Each well was incubated with 10 μL CCK8, 37 °C for 90 minutes. 90 μL of each well were transferred to a new 96 well plate for Absorbance Microplate Reader (ELX808 Biotek, Vermont, USA) at 450 nm. 3 wells data were used to calculate cell survival rate and expressed as percentage compared with untreated cells (100%). Each experiment was repeated three times. At the same time, 5.25% NaClO was added to test its toxicity to HGF cells.

After CCK-8 test, each well was washed by PBS for three times, and then added LIVE/DEAD Viability /Cytotoxicity Assay Kit (KGAF001, keygen, China), which contains Calcein AM (green florescence, Ex/Em: 495 nm/520 nm) for showing living cells and PI (red florescence, Ex/Em: 530 nm/620 nm) for showing dead cells. Cells were observed under fluorescence microscopy (ECLIPSE Tis, Nikon, Tokyo, Japan). The groups were as Table 9:

2.9 Mechanistic experiments

Starch test for iodine—MB (0.4 μM) and KI (100 mM) were irradiated with 660 nm LED for 0–18 J/cm^2[43]. 50 μL of each sample after illumination was added to 50 μL starch indicator (DI0636–100mL, Leagene, China) and immediately measured by microplate reader (ELx800, Biotek, USA), at 630 nm. Control groups were MB (0.4 μM), KI (100 mM), and PBS alone. Each group was retested at least three times.

Amplex-red assay for H_2O_2 production—Amplex Red (A22188, Invitrogen, USA) was used to detect H_2O_2 produced. H_2O_2 can react with the peroxidase in the kit to oxidize Amplex Red to form resorufin. Detection was performed according to the manufacturer's instructions. The reaction misture contained MB (0.4 μM) combined with KI (100 mM) and irradiated with an increasing fluence of 660 nm LED as above. 50 μM Amplex red reagent in Krebs-Ringer phosphate and 0.1 U/ml horseradish peroxidase (HRP) were added. The incremental fluorescence after the LED light exposure was measured using Enspire Multimode Plate Reader (Perkin Elmer, Waltham, USA) with excitation of 570 nm and emission of 585 nm. Controls were 3 groups with MB (0.4 μM), KI (100 mM), or PBS. Each experiment was performed at least three times.

Nitroblue tetrazolium (NBT) assay for superoxide—20 mM NBT, MB (0.4 μM) and KI (100 mM) were dissolved in PBS; the absorbance of the blue product (distinguishable from MB peak at 660 nm) after increasing LED light exposure as above, was measured using an absorbance microplate reader at 570 nm absorbance. Controls were MB (0.4 μM), KI (100 mM), and PBS alone. Each experiment was performed at least three times. A positive control using C60-fullerene with β-NADH, excited by 375 nm LED light was used to demonstrate the production of superoxide.

2.10 Statistical analysis

Graphs were shown as the mean ± standard error. When necessary, one-way analysis of variance (ANOVA) and student-t tests[56–58] was carried out by SPSS 24.0. (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001).

3 Results

3.1 MB plus KI aPDT parameter exploration

In order to find a suitable parameter of *MB-PDT + KI*, we fixed two of the three factors (KI, MB, and light dosage), according to existing data, in order to test the dose-response relationship of the other parameter.

Figure 2 (A–C) shows that KI could dramatically reduce the concentration of both MB and the light dose necessary for sterilization of *E. faecalis*.

Firstly, when KI (100 mM) [48] was added, the MB concentration could be reduced by 5 times (from 2 μM to 0.4 μM) to produce the same bactericidal effect against planktonic bacteria. The concentrations of MB from 0.1–0.4 μM combined with light could hardly kill any bacteria ($<10^1$ CFU), while with added KI (100 mM) the survival fraction of *E. faecalis* could be decreased to 0.00000001 ($10^{(8)}$ logs of killing). Moreover, MB (0.4 μM) combined with KI (100 mM) and 6 J/cm^2 could fully sterilize *E. faecalis* as efficiently as MB (2 μM) alone. The dark groups showed no obvious killing effect. (Fig. 2A)

The efficiency of the MB+KI aPDT combination was KI concentration dependent (Fig. 2B). When MB (0.4 μM) was excited with 6 J/cm^2 red light, a significant potentiation of 2 $\log_{10}$ was observed upon the addition of KI (50 mM), and complete sterilization (7 $\log_{10}$ potentiation) was seen with KI (100 mM).

As many researchers have used 5–30 J/cm(2)[50–52] as the energy density in aPDT, the addition of KI (100mM) to MB(0.1–0.4μM) aPDT could also dramatically reduce the light dose required for equivalent bacterial killing at different light dosage(6–24 J/cm^2). (Fig. 2C)

3.2 Long lasting killing effect, hypoxia effect and biofilm studies

KI plus MB could produce a long-lasting killing effect beyond the end of the illumination period. If the mixture was incubated for an extra 5 minutes after the end of the light, an extra 3 Log_{10} of killing was observed. If it was incubated for an extra 10 minutes after the light, complete sterilization (8 $\log_{10}$) was obtained. (Fig. 2D)

The addition of KI could preserve some of the antimicrobial killing effect of MB aPDT in an hypoxic environment as shown in Fig. 2E. In the case of MB aPDT alone, the removal of oxygen almost entirely quenched the killing. However, in the presence of added KI, there was still 1–2 $\log_{10}$ of killing remaining in the anaerobic chamber.

The effects of added KI on the killing of *E. faecalis* biofilm, which are known to be considerably more resistant to aPDT than planktonic cells. MB (10 μM) plus KI (100 mM)

and red light could sterilize the biofilm, giving almost complete killing effect compared to MB aPDT alone (Fig. 2F)

3.3 E. faecalis biofilm observed by CLSM

The fluorescence images of the live-dead cells in the biofilm are shown in Figure 3. *(A1-C1)* represent live cells, while ***(A2-C2)*** represent dead cells. ***(A3-C3)*** shows live and dead cells merged. Image J v. 1.80 (available as freeware from http://rsbweb.nih.gov/ij/) was used to measure the percentage of live or dead cells in each image.

