Genetic and Physiological Effects of Noncoherent Visible Light Combined with Hydrogen Peroxide on Streptococcus mutans in Biofilm

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Oral biofilms are associated with the most common infections of the oral cavity. Bacteria embedded in the biofilms are less sensitive to antibacterial agents than planktonic bacteria are. Recently, an antibacterial synergistic effect of noncoherent blue light and hydrogen peroxide (H₂O₂) on planktonic Streptococcus mutans was demonstrated. In this study, we tested the effect of a combination of light and H₂O₂ on the vitality and gene expression of S. mutans embedded in biofilm. Biofilms of S. mutans were exposed to visible light (wavelengths, 400 to 500 nm) for 30 or 60 s (equivalent to 34 or 68 J/cm²) in the presence of 3 to 300 mM H₂O₂. The antibacterial effect was assessed by microbial counts of each treated sample compared with that of the control. The effect of light combined with H₂O₂ on the different layers of the biofilm was evaluated by confocal laser scanning microscopy. Gene expression was determined by real-time reverse transcription-PCR. Our results show that noncoherent light, in combination with H₂O₂, has a synergistic antibacterial effect through all of the layers of the biofilm. Furthermore, this treatment was more effective against bacteria in biofilm than against planktonic bacteria. The combined light and H₂O₂ treatment up-regulated the expression of several genes such as gfbB, brp, smu630, and comDE but did not affect relA and jff. The ability of noncoherent visible light in combination with H₂O₂ to affect bacteria in deep layers of the biofilm suggests that this treatment may be applied in biofilm-related diseases as a minimally invasive antibacterial procedure.

Most bacteria in nature, particularly in the oral cavity, exist in communities known as biofilms (2, 31). Oral biofilms are associated with the most common infections in the oral cavity such as caries, gingivitis, and periodontal diseases (15). The constituents of the oral biofilm are embedded in an extracellular matrix (polysaccharides) synthesized mainly by extracellular enzymes such as glucosyltransferase (GTF) and fructosyltransferase (18). These enzymes are generated by several types of oral bacteria, especially mutants streptococci, which are highly associated with dental caries and oral biofilm formation. The biofilm structure serves as a physical barrier which limits the diffusion of agents, therefore severely limiting the effect of antibacterial treatment compared with the efficacy in planktonic bacteria (7, 36). In addition, bacteria embedded in biofilm express different genes, allowing acclimation to the biofilm microenvironment, which may also render these bacteria less sensitive to antibacterial agents (21, 27).

Interestingly, the use of light to kill bacteria growing on agar surfaces appears to be more effective than when they are grown in suspension (6). This is probably attributable to the scattering and absorption of light in the suspension, reducing penetration depth. Visible light wavelengths, mostly in the presence of a chemical photosensitizer, have been studied as a potential means of affecting bacterial vitality (6, 11, 16, 17, 23, 24, 34, 35, 39). Photosensitizers, molecules that are chemically excited by light of specific wavelengths, may cause biological damage or lead to the generation of reactive oxygen species (ROS) capable of reacting and affecting biological systems such as organelles, cells, and bacteria. More than 400 compounds are known to display photosensitizing properties. Most of them are dyes, drugs, and natural substances. Although many of these dyes may have inherent antibacterial effects, it is generally only during irradiation that the photodynamic bactericidal effect is elicited (20).

Recently, a synergistic antibacterial effect of noncoherent blue light, often used in restorative dentistry, and hydrogen peroxide (H₂O₂) on S. mutans under planktonic conditions was observed (5). Due to the widespread use of antibiotics and the emergence of more resistant and virulent strains of microorganisms, there is an urgent need to develop alternative sterilization technologies that affect biofilms. The aim of this study was to explore the synergistic effect of noncoherent blue light and H₂O₂ on bacterial viability and the effect of such treatment on gene expression of S. mutans in biofilm. (This study is part of the Ph.D. thesis of D. Moreinos.)

