Photodynamic Inactivation of Actinomyces naeslundii in Comparison With Chlorhexidine and Polyhexanide—A New Approach for Antiseptic Treatment of Medication-Related Osteonecrosis of the Jaw?

Sigurd Hafner, MD, DMD,* Michael Ehrenfeld, MD, DMD,† Enno Storz, MD,‡ and Andreas Wieser, MD§

Purpose: Local antimicrobial therapy is a fundamental principle in the treatment of lesions of medication-related osteonecrosis of the jaw. Antimicrobial photodynamic therapy (aPDT) as a local application for the treatment of microbial infections has become more widely used in recent years. In the mouth, the bone surface is in constant contact with saliva and thus cannot be kept sterile, making the development of strategies for disinfection even more important. Different methods currently in use include local rinses with chlorhexidine (CHX), polyhexanide (PHX), or aPDT. This study compared the efficiency of these 3 methods.

Materials and Methods: The in vitro activity of 3 different agents against slowly growing Actinomyces naeslundii isolated from a patient with osteonecrosis was evaluated. PHX 0.04% solution, CHX 0.12% solution, and methylene blue (MB) based dye with a laser light of 660-nm wavelength (aPDT) were compared.

Results: The decrease in colony-forming units by each method was measured using an in vitro killing assay based on a water-exposed surface in a well plate. MB dye with laser (10 seconds) decreased the bacterial load by more than 4 orders of magnitude and was superior to PHX and CHX exposure for 60 seconds.

Conclusion: Laser exposure alone and MB dye exposure alone decreased bacterial loads slightly, but less efficiently than 60-second exposure to PHX or CHX. The most effective means of decreasing colony-forming units was achieved by a combination of laser light and dye, which also can be used clinically.


*Senior Resident, Department of Oral and Maxillofacial Surgery, University of Munich (LMU), Munich, Germany.
†Professor and Department Head, Department of Oral and Maxillofacial Surgery, University of Munich (LMU), Munich, Germany.
‡Scientific Assistant, Department of Bacteriology, Max von Pettenkofer Institute (LMU), Munich, Germany.
§Senior Resident, Division of Infectious Diseases and Tropical Medicine, Medical Center of the University of Munich (LMU), Munich; Department of Bacteriology, Max von Pettenkofer Institute (LMU), Munich; German Center for Infection Research (DZIF), Munich, Germany; College of Public Health and Medical Science, Jimma University, Jimma, Ethiopia.

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Actinomyces species are known as primary colonizers inside the oral cavity and are well known as components of normal oral flora.\textsuperscript{1-6} Actinomyces species are among the major contributors for biofilm and plaque formation. In the past decade, many articles about the pathogenesis of and therapeutic options for bisphosphonate- or medication-related osteonecrosis of the jaw (MRONJ) have been published.\textsuperscript{7-15} The major clinical presentation of MRONJ is described as exposed necrotic jaw bone, often accompanied by an apparent infection. Although it is unclear whether this infection is primary or secondary, there is no doubt that the treatment of MRONJ should include the administration of systemic antimicrobial therapy.\textsuperscript{16} Despite the identification of microbial biofilms and Actinomyces species as the leading bacterial pathogen isolated from MRONJ lesions, adequate treatment is still unclear.\textsuperscript{17} Because antimicrobial resistance of biofilms is more pronounced than in aequously growing bacteria, they offer a therapeutic challenge. Clinically, the biofilms associated with necrotic bone in patients with MRONJ are resistant to systemic or local antibiotic therapy. Therefore, successful treatment of MRONJ often includes a combination of surgical removal of necrotic bone (including biofilms), careful wound closure, and systemic antimicrobial treatment.\textsuperscript{16} This combined treatment procedure frequently results in wound dehiscence that could be the consequence of inadequate local treatment of the infection. Sufficient intraoperative disinfection of bone, including the enclosed soft tissue, could minimize the problem of persistent infection and might speed up wound healing and lower the risk of reoccurrence of MRONJ.

