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ORIGINAL PAPER

Comparison of the Photosensitivity of Biofilms of Different Genera of Cariogenic Bacteria in Tooth Slices

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Abstract

This study compared the outcome of photosensitization on the viability of four different cariogens in planktonic form as well as biofilms in human dentine. Photodynamic therapy was carried out with a gallium aluminium arsenide laser (670 nm wavelength) using Toluidine blue O (TBO) as the photosensitizer. Cariogenic bacteria (*Streptococcus mutans*, *Lactobacillus casei*, *Streptococcus salivarius* and *Actinomyces viscosus*) were exposed to TBO and then to the laser for 1 minute in planktonic suspension. Then, tooth slices previously incubated for 24 hours with broth cultures of broth culture of the four cariogenic organisms were exposed to antimicrobial photosensitization. The control samples consisted of planktonic and sessile cells that were exposed to TBO alone, laser alone and the bacterial cells that were not treated with TBO or laser. The results showed significant reductions in the viability of *S. mutans*, *L. casei* and *A. viscosus* in both planktonic form (to 13%, 30%, and 55%, respectively) and sessile form hosted in dentinal tubules (to 19%, 13% and 52%, respectively), relative to the controls. *S. salivarius* was the least affected in planktonic (94% viability) and sessile form (86% viability). In conclusion, sensitivity to photosensitization is species-dependent and sessile biofilm cells are affected to the same extent as their planktonic counterparts.

Key words: cariogenic bacteria, planktonic, sessile cells, tooth slice, photodynamic therapy

Introduction

Despite major advances in dentistry, caries persists as a very common infectious disease of children and adults, and one of a major public health problem (Ten Cate 2013). Many bacteria are present in the mouth and assemble into a mass of accumulated bacteria on the tooth surface in the form of dental plaque, i.e., dental biofilm. Cariogenic bacteria become part of the dental biofilm during early childhood and in due course proliferate under a favorable milieu to cause disease (Smith 2002).

Although there is extensive ongoing research into development of a vaccine to prevent dental caries, there is currently no satisfactory vaccine available. In the search for an alternative approach to conventional methods of caries elimination, the antimicrobial photodynamic therapy (PDT) is becoming a popular possible choice. Different studies have reported that planktonic cells of cariogenic bacteria are sensitive to eradication by PDT (Burns et al. 1994; Williams et al. 2003; Paulino et al. 2005; Metcalf et al. 2006; Tonon et al. 2015). It remains true, however, that the causal agents of caries and periodontitis exist in biofilms on the surface of the teeth or within the tooth structure itself once the carious lesions have been initiated. Furthermore, bacteria in biofilms may be 1000 times more impervious to the action of antimicrobial agents and host defense systems compared to planktonic suspensions (Welin-Neilands and Svensater 2007; Jakubovics and Kolenbrander

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2010), and thus the effect of PDT on oral or cariogenic bacteria in biofilms has also been investigated (Zanin et al. 2006; Lee et al. 2012; Mang et al. 2012).

Appreciable destruction of *S. mutans* has been reported when the organisms were in a milieu similar to carious teeth encapsulated in collagen or inside carious bovine teeth (Williams et al. 2004; Giusti et al. 2008). Lethal photosensitization could also be achieved when *S. mutans* was enclosed in collagen and irradiated with light that first travelled through demineralized dentine (Burns et al. 1995). Studies on the effectiveness of PDT on cariogenic bacteria present as biofilms in root canals and human dentine, although limited, have also been reported. Indeed, considerable elimination of biofilms of *S. intermedius* in root canals as well as *Escherichia coli and Enterococcus faecalis* in the extensive layers of dentine has been reported (Seal et al. 2002; Schoop et al. 2004).

