Antimicrobial Activity and Cytotoxicity of 3 Photosensitizers Activated with Blue Light

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Abstract

Introduction: Pulp repair is less likely to occur when dentin or pulpal tissue remains infected after caries excavation. Yet there are currently few options to kill residual bacteria without damaging resident cells. The current study has evaluated the effect of 3 blue light–activated chemicals on the viability of lactobacilli, odontoblast-like cells (MDPC-23), undifferentiated pulp cells (OD21), and human embryonic stem cells (hESC H1). **Methods:** Bacteria were incubated for 15 minutes with curcumin, eosin Y, or rose bengal and then irradiated with blue light (240 seconds). Bacteria were labeled with LIVE/DEAD BacLight Bacterial Viability kit, and viability was assessed by fluorescence-activated cell sorting. Cytotoxicity assays were performed on MDPC-23 cells, OD21, and hESC H1 cells grown in 24-well plates and exposed to the same photosensitizer-light combination. After 24 hours, cellular response was measured by using the methyl-thiazol-diphenyl-tetrazolium assay. Results were statistically analyzed by using one-way analysis of variance and Tukey multiple comparison intervals (α = 0.05). **Results:** Bacterial viability was significantly reduced after exposure to different combinations of light and photosensitizers; mitochondrial activity of cultured cells remained unaffected when exposed to the same conditions, suggesting a good therapeutic index *in vitro*. **Conclusions:** Blue light–mediated disinfection is promising for the development of new treatment strategies designed to promote pulp repair after carious exposure. *(J Endod 2014;40:427–431)*

Key Words

Antimicrobials, cariously exposed pulp, *in vitro*, PDT, pulp repair

Direct pulp capping and partial pulpotomy are clinical procedures designed to maintain the vitality of the dentin-pulp complex exposed after trauma or deep caries excavation (1). However, the lower success rates reported for cariously exposed pulps compared with traumatically or mechanically exposed pulps have underscored the role of bacteria in the outcome of these treatments (2, 3). Unfortunately, most antimicrobial agents used for dentin and pulp disinfection also damage cells and growth factors involved in pulp repair (4–6). Thus, current clinical procedures recommend a complete amputation of pulp tissue despite the presence of stem/progenitor cells that retain their potential for tissue regeneration in presence of inflammation (7).

For many years, photo-activated disinfection (PAD) has been used for the treatment of dermatologic, gastrointestinal, genital, and oral infections with encouraging results (8–11). PAD is based on the interaction of a photosensitizer with a light source that produces reactive oxygen species (ROS) such as singlet oxygen (^{1}O2), hydroxyl radicals, and superoxide anions (12). ROS kill bacteria by damaging bacterial cell wall, nucleic acids, and membrane proteins. Research indicates that the degree of bactericidal effects depends on the amount of ROS produced (13).

Although red light–based PAD has been most investigated, blue light combined with appropriate photosensitizers was also shown to inactivate *Streptococcus mutans*, *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis,* and *Enterococcus faecalis* (14–17). Recently, lactobacilli freely dispersed in the pulp tissue beneath advanced carious lesions have been identified by fluorescence *in situ* hybridization (18). However, the ability of blue light–based PAD to inactivate *Lactobacillus* species, which dominate the flora of advanced carious lesions, remains poorly documented (19–21).

In the present study it was hypothesized that 3 blue light–activated photosensitizers can elicit a phototoxic effect on lactobacilli without affecting the viability of pulpal cells. Such a favorable therapeutic index would be needed if these compounds have clinical utility in pulpal disinfection and pulpal therapy.

Materials and Methods

Curcumin, eosin Y, and rose bengal (Sigma-Aldrich, Buchs, Switzerland) were used as photosensitizers because they absorb blue light (17). For each photosensitizer, a stock solution (400 μmol/L) was prepared in sterile distilled water. Curcumin was initially dissolved in ethanol to circumvent the relative insolubility of this chemical in water (22). Stock solutions were stored in the dark at -20°C and were diluted on

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the day of the experiments. The photosensitizers were activated for 240 seconds by using a dental light source (Optilux 501; KerrHawe SA, Bioggio, Switzerland) emitting blue light (380–500 nm). During experiments, the distance from the light guide tip to the target cells (bacteria or cells) was set at 20 mm to completely irradiate the surface of a culture dish.

Antibacterial Assay

Lactobacilli isolated from dental carious lesions (ATCC 4646; LGC Standards, Wesel, Germany) were cultured in 3 mL culture medium (MRS; AxonLab, Baden, Switzerland) and incubated anaerobically at 37°C (BD GasPak EZ; AxonLab). After 24 hours, the culture reached the late exponential phase of growth, and bacteria were harvested by centrifugation (3500*g*, 5 minutes) and resuspended into sterile 0.9% NaCl. Bacterial concentration was adjusted by measuring the turbidity of the suspension in a Biochrom WPA Biowave II spectrophotometer (OD_{600} 0.8, 10^8 cells/mL). For experiments, 0.5 mL of bacterial suspension was inoculated in 24-well plates with opaque walls to prevent light from conducting to the next well during irradiation (Genetix PetriWell; Molecular Devices, Sunnyvale, CA). The amount of 0.5 mL photosensitizers was added to the wells and incubated for 15 minutes before irradiation with blue light for 240 seconds. Each well was irradiated separately.

Bacterial suspensions were then labeled for viability measurements by using LIVE/DEAD BacLight Bacterial Viability kit (Life Technologies Europe BV, Zug, Switzerland). Two hundred microliters of the LIVE/DEAD stain (nucleic acid stains, SYTO 9 and propidium iodide [PI]) was added to the same volume of bacterial suspension, mixed thoroughly, and incubated at room temperature in the dark for 15 minutes. The excitation/emission wavelengths for SYTO 9 and PI are 480/500 nm and 490/635 nm, respectively. SYTO 9 is membrane permeable and can enter all cells, whereas PI only penetrates membrane-damaged cells.