Fig. 3 A1–A3 shows the untreated control group, with 20.4% area of live cells (99.5%) and 0.1% area for dead cells (0.5%).

Fig. 3 B1–B3 is aPDT using MB (10 μM) alone showing 12.4% area for live cells (38.9%) and 19.5% area for dead cells (61.1%).

Fig. 3 C1–C3 is aPDT with MB (1 μM) plus KI 100 mM showing only a 1.1% area for living cells (3.2%) but a 33.3% area for dead cells (96.8%).

3.4 Bacterial morphology by SEM

The SEM images are shown in Figure 4. Untreated *E. faecalis* cells (Fig. 4A) have intact membranes, while MB (10 μM) with light (Fig. 4B) has caused *E. faecalis* cell membranes were being damaged and atrophied. However, in aPDT with MB (10 μM) + KI (100 mM) (Fig. 4C), bacterial membranes were totally ruptured.

3.5 Photobleaching

As can be seen from Figure 5, a light blue color is visible if the MB concentration was 10 μM. A deep blue color could be seen when the concentration was 100 μM. After application of light, the reduction of the blue color in the case of 10 μM MB was minor without KI, but more pronounced with the addition of KI (100 mM). In the case of 100 μM MB alone plus light showed a reduction of the blue color, but when KI was added there was overt precipitation to give an insoluble dark solid.

3.6 HGF cell safety testing

Unlike NaClO, which killed all the cells, the ideal PDT treatment should provide effective sterilization of *E. faecalis* without significant damage to normal HGF cells. Figure 6 has shown that aPDT with MB (2 μM) alone or MB (0.4 μM) plus KI (100 mM), which both had similar killing ability for *E. faecalis,* do not cause any significant loss of viability of HGF cells. In contrast a bactericidal concentration of NaClO produced 100% loss of viability of HGF cells.

The fluorescence microscopy images of the HGF cells are shown in Figure 7. The aPDT MB alone group, MB+KI aPDT group showed no obvious differences. Green fluorescence means live HGF cells and red fluorescence means dead HGF cells. The MB group and MB +KI group showed no obvious cytotoxicity compare to the untreated group. But NaClO destroyed all the cells so that no red or green fluorescence was visible.

3.7 Mechanistic experiments

A previous study showed that MB plus light primarily works via a Type 1 photochemical mechanism. In other words, the production of singlet oxygen is less important compared to hydroxyl radicals and hydrogen peroxide when it comes to killing bacteria. The mechanism by which photoexcited MB interacts with inorganic ions such as iodide was originally proposed to be a Type I electron transfer process[44], but findings with other photosensitizers (Photofrin and Rose Bengal) showed it could also be a type II process involving singlet oxygen [50, 59]. Both Type I and Type photochemical processes can oxidize iodide to produce free molecular iodine. In Figure 8 we shown the results of the mechanistic studies.

Fig. 8A shows that increasing amounts of light produced more free iodine from KI (100 mM) in the presence of MB assessed by the absorption of the iodine starch complex at 630nm. Higher MB concentration produced more free iodine with increasing light dose

Fig. 8B shows the Amplex red assay demonstrating that hydrogen peroxide (H_2O_2) was generated in much higher quantities when KI was added. A previous study has shown that Photofrin or Rose Bengal with added KI could also generate more H_2O_2 than without KI.

There are two possible ways to form H_2O_2. One possibility is that iodide anion underwent a one-electron transfer to singlet oxygen to produce an iodine radical and superoxide radical anion. Superoxide could then undergo dismutation to give H_2O_2.

Fig. 8C shows superoxide production using nitro blue tetrazolium (NBT). If no superoxide was found, the second reaction pathway, singlet oxygen addition to iodine anion to produce peroxyiodide, which decomposes to produce H_2O_2 and iodine, is more likely. The results showed that compared to the positive control group (photoactivated fullerene plus NADH), MB plus KI did not form superoxide therefore the second pathway was more likely.

4 Discussion

aPDT is a treatment that could be employed in root canal therapy[20, 21]. Recently, researchers have found that KI could enhance the aPDT effect of photoactivated MB to kill bacteria and fungal infection. However, whether KI can still function in a hypoxic environment or could produce a long-lasting effect after light, and whether it could affect photobleaching of the MB color as well as biosafety were still unknown.

First, this study has carried out a lot of experiments and explored the suitable parameters of this complex system. The planktonic bacteria experiments showed that KI significantly enhanced the effect of aPDT with MB on *E. faecalis*. The killing effect was much better with increased concentrations of MB, KI, and increasing illumination time. MB with KI still has a continuing bactericidal effect after stopping the illumination, while carrying out the aPDT with the same concentration of MB had no obvious long-lasting bactericidal effect. In a hypoxic environment, the bactericidal effect of aPDT MB was completely abolished, but MB with added KI was still able to exert a bactericidal effect, although the killing was reduced compared to that found in the presence of oxygen. That observation suggests that

aPDT with MB plus Ki could damage *E. faecalis* in an hypoxic environment, as in the deeper dentinal tubules[54]. The explanation is probably that photoactivated MB could carry out electron transfer reactions not involving oxygen, thus producing iodine radicals from iodide anions.

We observed significant damage to the *E. faecalis* biofilm using confocal laser scanning microscopy and scanning electron microscope. This observation shows that photoactivation of 10μM MB plus KI may be a suitable method to destroy biofilm.

MB is a colored photosensitizer that can change the color of teeth by staining them blue [38]. We found that adding KI to the MB could increase the photobleaching effect thus reducing the blue color of MB.