Despite the fact that *S. mutans* plays a principal role in the induction of caries, many other microorganisms present in dental biofilms have additionally been implicated in the evolution of the lesions (Tanzer et al. 2001). These include *S. salivarius*, *S. sanguis*, *Lactobacillus casei* (progression of lesions) and *A. viscosus* (root surface caries) (Edwardsson 1987; Tanzer et al. 2001). Since the susceptibility of biofilms of different bacterial species to PDT may vary, the objective of the present study was to investigate whether different cariogenic bacteria present as biofilms in tooth slices are susceptible to photosensitization and to compare this susceptibility to that of the same bacteria in planktonic suspension using Toluidine blue O (TBO) as the photosensitizing agent activated by light from a laser diode.

Experimental

Materials and Methods

Light source and photosensitizer. Light for photosensitization was generated with a laser diode (gallium aluminium arsenide laser) (GaAlAs) (Q-beam 2001-A, Quantum devices Inc., Barneveld, Wisconsin, USA) with a central wavelength of 670 nm, which covered the absorbance of the photosensitizer, with an output power of 65W and photon flux density of 2000 μmol of photons $m^{-2}s^{-1}$. The distance between the bacterial suspensions or tooth slice specimens and the center of the GaAlAs laser was 13.5 cm, and appropriate spot sizes (1.5 cm^2) were made with an objective lens to cover the sample.

Toluidine blue O (TBO) (Sigma Ltd., Poole, UK) was used as the photosensitizer at a concentration of 10 mg/ml in distilled water.

Bacterial strains and culture. The cariogens consisted of: *S. mutans* (NCTC 10449), *L. casei* (NCTC 6375), *A. viscosus* (ATCC 43146) and *S. salivarius* (NCTC 8606). The bacteria were routinely cultivated on blood agar (Fluka Biochemica, Buchs, Switzerland). They were also cultivated in tryptone soya broth (Oxoid Ltd, Basingstoke, UK) and incubated for 24 h aerobically at 37°C and then used for the experiments at a density of 1×10^7 colony forming units/ml.

Photosensitization of planktonic cultures. Equal volumes of cultures of each of the four bacteria (triplicate samples) in tryptone soya broth were mixed with TBO (50 μg/ml) and incubated in the dark for 5 minutes. Then, they were exposed to laser light for 1 min, after which serial dilutions were prepared to determine bacterial viability. Aliquots of 100 μl from each dilution were plated onto blood agar and incubated at 37°C under aerobic conditions for 48 h, and then the number of visible colonies was counted. Cultures that had not been exposed to photosensitizer or laser were used to determine the total number of bacteria present in the cultures at the beginning of the experiment. The experiments were repeated three times and the average of each experiment was taken and the percentage viability of each culture was calculated relative to controls (controls consisted of cultures of bacteria without being exposed to photosensitizer or laser).

The cultures of the cariogenic bacteria exposed to the laser alone for 1 min and the bacterial cultures exposed to TBO for 6 min served as additional controls in order to evaluate any light toxicity and any toxicity from the photosensitizer, respectively.

Preparation of tooth slices. The teeth used in this study were third molars (wisdom teeth) extracted from adult patients who were 20 to 35 years of age. The teeth were vital but indicated for extraction due to the fact that they were malpositioned. The extracted molars were collected and stored in 0.2% thymol (Sigma Ltd., Poole, UK). The teeth were washed and cleaned with a brush using soap and water, and the roots of the teeth were removed and the buccal and lingual surfaces were then flattened to reach the dentine using a diamond disc on a slow-speed hand piece. Following this, the teeth were sliced in the middle from the mesial to the distal surface. The slices were approximately equal in shape and size ($10 \times 6 \times 3$ mm). The tooth slices were brushed again, and exposed to 17% EDTA, followed by 5.25% NaOCl, to remove the smear layer and washed with distilled water and autoclaved for 20 minutes and stored under aseptic conditions for later use.