MATERIALS AND METHODS

H₂O₂. H₂O₂ (MP Biomedicals, Solon, OH) was used at final concentrations of 3, 30, and 300 mM. These concentrations are significantly lower than the MIC for S. mutans reported by Feuerstein et al. in 2006 (4). Control samples, in the absence of H₂O₂, were prepared by substituting phosphate-buffered saline (PBS) for the 50 μl of H₂O₂.

Light source. A xenon lamp with a combined filter for the transmission of noncoherent blue light (wavelengths of 400 to 500 nm; MSQCaesarea1, Israel) was used. The distance between the light source tip and the exposed sample was...
set at 1.5 cm in order to obtain a constant power density of 1.14 W/cm². An average light power of 440 mW was measured with a power meter (Optiph, Jerusalem, Israel) over a spot 0.7 cm in diameter. The calculated power density was obtained by dividing the average power by the light-exposed area.

**Bacterial biofilm formation.** Biofilms were formed on a 96-well microplate by a method similar to that described by Nobbs et al. in 2007 (22). Briefly, S. mutans UA 159 cells were grown in brain heart infusion broth (BHI; Acumedia Manufacturers, Baltimore, MD) and incubated under aerobic conditions at 37°C in 5% CO₂. All bacteria were subcultured at least twice before biofilm growth. A 20-µl volume of bacteria suspended in PBS was placed in wells of a 96-well microplate (Nunc, Roskilde, Denmark). The plate was then incubated for 24 h, in the course of which biofilm was formed. After biofilm formation, the wells were washed with PBS to remove loosely bound bacteria.

**Bacterial viability: microbial assays.** Bacterial biofilm samples in the presence of 3, 30, and 300 mM H₂O₂ were exposed to noncoherent blue light for 30 and 60 s, which is equivalent to 34 and 68 J/cm², respectively. Following light exposure, the samples were washed twice with PBS, followed by the addition of 200 µl PBS to each well. The immobilized bacteria were then separated from the wells with two pulses of 2 s each from a probe sonicator (Vibracell; Sonics and Materials, Westbury, NY) (33). The samples were serially diluted (10⁻² to 10⁻⁷) in sterile PBS and then applied to mitis salivarius supplemented with tellurite-bacitracin agar plates. The number of viable bacteria was determined by counting the CFU on the plates after 48 h of incubation at 37°C with 5% CO₂. All experiments were conducted in triplicate and repeated six times (n = 18). Control groups consisted of samples undergoing the same procedure except that they were only exposed to light without H₂O₂ or to H₂O₂ at the different concentrations with no light exposure.

A synergistic, additive, or antagonist effect of H₂O₂ and light exposure was determined as follows. The minimum inhibitory dose (MID) of light exposure and the MIC of H₂O₂ required to inhibit bacterial growth (at least at a magnitude of 99%) were examined separately. Synergistic, antagonistic, or additive effects of light and H₂O₂ were determined by calculating the fraction inhibiting concentration index (FIC) as follows: FIC = Antibacterial agent (MIC) in combination with light exposure/[Antibacterial agent (MIC) + Antibacterial agent (MIC) in combination with light exposure]. An index value of 0.5 indicates that a synergistic effect has taken place. An index higher than 4 indicates an antagonist effect (10, 13).