The specimens were analyzed by flow cytometry (Accuri C6; BD Accuri Cytometers, Ann Arbor, MI). Forward scatter and side scatter have been adjusted to ensure the screening of the whole bacterial population and to exclude debris from analysis. To optimize fluorescence-activated cell sorter settings, calibration samples containing SYTO 9 alone or PI alone have been used; cytometer run limits have been set to 20,000 events. Live cells emit in the FL-1 channel (520 nm) and dead cells in the FL-3 channel (≥630 nm). Threshold on FL-1 has been used to fine-tune the acquisition. Further improvement was achieved by adjustment of photomultiplier tube voltages; FL-3 has been corrected by subtracting 3% of FL-1. Although bacteria labeled with SYTO 9 and PI produce distinct "live" (FL-1 positive) and "dead" (FL-3 positive) populations, pilot analysis has confirmed that approximately 15% of PAD-treated bacteria exhibited intermediate states (also referred to as "unknown"). Flow cytometric dot plots illustrating the different bacterial populations are shown in Supplemental Figure S1 (available online at www.jendodon.com). To verify whether unknown populations retain their proliferation capacity, they have been isolated by cell sorting (FACS Aria; Becton Dickinson, Franklin Lakes, NJ) and then cultured on agar plates for 48 hours. Because a fraction of the unknown population (approximately 1%) has been able to grow, unknown populations have been considered as live in the results.

Pilot studies were performed to estimate concentrations of photosensitizers exhibiting antimicrobial properties on light exposure. A wide concentration range (0–50 μmol/L) was first assessed. Curcumin exposed to blue light induced a progressive reduction in bacterial viability in the 0.5–10 μmol/L range, whereas eosin Y and rose bengal caused a rapid drop (<5 μmol/L) in bacterial viability on light exposure. For these photosensitizers, narrower ranges (0.2, 0.5, and 1 μmol/L) were investigated. Results from pilot studies are presented in Supplemental Figure S2 (available online at www.jendodon.com). On the basis of these pilot results, final concentrations of photosensitizers tested in the current study ranged from 0–20 μmol/L. Control cultures received blue light alone or photosensitizers alone (n = 4 per condition). Each experiment was repeated 3 times.

Cytotoxicity Assay

The cell lines used were murine odontoblast-like cells (MDPC-23) and undifferentiated dental pulp cells (OD-21) (23). Human embryonic stem cells (hESC H1; WiCell Research Institute, Madison, WI) were also used because they are totipotent and have the ability to generate all tissues and organs.

The MDPC-23 and OD-21 cells were cultured in Dulbecco modified Eagle medium (Gibco BRL, Grand Island, NY), supplemented with 5% fetal bovine serum, 200 mmol/L L-glutamine, 50 units/mL penicillin, and 50 μg/mL streptomycin (all obtained from Gibco, Zug, Switzerland) in a humidified 5% CO_2 incubator at 37°C. The cells were plated at a density of 25,000 cells/cm^2 in 24-well plates (Genetix, PetriWell) containing 1 mL culture medium and allowed to attach overnight. H1 cells were cultured on matrigel-coated 24-well plates (Becton Dickinson AG, Basel, Switzerland) containing 1 mL NutriStem medium (Stemgent, Cambridge, MA) supplemented with 10 ng/mL basic fibroblast growth factor. Cells were allowed to grow for 48 hours before experiments.

Various concentrations (0–400 μmol/L) of photosensitizers (in phosphate-buffered saline [PBS]) were added to the cell cultures and incubated for 15 minutes before light irradiation. Control cultures received blue light alone or photosensitizers alone (4 replicates for each experimental condition). The entire experiment was repeated 3 times.

The cells were then incubated for 24 hours in a humidified 5% CO_2 incubator at 37°C, and cellular response was estimated by measuring mitochondrial activity by using the methyl-thiazol-diphenyl-tetrazolium (MTT) assay. With this assay, the amount of formazan (indicative of mitochondrial activity) is measured at 560 nm; results are expressed as a percentage of the PBS controls (24). Results of both antibacterial and cytotoxicity assays were compared statistically by using one-way analysis of variance (ANOVA) and Tukey multiple comparison intervals (α = 0.05).

Results

Blue light irradiation alone had no significant (P > .05) effect on either bacteria or cells. Furthermore, curcumin and eosin Y had little or no effect on bacteria and cell viability in absence of light activation.

Curcumin 0.5–10 μmol/L (Fig. 1) had no effect without light activation (dark toxicity) but significantly suppressed bacterial viability when light-activated (P < .05). Mitochondrial activity of OD 21, MDPC23, and H1 cells was significantly reduced after exposure to 10 μmol/L, 10 μmol/L, and 20 μmol/L light-activated curcumin, respectively. The TC50 concentration, or concentration required to reduce mitochondrial activity by 50%, was approximately 60 μmol/L for MDPC cells, 30 μmol/L for OD 21 cells, and 35 μmol/L for H1 cells.

Eosin-Y (Fig. 2) also did not exhibit dark toxicity at concentrations through 20 μmol/L but decreased bacterial viability to near 0 at only 1 μmol/L after light irradiation (P < .05). Eosin Y 100 μmol/L plus light significantly reduced mitochondrial activity of H1 cells, whereas no significant reduction was observed in OD 21 and MDPC23 cells below 400 μmol/L (P < .05). The TC50 concentrations were approximately 400 μmol/L for MDPC cells, 300 μmol/L for OD 21, and 100 μmol/L for H1 cells.

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