For the biosafety study, we compared MB, MB with KI and NaClO added to HGF cells, and found that aPDT with MB plus KI showed no obvious toxicity to HGF cells, while NaClO could destroy almost all the cells.

The synergistic mechanism by which KI and MB-PDT may involve the production of short-lived reactive iodine species, such as iodine radicals, in addition to the bactericidal long-lived free molecular iodine. Previous studies have found that MB+KI does not produce more singlet oxygen, but the formation of hydroxyl radicals is reduced. Therefore, it is not believed that ROS are increased by the addition of KI [44]. In the present study, it was confirmed that the addition of KI to aPDT with MB could increase the production of free iodine and increase the production of hydrogen peroxide. Hydrogen peroxide has a bactericidal effect, and its production is believed to be caused by the addition of singlet oxygen to the iodide anion to produce peroxyiodide (Equation 1). Peroxyiodide then gains a proton to form iodine hydroperoxide (Equation 2). Iodine hydroperoxide then reacts with an additional iodide anion to form $HOOI_2^-$ (Equation 3). Dalmazio et al. [60] from Brazil used mass spectrometry and *ab initio* free energy calculations to study the decomposition of hydrogen peroxide in the presence of iodide anions. They detected a species with $m/z = 287$ that was proposed to be $HOOI_2^-$. They carried out free energy calculations revealing that the most thermodynamically favored decomposition pathway of $HOOI_2^-$ was via Equation 4, to give two radicals, $I_2^{\bullet -}$ and HOO•. Both these short-lived radicals would be expected to cause significant damage to the bacteria. The net result of these steps is the production of free iodine (actually, tri-iodide anion I_3^- in the presence of excess iodide) plus H_2O_2 as shown in Equation 5. These two bactericidal species are likely to be responsible for the long-lasting bactericidal effect that continues after cessation of illumination.

$$^1O_2 + I^- \rightarrow IOO^- \quad (1)$$

$$IOO^- + H^+ \rightarrow HOOI \quad (2)$$

$$IOOH + I^- \rightarrow HOOI_2^- \quad (3)$$

$$HOOI_2^- \rightarrow I_2 \bullet^- + HOO \bullet \quad (4)$$

$$^1O_2 + 3I^- + 2H_2O \rightarrow I_3^- + 2H_2O_2 \quad (5)$$

In the absence of oxygen (in the anaerobic incubator) we propose that the excited state of MB can undergo a 1-electron transfer to form the MB radical cation and the iodine radical anion as shown in Equation 6. These two highly reactive radicals could damage the bacterial cells.

$$MB^* + 2I^- \rightarrow MB \bullet^+ + I_2 \bullet^- \quad (6)$$

In conclusion, we found that addition of KI (100 mM) to MB PDT (0.4μM for planktonic, and 10μM for biofilm) dramatically increased the sterilization effect against *E. faecalis,* while still preserving the safety of the treatment towards human cells. The combination was able to sterilize bacterial biofilm growing on human teeth, was able to preserve a bactericidal effect after switching off the light and could still produce bacterial killing in the absence of oxygen. If lighting time can be increased, the aPDT effect can be more. These data suggest the combination approach could be developed for root canal disinfection in root canal therapy. These data suggest the combination approach could be developed for root canal disinfection in root canal therapy. This would likely be carried out after conventional chemo-mechanical debridement had removed the bulk of the infected material [61]. Moreover considering the lack of toxicity towards gingival fibroblasts, the addition of KI to MB aPDT alone[62, 63] could be tested in other dental indications such as peri-implantitis or periodontitis.

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Highlights

- KI potentiates MB aPDT of *E. faecalis* in planktonic cells and dentine biofilm.
- MB + KI aPDT produces long-lasting effects and could work in hypoxic root canals
- The combination is safe for human cells compared to 5.25% NaClO.
- KI accelerates MB photobleaching avoiding tooth staining.
- Mechanism involves production of free iodine and hydrogen peroxide