Antimicrobial photodynamic therapy (aPDT) is an increasingly established method for the treatment of bacteria-associated oral diseases, such as periodontitis, peri-implantitis, and other local infections in the mouth.\textsuperscript{20-25} Inactivation of Actinomyces species with aPDT has been reported previously, but none of these studies have presented detailed comparisons with other methods.\textsuperscript{25-28} Since the observation in 1899 by O. Raab at the Pharmacological Institute of Munich, that paramecia are killed more efficiently by acridine in combination with sunlight, PDT has been used.\textsuperscript{29,30} Different commercial “aPDT systems” are currently available on the market for the treatment of periodontitis and other local infections.\textsuperscript{31} The most commonly used aPDT system with methylene blue (MB) containing dye and a mobile diode laser (HELBO) has shown good results during surgical procedures or in cases of postoperative wound dehiscence as adjuvant therapy in patients with MRONJ. There are no data regarding the bactericidal activity of aPDT against the commonly isolated Actinomyces species in MRONJ lesions. In this study, an Actinomyces naeslundii strain isolated from a patient with MRONJ was used to determine the bactericidal activity of aPDT against A. naeslundii.

Materials and Methods

LASER MODEL AND DYE COMPOSITION

HELBO MB was used as a photosensitizer (PS; Bredent Medical, Senden, Germany). This special dye is a sterile, isotonic, deep blue, and odorless aqueous solution. It contains 1% MB (3.7-bis-(dimethylamino)phenothiazin-5-ium chloride), glucose for isotonic properties, methylhydroxypropylcellulose to set the viscosity, and citrate to adjust the pH of the solution. The HELBO laser (660-nm wavelength, 100-mW low-power continuous-wave diode laser) in combination with the HELBO 2D Spot Probes as optical fibers were used as the light source (Bredent Medical). This system was used because it is currently the only system available on the commercial market with sterile dye, sterile spot probes, and approval for use in oral and maxillofacial surgery in Europe.

BACTERIAL CULTURE, KILLING, AND COLONY-FORMING UNIT DETERMINATION

As reference antibacterial solutions, chlorhexidine (CHX; 0.12%; Paroex, Sunstar Suisse SA, Etoy, Switzerland) and polyhexanide (PHX; 0.04%; 20% PHX [m/V] 0.208 g, sodium chloride 0.86 g, potassium chloride 0.03 g, calcium chloride dihydrate 0.033 g, water 100 g; Pharmacy of LMU, Munich, Germany) were used. Solutions were used shortly after preparation and stored at room temperature. Before the experiments, Actinomyces bacteria were thawed from frozen stock cultures prepared with brain heart infusion medium (Becton Dickinson, Heidelberg, Germany) supplemented with 25% glycerol. Colony-forming units (CFUs) were determined after a washing step in Dulbecco phosphate buffered saline (dPBS; Sigma Aldrich GmbH, Munich, Germany) using serial dilutions of 5% Columbia sheep blood medium (Becton Dickinson, Heidelberg, Germany). All measurements were performed independently in triplicate and yielded a stock solution concentration of 132,000 CFUs/μL of A. naeslundii.

For killing experiments, frozen stocks were thawed and pelleted by centrifugation (5,000 g, 15 minutes, 4°C). Bacterial pellets were suspended in sterile dPBS and 50-μL aliquots were used for the individual experiments in parallel. One group was tested with PHX (0.04%) 20 μL and with CHX (0.12%) 20 μL. Two groups were tested with MB 20 μL and one control group was tested with dPBS only. Solutions were incubated for 60 seconds at room temperature (21 ± 1°C). Bacteria were pelleted by centrifugation at 10,000g at 4°C for 60 seconds. The supernatant was gently removed and the remaining pellet was dissolved in...
dPBS 50 μL by pipetting up and down and brief vortex mixing. The bacteria were subsequently pipetted into individual wells of sterile 24-well plates (Thermo Fisher Scientific, Braunschweig, Germany). Homogeneous dispersion was achieved by shaking. Wells were subsequently stored in the dark or illuminated by laser light for 10 seconds under standardized conditions with the HELBO 2D Spot Probe, depending on the experimental group. After completion, the well plates were mixed by shaking and the bacteria were enumerated by plating in serial dilutions in triplicate. All experiments were independently repeated at least three times and were performed in parallel to avoid different incubation times.