Tests similar to those described above were performed on planktonic bacteria in order to compare the combined light and H₂O₂ effects on immobilized and planktonic bacteria. Briefly, S. mutans bacteria were grown on plates containing 30 mM H₂O₂ and 60 s of light exposure (equivalent to 68 J/cm²). They were then transferred to test tubes containing 10 ml BHI and incubated at 37°C with 5% CO₂ for 24 h. After incubation, 3 ml of each sample (optical density at 650 nm = 0.5) was transferred to a test tube and 3 ml of RNA protect (Qiagen, Hilden, Germany) was added to each test tube, which was then incubated at room temperature for 10 min. Cells were collected by centrifugation (4,000 rpm for 8 min at 4°C). Total RNA extraction, reverse transcription (RT)-PCR, and real-time quantitative PCR were performed as described by Shemesh et al. (28). In short, collected cells were resuspended in Tri-reagent (Sigma-Aldrich) and disrupted with a Fast Prep Cell Disruptor (Bio 101, Savant Instruments, CA; Holbrook, NY). A 100-µl volume of the suspension was transferred to a test tube and incubated at 65°C for 10 min. The RNA was precipitated with isopropanol. After centrifugation, the pellet was washed with ethanol and resuspended in diethyl pyrocarbonate-treated water. Residual DNA was eliminated by DNase, and the RNA was precipitated with ethanol and resuspended with diethyl pyrocarbonate-treated water. The RNA concentration was determined spectrophotometrically by measuring the A₂₆₀/A₂₈₀ ratio with a NanoDrop instrument (NanoDrop Technologies, Wilmington, DE). The integrity of the RNA was assessed by agarose gel electrophoresis. An RT reaction mixture containing random hexamers, a deoxyribonucleic triphosphate mixture, and a total RNA sample was incubated to remove any secondary structure. RT buffer, MgCl₂, dithiothreitol, RNaseOUT recombinant RNase inhibitor, and SuperScript II RT (Invitrogen, Life Technologies, Carlsbad, CA) were added to each reaction mixture. After incubation, the reaction was terminated and the cDNA samples were stored at −20°C until used. Real-time quantitative PCR was performed with a GeneAmp 7000 Sequence Detection System (PE Applied Biosystems, Foster City, CA) with SYBR green PCR Master Mix (PE Applied Biosystems). The reaction mixture contained the cDNA sample and the appropriate PCR primer (Table 1). The cycle profile was as follows: 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 1 min, and 30 cycles of 95°C for 15 s and 60°C for 1 min. After the last cycle, the following dissociation protocol was followed: a hold at 95°C for 15 s, a hold at 60°C for 20 s, and a slow ramp (20 min) from 60°C to 95°C. The critical threshold cycle (Ct) is defined as the cycle at which fluorescence is detectable above the background and is inversely proportional to the logarithm of the initial number of template molecules. A standard curve was plotted for each primer set with Ct values obtained by the amplification of known quantities of cDNA from S. mutans MT8148. The standard curves were used for transformation of the Ct values to the relative number of cDNA molecules. The data are expressed as the mean ± standard deviation of triplicate experiments. In this study, we examined the differential expression of several genes associated with...
noncoherent light and 30 mM H₂O₂ on bacteria in biofilm and versus that on bacteria in biofilm. The combined effects of light exposure periods and samples exposed to light for 30 s (equivalent to ∼34 J/cm²) or 60 s (equivalent to ∼68 J/cm²) in the absence or presence of H₂O₂ at a concentration of 3, 30, or 300 mM is expressed as CFU counts on a logarithmic scale. Asterisks indicate statistically significant differences (P < 0.001).

FIG. 1. Bacterial growth following exposure of bacterial biofilm to noncoherent visible light (at wavelengths of 400 to 500 nm) in combination with different concentrations of H₂O₂. The growth of nonexposed (control) bacterial samples and samples exposed to light for 30 s (equivalent to ∼34 J/cm²) or 60 s (equivalent to ∼68 J/cm²) in the absence or presence of H₂O₂ at a concentration of 3, 30, or 300 mM is expressed as CFU counts on a logarithmic scale. Asterisks indicate statistically significant differences (P < 0.001).

Biofilm formation, i.e., ftf, gtfB, comDE, relA, brpA, and smu630 (Table 1). The expression levels of all tested genes were normalized using the 16S rRNA as internal standard.

Statistical analysis. Kruskal-Wallis analysis was performed by comparing the bacterial samples under the tested conditions with the controls. P < 0.05 was considered statistically significant.