Photosensitization of bacteria present as biofilms in tooth slices. The different cariogenic bacteria were cultured in tryptone soya broth for 24 h at a temperature of 37°C. The tooth slices were immersed in one ml suspensions of each of the bacterial strains in sterile test tubes. The tooth slices were incubated with the bacteria for 24 h at 37°C to allow the formation of biofilms

in the dentine. Bacterial suspensions were then aspirated and the tooth slices were washed once with distilled water (1 ml). Following this, 1 ml of TBO (50 μg/ml) was applied to the tooth slices and left for 5 min in the dark. For each bacterial strain nine tooth slices were exposed TBO and laser. Furthermore, nine tooth slices were exposed to laser only, another nine were exposed to TBO only, and the controls consisted of nine tooth slices that were exposed neither to laser nor TBO. Exposure to laser was carried out in the dark. The light emitted from the diode laser was focused onto the tooth specimen, which was inside the tube so that the whole tooth surface was exposed to the light. The tooth slices were illuminated for 1 min on each side. Following this, aliquots of fresh tryptone soya broth were added to each tube and the tooth slices were agitated using a vortex mixer. Serial dilutions were then prepared and 100-μl aliquots inoculated onto blood agar. The number of colonies was then enumerated and the percentage viability of each culture was calculated relative to controls (controls consisted of tooth slices without exposure to laser or TBO).

Data and Statistical Analysis. The effect of laser alone, TBO alone, and laser and TBO on the viability of the bacteria was determined relative to the controls (100% viability). Two-way analysis of variance (ANOVA) was used to determine any significant effects of the treatment protocol or bacterial species on the percentage viability of the cariogenic bacteria. Follow up comparison between the groups were then carried out using Tukey multiple comparison test ($\alpha = 0.05$).

Results

Figures 1–4 show the results of exposure of both planktonic and sessile biofilm cells of *S. mutans*, *L. casei*, *A. viscosus*, and *S. salivarius* to laser, TBO, and laser with TBO. The results show that there was little direct toxicity with TBO as a sensitizer for the cariogenic bacteria. Similarly, irradiation with the diode laser alone did not cause any significant changes in the viability of planktonic or sessile biofilm cells.

On the other hand, treatment with TBO together with irradiation with the laser resulted in reductions in the viability of *S. mutans* to levels of 13% in planktonic cells and 19% in biofilm cells (Fig. 1). Furthermore, exposure to the laser and TBO caused reductions in the viability of *L. casei* to levels of 30% in planktonic and 13.29% in sessile biofilm cells (Fig. 2). With *A. viscosus* planktonic cells showed a viability of 55% compared to 52% in biofilm cells (Fig. 3). Interestingly, exposure of *S. salivarius* to TBO and laser did not have much effect, with 95% of the planktonic cells remaining viable compared to 86% viability of biofilm cells (Fig. 4).

Two-way analysis of variance (ANOVA) revealed that both the treatment protocol and the species of bacteria as well as the interaction between treatment and species of bacteria had highly significant effects on the viability of the cariogenic bacteria in planktonic and biofilm form ($p < 0.001$). Follow up multi comparison was carried out using Tukey's pairwise comparison to determine any significant differences between the viability of the bacterial species at each treatment protocol as well as the differences between the

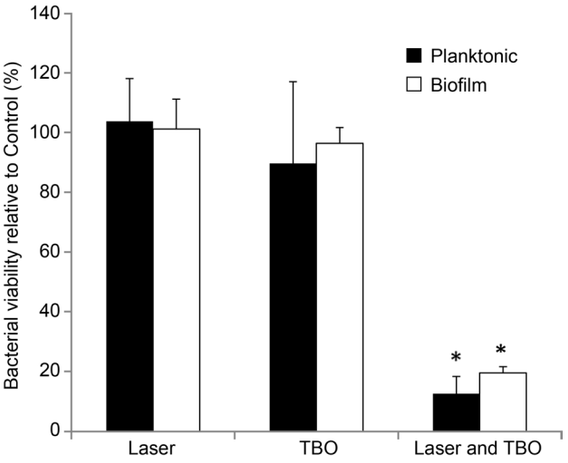

Fig. 1. Viability (mean + SD) of *S. mutans* as planktonic cells in suspension and sessile cells (biofilms) on tooth slices treated with; Laser, TBO, and Laser with TBO relative to controls (100% viability). Asterisk symbol represents statistically significant differences in viability in comparison to treatment with laser alone and TBO alone.