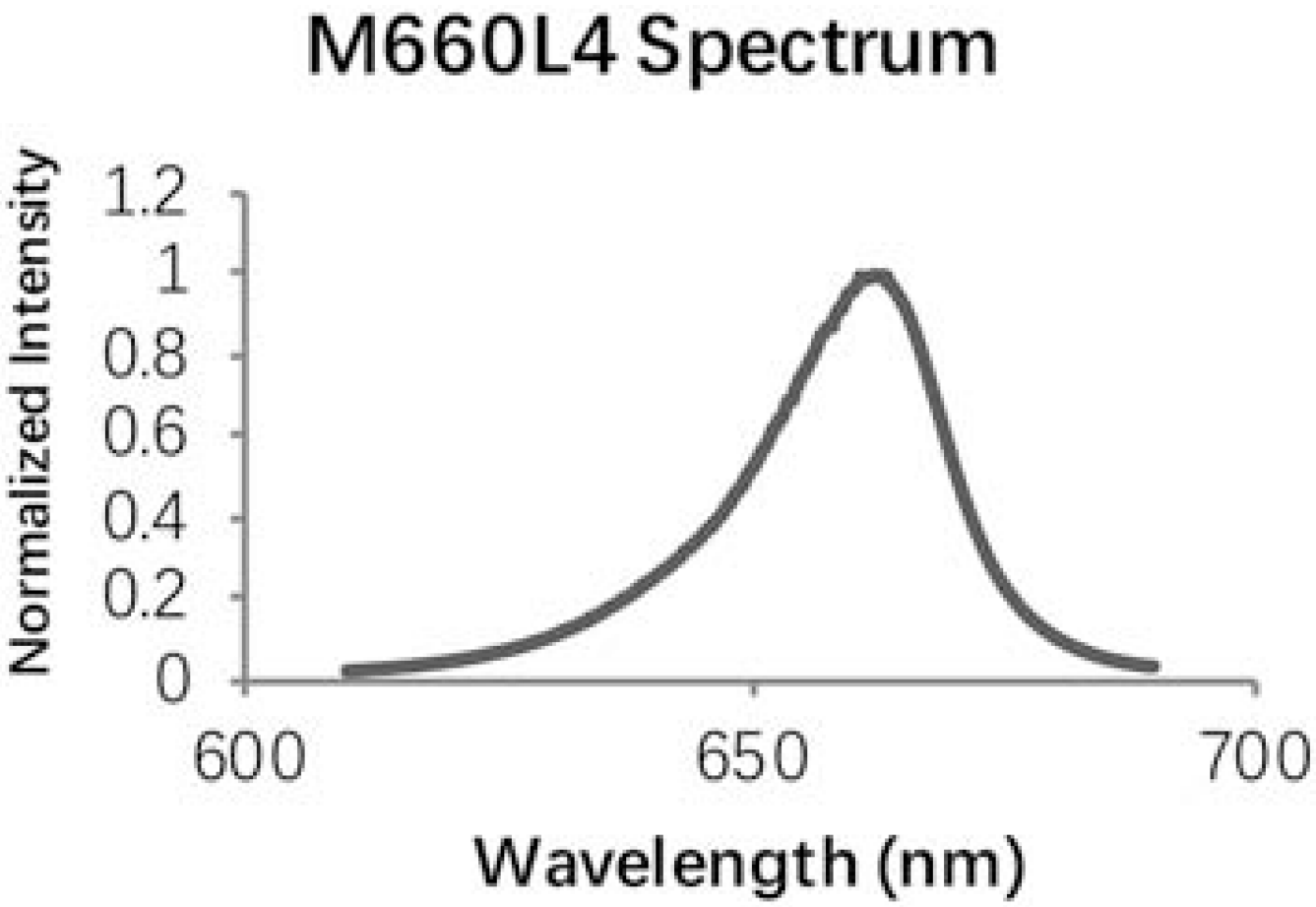

Figure 1. Spectrum of 660 nm deep red led light source
(Data taken from Thorlabs.com).

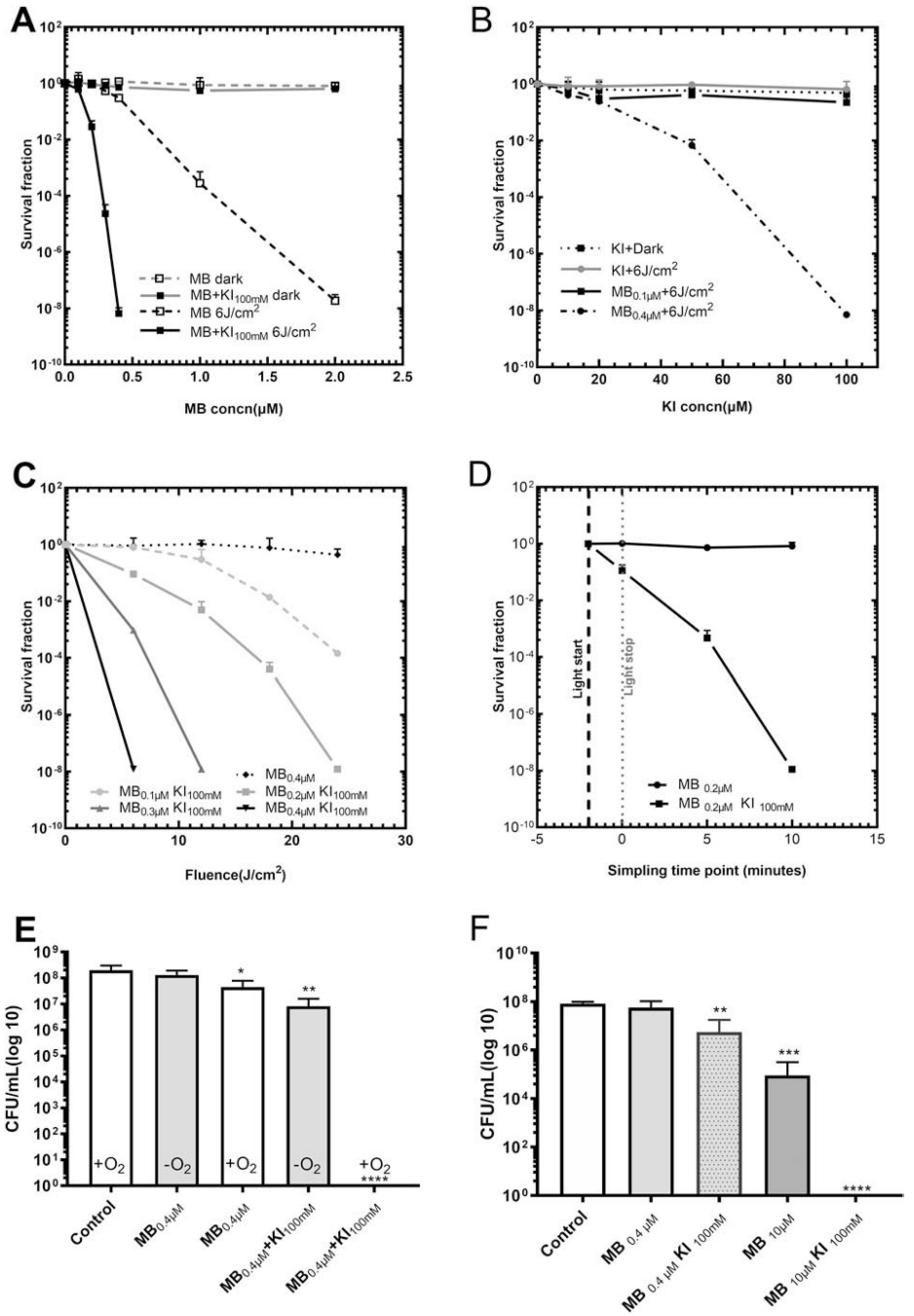

Figure 2. MB plus KI enhanced aPDT of *E. faecalis*.