RESULTS

Effect of noncoherent light and H₂O₂ on bacterial viability in biofilm. Bacterial growth in biofilm was assessed after treatment with different combinations of light exposure periods and H₂O₂ concentrations. Bacterial growth is expressed as the log value of the CFU count. Exposure of bacterial samples to light in the absence of H₂O₂ showed no statistically significant effect on growth (Fig. 1). Supplementing the bacterial biofilm with H₂O₂ at concentrations of 3, 30, and 300 mM without light exposure caused a slight reduction in the CFU count. However, a combination of the low H₂O₂ concentration (3 mM) with light exposure (30 and 60 s) reduced bacterial growth by 1.5 and 2.3 logs, respectively, compared with that of the control (P < 0.001). Similarly, combinations of 30 and 300 mM H₂O₂ with light exposure for 30 and 60 s caused a statistically significant reduction in bacterial counts (P < 0.001). In addition, a dose-dependent reduction in the number of S. mutans bacteria in the biofilms was observed with increasing light exposure and H₂O₂ concentrations.

The calculated FIC of all of the combinations described was lower than 0.5, suggesting that a synergistic effect took place under these experimental conditions.

Effect of noncoherent light and H₂O₂ on planktonic bacteria versus that on bacteria in biofilm. The combined effects of noncoherent light and 30 mM H₂O₂ on bacteria in biofilm and under planktonic conditions were further compared in relation to the untreated control samples. Bacteria under planktonic conditions showed CFU count reductions of 0.72 and 1.19 logs after light exposure for 30 and 60 s, respectively. Bacteria in biofilm showed greater CFU count reductions of 2.12 and 2.6 logs after light exposure for 30 and 60 s, respectively (Fig. 2) (P < 0.02).

Effect of noncoherent light and H₂O₂ on bacterial viability in different layers of the biofilm. S. mutans in biofilms were examined under the CSLM immediately and 3 and 6 h after noncoherent light exposure for 60 s in the presence of 30 mM H₂O₂. Images of the biofilm captured immediately after treatment showed that most of the bacteria in all of the layers of biofilm were not viable (Fig. 3a). Images captured 6 h following treatment showed that most of the bacteria in all of the layers of biofilm were not viable (Fig. 3c).

Effect of noncoherent light and H₂O₂ on bacterial metabolic activity in biofilm. ATP levels of bacteria in biofilm were examined immediately and 1, 3, and 6 h following exposure to noncoherent light for 60 s (equivalent to 68 J/cm²) in the presence of 3 mM H₂O₂ (Fig. 4). Immediately after treatment, a sharp increase in ATP levels was recorded that was significantly different from that of the H₂O₂ controls and irradiation-alone controls (P < 0.05). After 1 h, the ATP levels dropped to a level similar to that of the nonexposed control samples. They continued to drop after 3 and 6 h compared with those of the control samples (P < 0.05). Samples exposed to light or H₂O₂ alone showed no significant change in ATP levels at all of the measured time points compared with those of the nontreated control samples.

Genetic effects on bacteria in biofilm. Quantitative real-time RT-PCR showed significant up-regulation of the expression of brpA, gtfB, smu630, and comDE following exposure to light for 60 s (equivalent to 68 J/cm²) alone and in the presence of 30 mM H₂O₂ (P < 0.05) (Fig. 5). The up-regulation of gtfB, smu630, and comDE after light exposure was about eight, four, and six times, respectively, higher than that of the nontreated samples (P < 0.05). The combination of light and H₂O₂ resulted in the up-regulation of those genes (15-, 10-, and 10-fold, respectively). The expression of brpA was up-regulated following exposure to light approximately five times more than that of the control, regardless of the presence or absence of
H₂O₂. The expression of *relA* and *ftf* was not affected by the treatment. Exposure to H₂O₂ alone did not affect the expression of the genes tested.