**Microscopy and Live and Dead Staining**

As a second line of evidence, one aliquot of *Actinomyces* species was prepared and treated with MB and laser light. Thereafter, instead of CFU determination, bacteria were stained with the LIVE/DEAD BacLight Bacterial Viability Kit L7007 according to the manufacturer’s instructions (Life Technologies, Thermo Fisher Scientific, Waltham, MA). After a short staining period, the bacteria were pipetted into a microscopic chamber and pictures were taken with a confocal laser-scanning microscope (SP-5, Leica, Wetzlar, Germany).

**Results**

*Actinomyces* species are by nature relatively stable organisms that grow slowly and are not easily killed.32 Experiments were performed with 0.04% PHX and 0.12% CHX solutions as the gold standard for current regimens in the local treatment of oral infections. One group was sham-treated with dPBS to allow for correction of dilution artifacts and possible effects of pipetting and washing. All comparisons were performed against the dPBS-treated group. There was a decrease in CFUs after 60 seconds of exposure to 0.04% PHX of a factor of approximately $10^2$ and to 0.12% CHX of approximately $10^{1.5}$. The decrease of the counted CFUs was highly significant for CHX and PHX ($P < .024$) compared with the dPBS-treated group. Interestingly, PHX was found to be significantly more efficient than CHX ($P < .004$). The difference was a factor of approximately 3 (Fig 1).

To elucidate the effect of the laser light and of the MB dye containing photodynamic solution, one group was incubated with laser light alone, one group with the dye alone protected from light, and one group with the dye in combination with laser light. The dye alone exhibited bactericidal activity, which was significant compared with the dPBS control ($P < .001$), but not dramatic, with a CFU decrease of less than 1 order of magnitude (factor 5; Fig 1). The group treated with laser only also was found to have a slightly smaller CFU count ($P < .007$). However, the magnitude was even less pronounced, with a decreased factor of approximately factor 2 (Fig 1). PHX and CHX were found to be more efficient than laser light or MB alone. The most efficient decrease of bacterial load was achieved by the combination of laser light and MB. CFUs were decreased by more than 4 orders of magnitude.

To visualize the killing effect and to observe whether there would be a benefit of potential bacterial clusters, bacteria were stained before and after killing with MB and laser light (illumination, 10 seconds). The LIVE/DEAD BacLight Bacterial Viability Kit displays viable bacteria in green fluorescent color (SYTO 9 stain) and indicates dead bacteria in red fluorescent color (after penetrating the permeable cell wall of dead bacteria, propidium iodide stains the DNA and SYTO 9 membrane staining is decreased; Fig 2). The vast majority of bacteria in the sample died after laser irradiation (illumination, 10 seconds). In all experiments, *A. naeslundii* were found floating freely in the medium. In this condition, the microscopy-based assay could confirm the antimicrobial activity of the protocol.
DISCUSSION

The photo-physical background for photodynamic inactivation can be described by three different reactions based on the photochemical transfer of luminous energy (eg, photons of laser light) to a dye that is a PS with light-absorption properties, resulting in higher energy levels of the dye (3PS*, excited triplet state of PS). The type I mechanism is described as an electron transfer between 3PS* and a substrate that generates free radicals and subsequently active oxygen species and hydrogen peroxide and oxygen or hydroxyl radicals. The type II reaction is described as a direct energy transfer from 3PS* to 3O2, leading to highly reactive singlet state oxygen (1O2). Bacterial cell wall components can be oxygenized and thus destroyed by 1O2 and free radicals, killing the microbes. Other antimicrobial mechanisms resulting from the interaction of 1O2 with bacterial enzymes have been described.

The photoactive dye is enriched in microbial cell walls compared with human tissue. Combined with the very transient singlet state of oxygen (<0.04 μs) and the resulting very short distance of effective action of activated oxygen (<0.02 μm), the main effect is concentrated around bacterial cell walls. Natural antioxidant agents of human somatic cells neutralize excess radicals, thus mitigating the toxic effect on human body cells relative to microbes, when this kind of therapy is performed in the local treatment of infections. Type III photo-oxygenation is characterized by an electron transfer between 3PS* and the substrate leading to an anionic PS (PS−) and cationic substrate resulting in an electron transfer to 3O2 to form O2−.