(A) KI (100mM) reduced MB concentration needed for *E. faecalis* aPDT using 6 J/cm^2. (B) KI concentration effect on potentiation of MB (0.1 or 0.4μM) aPDT using 6 J/cm^2. (C) KI (100mM) reduces light dose needed for *E. faecalis MB* (0.1–0.4μM)-aPDT. (D) MB plus KI aPDT for *E. faecalis* sampled at different time after the end of illumination. (E) Hypoxic antimicrobial effect of MB (0.4μM) plus KI (100mM) aPDT for *E. faecalis* using 6J/cm^2. (F) Photoactivation of MB with or without KI to kill *E. faecalis* biofilm at 30 J/cm^2 of light, the

biofilms were formed in 6 well plates. (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001).

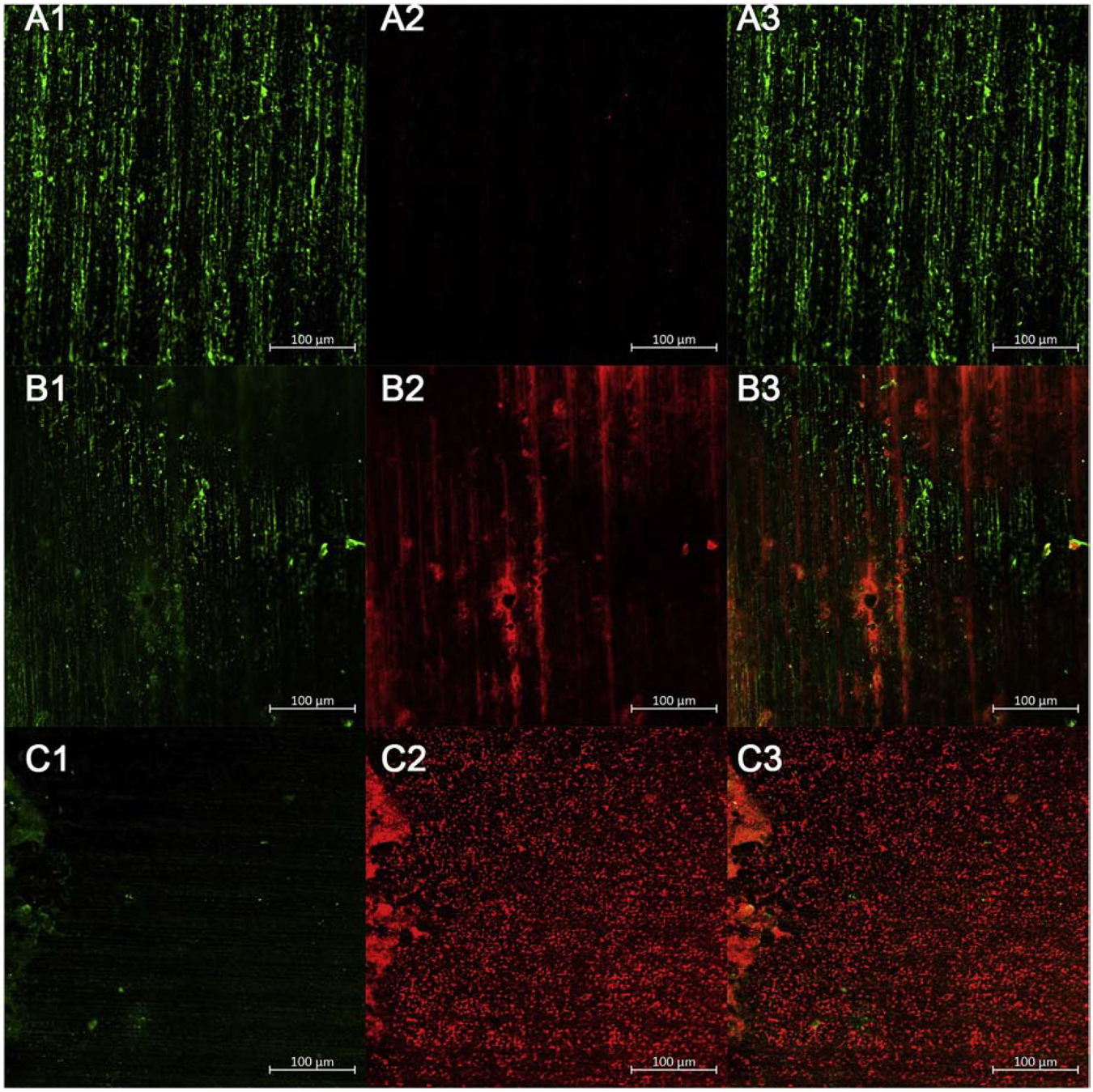

Figure 3. CLSM images for *E. faecalis* biofilm growing on dentin blocks.

Biofilm were treated after 21 days using the following groups. (A1-A3) Control group; (B1-B3) MB (10 μM) plus 30 J/cm^2; (C1-C3) MB (10 μM) plus KI (100 mM) plus 30 J/cm^2. Images are representative examples of three separate images