**DISCUSSION**

It has been reported that most bacteria embedded in various biofilms are far less sensitive to antibacterial treatment than planktonic bacteria are (36). The low capability of the agents to penetrate the deep layers of the biofilm is considered a pivotal factor in this effect. Affecting bacteria by light energy may be an alternative means of action to conventional antibacterial therapy and may also be applied to oral diseases (3, 19, 20). In this era, when the use of antibacterial agents is being reduced because of their numerous side effects, alternative antibacterial measures such as light irradiation procedures have several advantages over conventional antibiotic treatment (37). First, it is conceivable that light energy has the capability to penetrate the deeper layers of the biofilm relatively more than chemical agents do, although some reduction in penetration may occur in deeper layers of biofilm. Second, so far no reports have been published regarding bacterial resistance to light irradiation. Third, by localization of therapy, the light beam can be confined to treated areas, not affecting areas adjacent to the infected regions. Lastly, the intensity of the irradiation can be readily monitored.

Previous findings demonstrated the mutual antibacterial effect of noncoherent light and H₂O₂ on planktonic oral bacteria (5). Since it is now well established that the virulence characteristics of bacteria can be markedly altered when they exist in biofilm, in the present study, we explored the potential synergistic effect of irradiation by noncoherent blue light and H₂O₂ on *S. mutans* embedded in biofilm. The results show that non-
coherent blue light alone at fluences of up to 68 J/cm² had a limited effect on *S. mutans* viability in biofilm, as did H₂O₂ at concentrations of up to 300 mM. However, a dose-dependent synergistic antibacterial effect on bacteria embedded in biofilm was demonstrated when noncoherent blue light and H₂O₂ were applied simultaneously. It was previously shown that the phototoxic effect of noncoherent blue light on planktonic bacteria involves the formation of ROS and that hydroxyl radicals (OH) play an important role in this process (4, 5). It is assumed that the synergistic effect on bacterial vitality in biofilm is the result not of direct fission of H₂O₂ by light, as described for the mechanism of action of UV light, but of the generation of the highly reactive OH radical when H₂O₂ encounters “free Fe(II),” via the Fenton reaction (5).

The OH radical is a potent oxidant that can react readily with macromolecules such as DNA or lipids in the cell membrane. However, penetration of the cell wall by H₂O₂ may be the rate-limiting factor in its antibacterial activity (1). Wood et al. (39) showed that the antibacterial effect of white light combined with photosensitizers is directly associated with the age of the biofilm and its architecture. Using lethal photosensitization, Williams et al. (34) found that the antibacterial effect of red light, in combination with the photosensitizer toluidine blue O, on *S. mutans* embedded in collagen was less than that obtained for planktonic suspensions. Using a Q-switched ruby laser, Soukos et al. (29) showed that photomechanical energy enhanced the penetration into a biofilm of the oral pathogen *Actinomyces viscosus* by methylene blue.

As limitation of diffusion into deep layers of the biofilm is one of the main problems in affecting bacteria, alternative means of light and sound energy have been used to overcome this obstacle. Our assumption was that light energy together with small molecules such as H₂O₂ would be capable of affecting bacteria in deeper layers of the biofilm. The combination of noncoherent light and H₂O₂ may also induce disruption of the biofilm by photo-oxidation along the light path, allowing greater permeation of the interbacterial void volume of the biofilm by H₂O₂ and eventually also of the cells. This increase in the local concentration of H₂O₂ in the microenvironment of the biofilm, and the fact that H₂O₂ molecules are small compared with the photosensitizer molecules, may explain the antibacterial activity observed in the deep layers of the biofilm.

Although it appears that the combination of H₂O₂ and noncoherent light has an advantage in penetrating the deep layers of the biofilm, its kinetics of action at those dosages and exposures is not high. Our CLSM results show that the antibacterial effect on bacteria embedded in biofilms is delayed for several hours after treatment. This observation is further supported by analysis of ATP levels. Our results clearly show a reduction in bacterial physiology expressed by ATP levels, following an increase in ATP levels immediately after light and H₂O₂ treatment. Visible light exposure in the presence of H₂O₂ resulted in accumulation of ROS in the bacterial surroundings. We assume that such a change creates environmental conditions stressful to the exposed bacteria. In response, the bacteria adapt by enhanced production of ATP. When these attempts fail, the number of live bacteria is reduced and the total amount of ATP in the sample is reduced gradually over time.