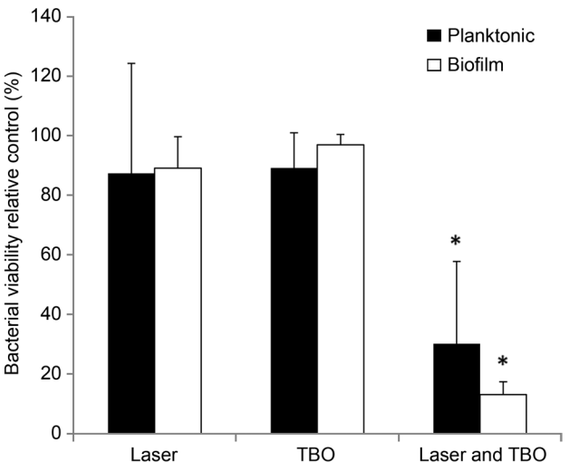

Fig. 2. Viability (mean + SD) of *L. casei* as planktonic cells in suspension and sessile cells (biofilms) on tooth slices treated with; Laser, TBO, and Laser with TBO relative to controls (100% viability). Asterisk symbol represents statistically significant differences in viability in comparison to treatment with laser alone and TBO alone.

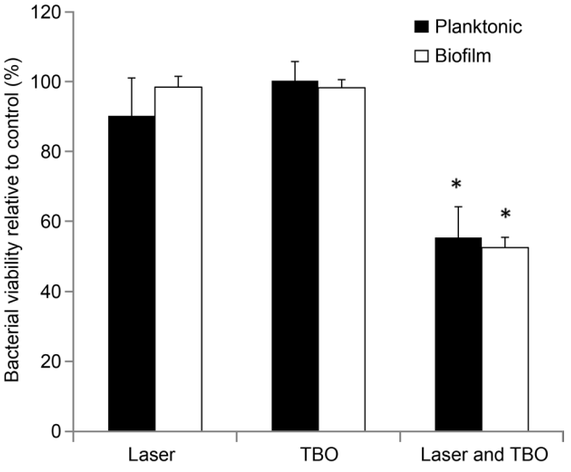

Fig. 3. Viability (mean + SD) of *A. viscosus* as planktonic cells in suspension and sessile cells (biofilms) on tooth slices treated with; Laser, TBO, and Laser with TBO relative to controls (100% viability). Asterisk symbol represents statistically significant differences in viability in comparison to treatment with laser alone and TBO alone.

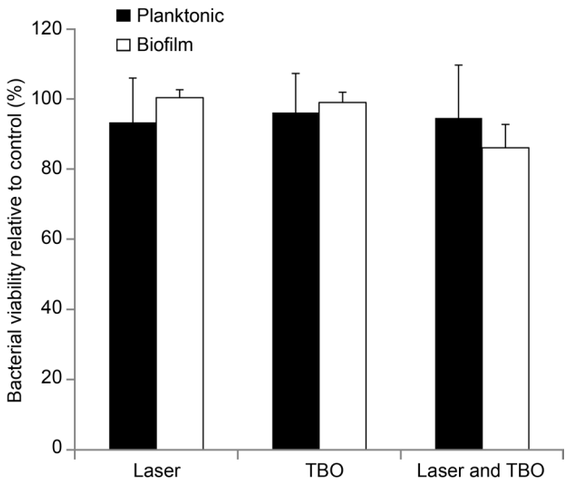

Fig. 4. Viability (mean + SD) of *S. salivarius* as planktonic cells in suspension and sessile cells (biofilms) on tooth slices treated with; Laser, TBO, and Laser with TBO relative to controls (100% viability).

treatment protocols for each bacterial species. When the effects of exposure of planktonic or biofilm cells to laser alone or TBO alone, were examined, no significant reductions in viability of any of the cariogens were observed ($p > 0.05$). However, treatment with TBO and laser caused significant reductions in bacterial viability ($p < 0.05$) in comparison to treatment with laser alone and TBO alone for both the planktonic and biofilm cells for all the bacterial species investigated. Interestingly, Tukey's pairwise comparison showed that differences in viability between planktonic and biofilm cells treated with TBO and laser were not significant ($p > 0.05$).