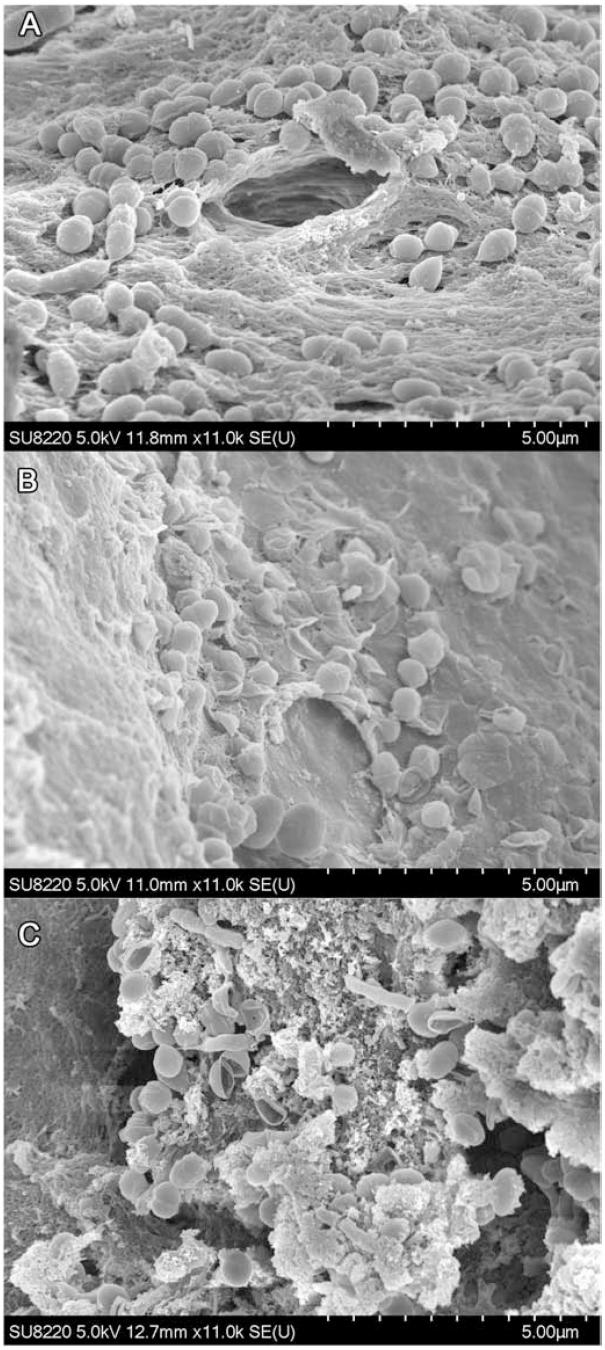

Figure 4. SEM images of *E. faecalis* biofilm.

(A) Control group; (B) MB (10 μM) plus 30 J/cm^2; (C) MB (10 μM) plus KI (100 mM) plus 30 J/cm^2. Images are representative examples of three separate images

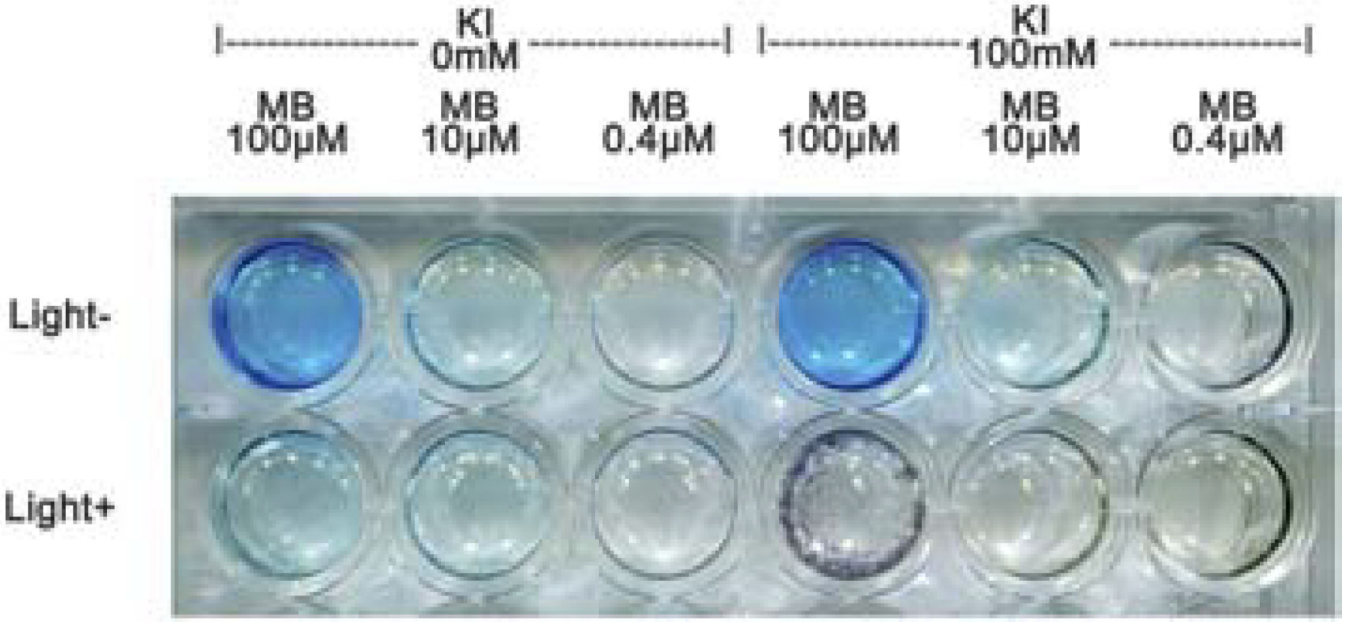

Figure 5.
Light-induced color changes of MB ± KI with or without 6 J/cm^2.