CLSM images indicate that the antibacterial effect begins in the middle layers of the biofilm (35 to 65 µm) and spreads to the remaining layers over a period of several hours. It is likely that the heterogeneity of cell vitality through biofilms is due to nutrient and/or physicochemical (e.g., pH, redox potential) gradients and subsequent limitation (38). Here, the depth-related vitality profile could be explained by ROS formation, which does not take place under anaerobic conditions such as those existing in the deepest layers of the biofilm. In the upper layers, the ROS that do form following 60 s of light treatment are either washed out by PBS or react with the air surrounding the wells and are neutralized before they can react with the bacteria, similar to the “oxygen inhibition layer” phenomenon, referred to as an unpolymerized surface layer observed when using blue light in room air to cure resins used for restorative dentistry (25). This phenomenon is not in agreement with another study, where lethal photosensitization occurred predominantly in the outermost layers of the biofilm (40). However, unlike our study the *S. mutans* biofilms in that study were exposed to larger and less diffusible molecules of toluidine blue O in combination with red light for longer periods of time (5 to 30 min).

Light has been shown to affect the regulation of gene expression. It may decrease gene expression (8) or induce regulation (26), depending on the environmental conditions and type of microorganism. Intriguing is the fact that a combination of noncoherent light irradiation and H₂O₂ results in the up-regulation of several genes of *S. mutans*, especially some that are associated with biofilm formation. The *gfb* gene, which encodes the GTF enzyme that synthesizes the extracellular glucans which play a pivotal role in sucrose-dependent bacterial adhesion, is highly up-regulated in the presence of light and H₂O₂. These findings suggest that the combination of light and H₂O₂ has a great potential to enhance bacterial adhesion. However, it should be noted that sucrose is required as a substrate for GTF activity to synthesize those glucans. On the other hand, the combination of noncoherent light irradiation and H₂O₂ does not alter mRNA levels of *fzf*, which encodes the fructosyltransferase enzyme that synthesizes extra-cellular fructans. The different effects on *gfb* and *fzf* indicate that the influence of noncoherent light in combination with H₂O₂ is of a specific nature, *brp*, which is a regulatory gene responsible for biofilm formation, and *smut630*, which is associated with biofilm formation, are also up-regulated, indicating again the potential of combined noncoherent light and H₂O₂ to induce biofilm formation. *comDE*, which encodes a histidine kinase receptor (*comD*) and a cognate response regulator (*comE*) of the competence-stimulating peptide, which are part of the quorum-sensing cascade of *S. mutans* (32), is up-regulated too. This effect may enhance the ability of the bacteria to form biofilm if other environmental conditions are adequate. On the other hand, *relA*, which is associated with bacterial physiology by acting as a guanosine tetra (penta)-phosphate synthetase, is not significantly affected by noncoherent light and H₂O₂. As the genes tested are only selected genes of the *S. mutans* genome, additional investigation of other genes which are associated with biofilm formation, inhibition, or quorum sensing may further elucidate the broader spectrum of effects of the combined therapy on biofilm formation and bacterial physiology.

Overall, our results are in agreement with those of Khaen-
grae and Reed (14), who suggested that the sublethal damage to bacterial cells caused by light leads to an ROS-sensitive state since it imposes an additional stress on the bacteria. Affecting bacteria in biofilm is one of the greatest challenges in the prevention and treatment of infectious diseases such as those associated with dental biofilm. This study presents a potential new means to beneficially alter oral biofilm: a synergistic effect obtained by the simultaneous harnessing of non-coherent blue light and H2O2.

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REFERENCES