Discussion

Various studies have reported that successful lethal photosensitization can be achieved when oral bacteria are grown as planktonic cultures (Burns et al. 1994; Williams et al. 2003; Paulino et al. 2005; Metcalf et al. 2006; Tonon et al. 2015). However, target organisms in oral infections are present within biofilms, which are known to be impervious to the action of many antimicrobial agents, since they are protected by a network of polymeric substances and exhibit differences in structure, metabolism and gene expression (Marsh 2004; Chávez De Paz et al. 2008; Jakubovics et al. 2008; Decker et al. 2014; Van Acker and Coenye 2016).

Different studies have reported that lethal photosensitization of biofilms of cariogenic bacteria could be achieved in root canals and human dentine. However, according to the authors' knowledge, comparative studies of different genera of cariogenic bacteria present as biofilms in the tooth structure are lacking. Thus, the current study compared the sensitivity to photosensitization of three different genera of cariogenic bacteria present as biofilms in coronal tooth slices with that of their planktonic counterparts, to see if there are differences in sensitivity to this mode of therapy. The enamel was removed from the tooth specimens to expose the dentinal area, which is the part of the tooth that the cariogenic bacteria colonize after the enamel surface breaks away in the process of cavity formation. The results indicated a similar level of reduction in the viability of both planktonic and sessile biofilm cells.

Toluidine Blue O was used as the photosensitizer not only because it is very effective in sensitizing bacteria (Williams et al. 2003) but also since its absorption maxima falls within the range of the wavelength of light emitted by the diode (GaAlAs) laser chosen. Exposure of the bacteria to TBO alone had no effects on the viability of planktonic bacteria as well as their biofilm counterparts, in agreement with previous reports (Burns et al. 1994; Wilson et al. 1995; Williams et al. 2003). Furthermore, exposure to laser alone had no effects on the viability of the planktonic or biofilm cells, in contrast to a previous report where exposure of different oral bacteria to light from a diode laser for 1 min resulted in a significant decrease in viability of various oral bacteria (Chan and Lai 2003).

The whole tooth slice specimen was immersed in the culture medium containing the bacteria in order to avoid any variation in the penetration of the bacteria into the dentinal tubules and the tooth slices were incubated with the bacteria for 24 h to allow the formation of a biofilm. The procedure for preparation of the biofilm was in accordance with a previous study (O'Neill et al. 2002).

Biofilms of four species of cariogenic bacteria, (*S. mutans*, *S. salivarius*, *L. casei* and *A. viscosus*), were used since it is well established that they are important in dental caries (Samaranayake et al. 2012). Indeed, *S. mutans* is the initiator of dental caries due to its acidogenic activity that culminates in the degradation of the enamel matrix (Love et al. 2000; van Ruyven et al. 2000). Expansion from this primary focus of enamel degradation results in exposure of the underlying dentine allowing access of microorganisms to the dentinal tubules and subsequently the dental pulp (Love et al. 2000). In succession, lactobacilli play an important role in the progression to a more caries inducing plaque. Lactobacilli are frequently located in the deepest part of the lesion (dentine), under conditions of high acidity for extended periods of time (Munson et al. 2004; Aas et al. 2008). *S. salivarius* is believed to persevere in dental biofilms, colonizing teeth and soft tissues and reported to be intimately involved in health and disease of the oral cavity (Chen et al. 1996; Chen et al. 2000; Gross et al. 2012; Krzyściak et al. 2017). Endogenic Actinomyces constitute between 40 to 80% of the normal flora on adjacent tooth surfaces participating in the aggregation of different species of bacteria during dental biofilm formation and contributing to root caries and periodontal infections (Whittaker et al. 1996; Socransky and Haffajee 1997; Ruby et al. 2002; Do et al. 2017).