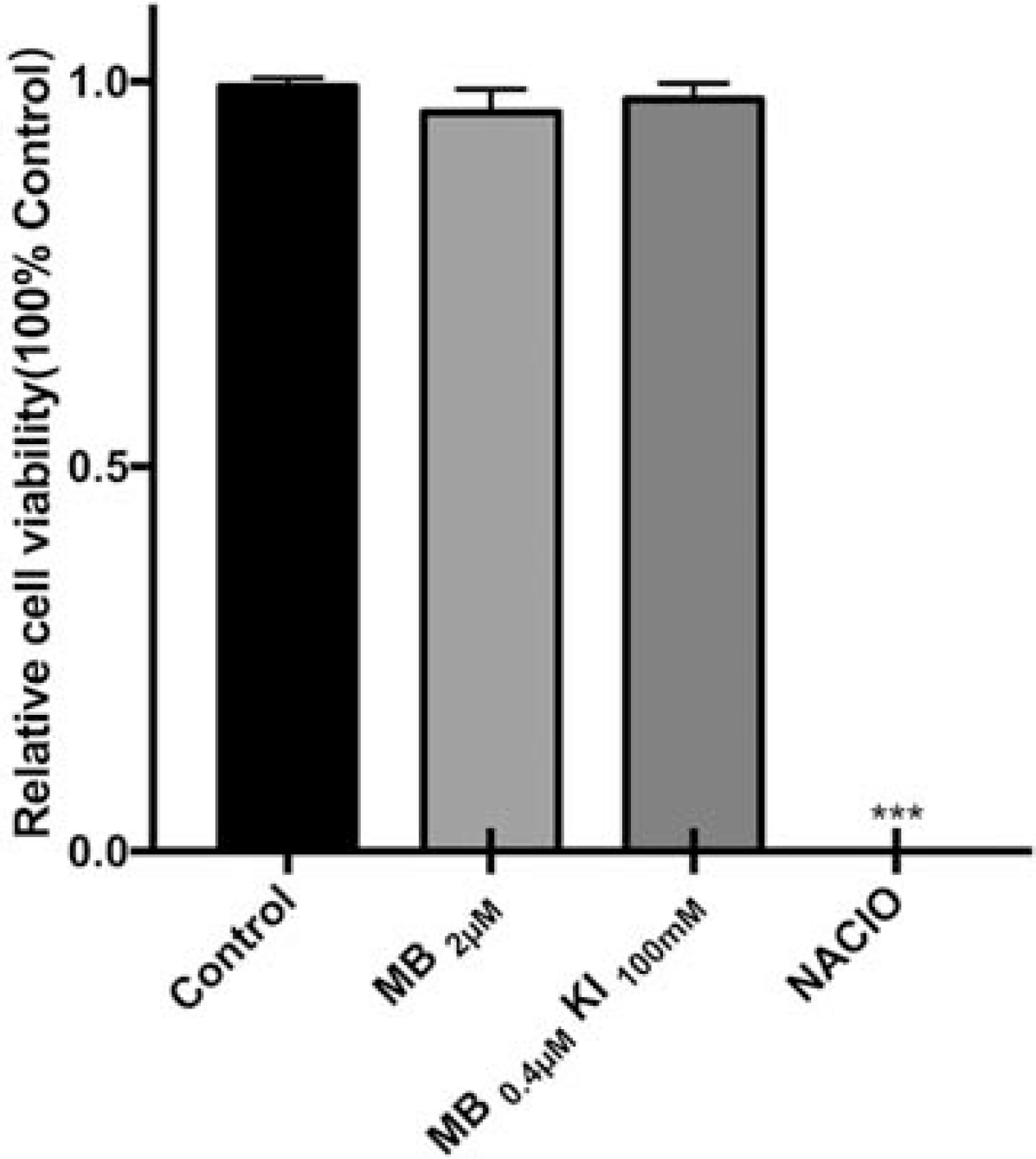

Figure 6. Survival fractions of HGFs.

Untreated control group; MB (2 μM) plus 6 J/cm^2 light: MB (0.4 μM) plus KI (100 mM) plus 6 J/cm^2 light; NaClO (5.25%)

