Eur J Oral Sci 2006; 114: 64–69
Printed in Singapore. All rights reserved

European Journal of
Oral Sciences

Photosensitization of *in vitro* biofilms by toluidine blue O combined with a light-emitting diode

Iriana C. J. Zanin[1], Maristela M. Lobo[1], Lidiany K. A. Rodrigues[2], Luiz A. F. Pimenta[1], José F. Höfling[1], Reginaldo B. Gonçalves[1]

[1]Dental School of Piracicaba, State University of Campinas, Piracicaba, SP, Brazil; [2]Faculty of Pharmacy, Dentistry and Nursing, Federal University of Ceará, Fortaleza, CE, Brazil

Zanin ICJ, Lobo MM, Rodrigues LKA, Pimenta LAF, Höfling JF, Gonçalves RB. Photosensitization of in vitro *biofilms by toluidine blue O combined with a light-emitting diode. Eur J Oral Sci 2006; 114: 64–69.* © Eur J Oral Sci, 2006

In natural ecosystems, micro-organisms grow preferentially attached to surfaces, forming matrix-enclosed biofilms. The aim of this study was to determine photodestruction levels in biofilms after subjecting them to photodynamic therapy. Biofilms of *Streptococcus mutans*, *S. sobrinus*, and *S. sanguinis* were grown on enamel slabs for 3, 5 or 7 d. Both the number of viable micro-organisms and the concentration of water-insoluble polysaccharides were analysed, and mineral loss (ΔZ) analyses were performed on the enamel slabs. The antimicrobial potential of toluidine blue O (0.1 mg ml^{-1}), associated with 85.7 J cm^{-2} of a light-emission diode, was evaluated on the viability of 5-d biofilms. Both the number of micro-organisms and the concentration of water-insoluble polysaccharide increased with the age of the biofilms. A significant reduction ($\approx$ 95%) in viability was observed for *S. mutans* and *S. sobrinus* biofilms following photosensitization, with a $>$ 99.9% reduction in the viability of *S. sanguinis* biofilms. In conclusion, a biofilm model was shown to be suitable for studying changes in bacterial numbers and enamel mineralization and for demonstrating the potential value of photosensitization in the control of *in vitro* biofilms.

Reginaldo B. Gonçalves, Dental School of Piracicaba – UNICAMP, 901 Limeira Avenue, Piracicaba, Brazil 13414–900

Telefax: +55–19–34125218
E-mail: reginald@fop.unicamp.br

Key words: antimicrobial agents; cariogenic bacteria; photodynamic therapy; *Streptococcus mutans*

Accepted for publication September 2005

Microbial biofilm can be defined as a diverse community of micro-organisms found on a surface, usually with a liquid interface (1). Dental plaque has the properties of a biofilm because it is formed on the tooth surface, is bathed by saliva, and contains a community of numerous bacteria embedded in an extracellular matrix of polymers (2). Clear evidence is now available showing that cells in biofilms are in a physiological state that differs from their planktonic counterparts, and tend to be less susceptible to antimicrobial agents (3, 4).

The complex bacterial biofilm accumulation on non-shedding tooth surfaces and the tongue allows the resident bacteria to survive removal forces and also contributes to their protection against host-specific and non-specific defence mechanisms and harmful agents from external environments (5). The resistance mechanisms also include physical or chemical diffusion barriers to antimicrobial agent penetration into the biofilm, slower growth of the biofilm owing to regions of nutrient limitation, activation of general stress responses, and the emergence of biofilm-specific phenotypes (6).

It is well known that the accumulation of bacterial biofilms on tooth surfaces results in some of the most prevalent bacterial-induced human diseases, caries and inflammatory periodontal diseases (7, 8). Current treatment of subjects with plaque-related diseases involves mechanical removal of the biofilm and the use of antiseptics and antibiotics. However, the emergence of antibiotic resistance in pathogenic bacteria has led major research efforts towards discovering alternative antibacterial therapeutics to which bacteria would not develop resistance (9).