The current study found that planktonic cultures of *S. mutans* were the most sensitive (87% killing) followed by *L. casei* (70% killing), *A. viscosus* (45% killing) and finally *S. salivarius* (5% killing). Successful lethal photosensitization of cariogenic bacteria using similar conditions and time of exposure has been previously reported in other studies (Burns et al. 1994; Williams et al. 2003). Indeed, Williams et al. (2003) found 100% killing of *S. mutans* when exposed to lethal photosensitization for 1 min under similar conditions as the current study. Furthermore, Burns et al. (1994) observed appreciable destruction that had been achieved for *S. mutans*, *L. casei* and *A. viscosus* within 30–90 sec exposures to lethal photosensitization. Our results are also in agreement with those of Wilson et al. (1995) who found that when *S. mutans*, *L. casei* and *A. viscosus* were treated by PDT for an exposure time of 1 min, 76% reduction occurred in the viability of *S. mutans* with the same laser but a different sensitizer, while the viability of *A. viscosus* decreased by 37%.

The results also showed that photosensitization of biofilms of cariogenic bacteria in tooth slices resulted in a genus and species-dependent decrease in viability. The most susceptible cariogen was *L. casei* (87% death) followed by *S. mutans* (80 % death), *A. viscosus* (47% death) and *S. salivarius* (14% death). Our results agree with those of Zanin et al. (2006) who reported a slightly greater (95%) reduction in the number of viable *S. mutans* in biofilms cultured on enamel slabs, following PDT with TBO and diode laser. Furthermore, Ricatto et al. (2014) also found significant reductions in the viability of *S. mutans* and *L. casei* using a different sensitizer (Methylene Blue) and a diode laser. In addition, significant reductions in *S. mutans* (1.08 ± 1.20 log) and *Lactobacillus* spp. (1.69 ± 1.37 log) have been observed in dentine from deep carious lesions (Melo et al. 2015).

The results showed that *S. salivarius* was the most resistant strain. There are very few studies, if any, on the effects of PDT on biofilms of *S. salivarius*, according to the authors' knowledge. The reasons behind this resistance to PDT need further investigation.

The current study investigated the effectiveness of antimicrobial PDT on single-species biofilms cultured on coronal tooth slices and future work is in progress to investigate the outcome of PDT on polymicrobial infection encountered in the process of caries formation. The dye/laser combination was found to be effective in achieving significant elimination of biofilms of *L. casei*, *S. mutans* and *A. viscosus* in the tooth structure itself. Surprisingly, *S. salivarius* was comparatively resistant to treatment in both planktonic and sessile state.

Although it remains true that PDT can only be used superficially due to limited light penetration, we believe that PDT will be useful in the case of resin restorations or in fissure sealant application to eliminate any bacteria before applying resin-filling materials. Furthermore, in the case of deep caries with a risk of pulp exposure, the remaining layer that separates the pulp from the cavity can be exposed to PDT to eliminate any bacteria that could be present in this layer or bacteria that have penetrated into the dentinal tubules.

The advantage of PDT is in applying the photosensitizer locally, precisely to the lesion. Subsequent to the administration of the photosensitizer, light of the appropriate wavelength could be conveyed into the intended space specifically using a fiber optic cable. Therefore, with the use of PDT to treat carious lesions perturbation of the normal microbial community at other locations in the oral cavity would not occur (Gross et al. 2012; Lee et al. 2012).

Conclusion

The results of this in vitro study suggest that sensitization with TBO and exposure to light from a laser diode (GaAlAs) with a wavelength of 670 nm can kill most of *L. casei*, *S. mutans*, *A. viscosus*, and to a lesser extent *S. salivarius*, adhering to coronal tooth slices

and hosted in the dentine. Furthermore, the levels of antimicrobial photosensitization achieved with the cariogenic bacteria hosted in the dentine were similar to that achieved with planktonic cells in suspension.

Ethical Statement

Verbal consent was obtained from all patients from whom the extracted teeth samples were obtained. The Institutional Ethics Review Board of Jordan University of Science and Technology approved the consent procedure and the research. The work complied with the World Medical Association Declaration of Helsinki.

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Conflict of interest

Authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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