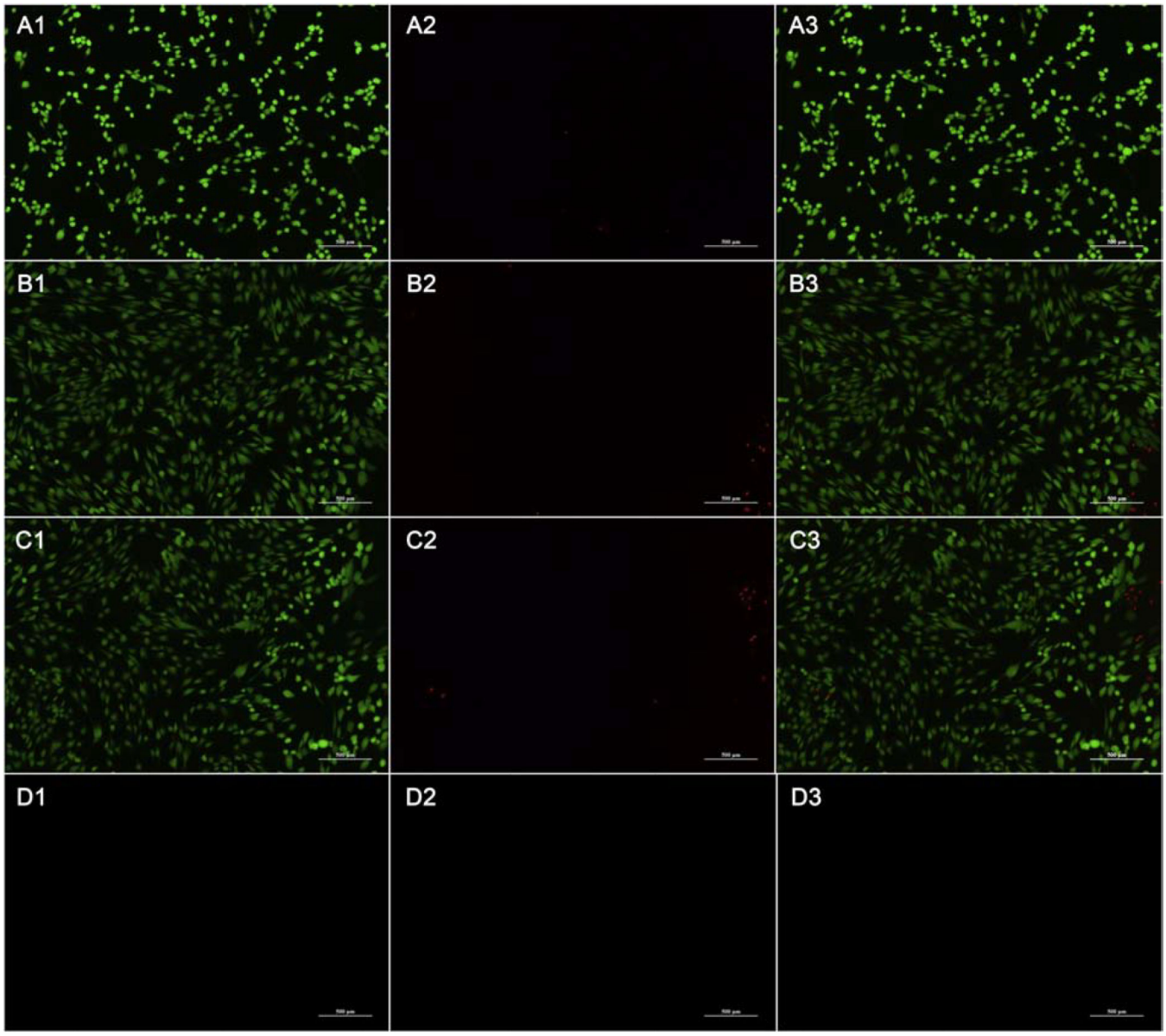

Figure 7. Fluorescence microscopic images for HGF cells in groups.

(A1-A3) untreated group; (B1-B3) MB (2 μM) + 6 J/cm^2; (C1-C3) MB (0.4 μM) + KI (100 mM) + 6 J/cm^2; (D1-D3) 5.25% NaClO. A1-D1, green cells are living cells; A2-D2, red cells are dead cells; A3-D3 are merged image.

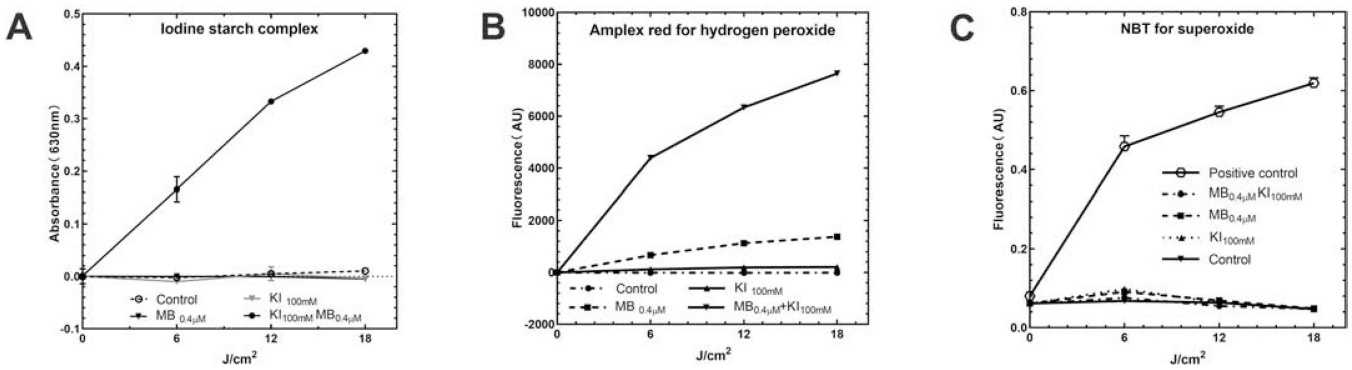

Figure 8. Mechanistic experiments.

A) Production of free iodine measured by starch indicator assay. B) Production of hydrogen peroxide by Amplex red assay. C) Production of superoxide anion by the NBT test.

Table 1

Parameters for varying MB concentration

Group A	MB (μM)	KI (mM)	660 nm (J/cm^2)
1	0, 0.1, 0.2, 0.3, 0.4, 1, 2	0	0
2			6
3		100	0
4			6

Table 2

Parameters for varying KI concentration

Group B	MB (μM)	KI (mM)	660 nm (J/cm^2)
1	0		
2	0.1	0, 10, 20, 50, 100	6
3	0.4		

Table 3

Parameters for varying light dosage

Group C	MB (μM)	KI (mM)	660 nm (J/cm^2)
1	0.4	0	0, 6, 12, 18, 24
2	0.1	100	
3	0.2		
4	0.3		
5	0.4		

Table 4

Parameters for long lasting killing effect

Group D	MB (μM)	KI (mM)	660 nm (J/cm^2)	Sampling time (minutes)
1	0.2	0	6	0, 5, 10
2		100		

Table 5

Parameters for aPDT effect in hypoxia

Group E	MB (μM)	KI (mM)	660 nm (J/cm^2)	O_2
1	0	0		+
2				−
3		0	6	+
	0.4			
4				−
5		100		+

Table 6

Parameters for biofilm experiment

Group F	MB (μM)	KI (mM)	660 nm (J/cm^2)
1	0	0	
2	0.4	0	
3		100	30
4	10	0	
5		100	

Table 7

Parameters for *E. faecalis* biofilm sterilization in tooth blocks

Group G	MB (μM)	KI (mM)	660 nm (J/cm^2)
1	0	100	
2	10	0	30
3		100	

Table 8

Parameters for photobleaching of MB + KI

Group H	MB (μM)	KI (100 mM)	660 nm (6 J/cm^2)
1	100		
2	10	±	±
3	0.4		

Table 9

Parameters for safety evaluation on HGF

Group I	MB (μM)	KI (mM)	660 nm (J/cm^2)
1	0	0	
2	2	0	6
3	0.4	100	
4	Only 5.25% NaClO		