In this context, photosensitization may constitute a suitable process to combat both biofilm-related resistance and antimicrobial resistance. In this process, a photoactive dye, called a photosensitizer, is taken up into cells and is irradiated with light of an appropriate wavelength. This may result in cell death through the production of active oxygen species (10). Generally, in photosensitization processes, the light or dye alone are non-toxic (11–14) and therefore only cells that contain photosensitizer and also receive light are affected by the treatment. Thus, the use of this treatment provides an opportunity to achieve selectivity and to target specific areas of the mouth or plaque (15).

Previous studies have shown that photosensitization using white light or conventional lasers, such as helium/neon (HeNe) and gallium aluminum arsenide (GaAlAs) and a suitable photosensitizer, such as aluminum disulfonated phthalocyanine ($AlPcS_2$), toluidine blue O (TBO) or cationic Zn (II) phthalocyanine, are capable of killing Gram-positive oral bacteria (12, 14–19). However, there have been no previous studies on the antimicrobial effects of TBO associated with a red light-emission diode

(LED) on the viability of single-species biofilms of oral streptococci. Moreover, TBO is a cell membrane active photosensitizer (20), which can absorb red laser light and is bactericidal for multiple species, including organisms such as streptococci that are implicated in dental caries (14, 15, 21). This photosensitizer was found to be effective *in vitro*, even when sensitizing substances from the oral environment, such as demineralized dentine and collagen, were present (21). This is particularly relevant when one considers that there is restricted penetration of substances into plaque biofilms during brief topical exposure, which may limit their anticaries efficacy (22). Moreover, it has been shown that the light dose required to kill bacteria treated with TBO is far lower than that causing toxicity in cultured human keratinocytes and fibroblasts (23).

Hence, this study investigated the cariogenic potential of single-species biofilms of oral streptococci *in vitro* and studied the antimicrobial effect of TBO associated with a red LED on the viabilities of these biofilms.

Material and methods

Preparation of enamel slabs

Enamel slabs ($4 \times 4 \times 2$ mm) were prepared from bovine incisors that had been stored in 0.1% (v/v) thymol solution at 4°C for 30 d (24). The enamel surface was polished for baseline surface microhardness determinations and subsequent selection of specimens (25) (Fig. 1). The prepared tooth slabs were randomly assigned to 12 different groups according to micro-organism (*Streptococcus mutans*, *S. sobrinus* or *S. sanguinis*) and biofilm age (3, 5 or 7 d of biofilm growth). Square-shaped adhesive tape (4×4 mm) was attached to the centre of the enamel slab. The remaining surfaces of the slab were painted with acid-resistant nail varnish (Colorama, CEIL Coml. Exp. Ind. Ltda., Sao Paulo, Brazil), so that a 16-mm^2 enamel surface area was exposed after removing the adhesive tape. The enamel slabs were fixed in the lids of glass container vessels with orthodontic wire, kept immersed in sterile distilled water, and then sterilized in a gamma radiation chamber (Gammacell 220 Excel, GC-220E; MDS Nordion, Ottowa, Canada) (26, 27).

Growth of biofilms

After sterilization, the dental slabs were removed from the distilled water and immersed in sterile brain-heart infusion broth (BHI; Merck, Darnstadt, Germany) containing 5% (w/v) sucrose (28). All BHI-containing glass vessels, except those in the control groups, were inoculated with 0.1 ml [1–2×10^8 colony-forming units (CFU) ml^{-1}] of an overnight culture of *S. mutans* ATCC 25175, *S. sobrinus* ATCC 27607 or *S. sanguinis* ATCC 10556. Inoculation of each BHI-containing glass vessel was performed only once, and the enamel specimens were transferred into fresh medium every 24 h (26). At each transfer time, samples of the cultures were streaked onto BHI agar plates and incubated at 37°C in order to check purity. The biofilms formed on the specimens were analysed after 3, 5 or 7 d of growth. At the end of each experimental period, the biofilms were washed twice in sterile saline solution in order to remove loosely bound material (29) and were collected with sterile plastic curettes