The use of MB dye as described in the patient samples of this study has been Communauté Européenne (CE)-licensed for use in dental medicine and maxillofacial surgery in Europe since 2003 and has been used for the treatment of infected wounds. The authors have used the technique in clinical practice for MRONJ lesions for at least 10 years for more than 200 patients in combination with systemic antimicrobial therapy with β-lactam antibiotics combined with β-lactamase inhibitors. They have found marked improvement especially in wounds with microbiologically proven colonization with Actinomyces species.

To generate realistic data on the antimicrobial efficiency of CHX, PHX, and aPDT, the authors developed a model based on a watery film on a solid surface in a well plate. This method, as in all in vitro studies, has shortcomings in describing a real-life scenario. As expected, the antimicrobial activity of CHX and PHX was shown (Figs 1, 2). Interestingly, MB alone also exhibited some antibacterial effects. These could be due to the redox potential of the cationic dye binding to the bacterial cell wall and thereby exhibiting some toxic effect. Although, to the authors’ knowledge, this has not been described for A. naeslundii, it is a plausible effect.

The clinically relevant penetration of laser light in an in vivo situation was expected to be relatively low, mainly because the surface is treated. In addition, penetration
owing to absorption of water or buffer saturated with bacteria can be hampered. To account for this and generate a more realistic situation, the illumination step was performed in a 24-well plate, which has a surface area of 1.9 cm². Using a volume of 50 μL, the average depth of the solution is approximately 260 μm. Penetration should be sufficient for this depth and absorption effects because water can be neglected at a 660-nm wavelength. An effective action on wet surfaces can be confirmed clinically. In patients, sufficient disinfection can be achieved, although standardization is not possible to the degree in an in vitro study (Fig 3).
In this study, a 100-mW diode laser with 660-nm wavelength was used to excite MB (excitation maximum, 664 nm). This device was used because it is the exact same sterile system that the authors have applied in clinical therapy for many years (Fig 3). It remains unclear whether biofilms hamper the effectiveness of the treatment, because they might limit the penetration of dye into individual cells. However, this also is true for other disinfectants and antibiotics. In the present study, 2 of 3 independent experiments showed a decrease of bacterial load to 0 in the aPDT group. In one experiment there were few CFUs remaining. There might have been formation of bacterial clumps in this particular experiment that blocked staining of the micro-organisms or some effect of shading of parts of the laser-illuminated field. The exact cause remains elusive. Illumination of the samples was performed in a standardized manner, as described earlier. Whether such effects are relevant in vivo, where longer laser exposure and exposure from different angles occur, is unclear. Promising results of an in vitro study by Rosa et al.\(^4\) showed effective inactivation of *Staphylococcus aureus* biofilms in compact and cancellous bone by using aPDT with MB dye and laser with a 660-nm wavelength. Penetration problems were not observed. Further investigations using biofilms of bacteria grown on bone graft material and treating them with different solutions to determine their resilience are warranted.

For the first time, this study has proved the effective killing of *A. naeslundii*, cultured from a patient with MRONJ, using aPDT (660-nm wavelength, 100-mW diode laser, MB dye). Using a standardized in vitro killing method, the present study showed a meaningfully higher killing rate (≥3 log\(_{10}\)) of aPDT compared with CHX and PHX, which are regularly used. These results show the effectiveness and additional benefit of aPDT for the killing of *A. naeslundii* compared with CHX and PHX in vitro. Further investigations should be performed to evaluate the killing effect on other *Actinomyces* species and other bacteria. In the treatment of periodontitis, aPDT has been used for longer than a decade and no side-effects or bacterial resistances have been reported.\(^4\) Moreover, the authors observed no permanent staining or irritation in the wound area even after prolonged use. Some researchers have reported that aPDT is efficient against antibiotic-resistant bacteria.\(^4\)–\(^9\)

The results of this study show that aPDT could have broader use as adjuvant therapy in the management of MRONJ and other kinds of septic surgery. The aPDT system (HELBO) is CE-certified in Europe for use in maxillofacial surgery. The authors’ clinical experience with this kind of adjuvant antiseptic treatment during the past 10 years has shown very good subjective results and has contributed to their treatment of septic wounds (Fig 3). Further investigations are needed to assess the effectiveness and penetration of the method when used on biofilm-containing tissues or implants.

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