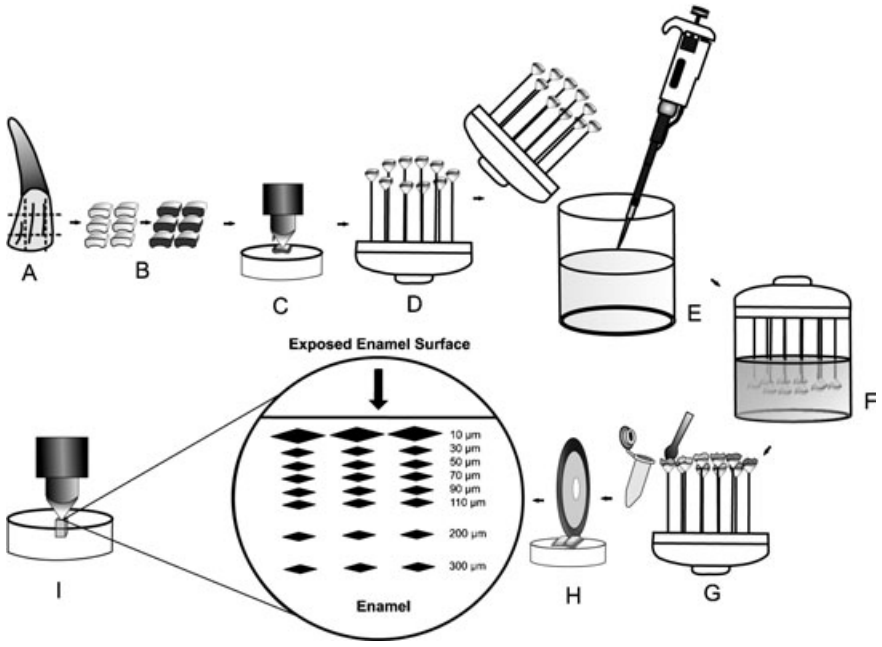

Fig. 1. (A) Enamel slabs prepared from bovine incisors stored in 0.1% thymol. (B) Flattened and polished slabs were painted with acid-resistant nail varnish, leaving a 16-mm^2 enamel surface area to be exposed to cariogenic challenge. (C) Baseline surface microhardness determination. (D) Enamel slabs fixed with orthodontic wires into the lids of glass container vessels. (E) Inoculation of medium with *Streptococcus mutans*, *S. sobrinus* or *S. sanguinus* cultures. (F) Biofilm growth; enamel specimens were transferred into fresh medium, containing 5% (w/v) sucrose, every 24 h. (G) Biofilms collected for microbiological and water-insoluble polysaccharide analyses after 3, 5 or 7 d of growth. (H) Tooth slabs were longitudinally sectioned through the centre of the exposed enamel area. (I) One of the halves was randomly assigned to cross-sectional microhardness measurements.

(Fig. 1). The collected material was placed into two pre-weighed microcentrifuge tubes (one for water-insoluble polysaccharide analysis and the other for microbiological counting), and the biofilm was analytically weighed.

Water-insoluble polysaccharide analyses

NaOH (1 M) was added to the biofilms (0.1 ml mg^{-1}), the samples were homogenized, subjected to continuous shaking for 3 h, and then centrifuged for 5 min at 13 000 *g*. The concentration of water-insoluble polysaccharide was determined in the supernatant using the phenol/sulfuric method (30).

Microbiological analyses

Biofilms were placed in 0.9% (w/v) NaCl (0.1 ml mg^{-1}) and sonicated on ice using a Branson Sonifier Cell disruptor S250 (Sonifier®, Danbury, USA) at 60 W for 15 s in order to obtain suspensions containing single cells (31). Suspensions were serially diluted, plated onto BHI agar, and the plates were incubated at 37°C, at a partial pressure of 10% CO_2, for 48 h. After incubation, the numbers of surviving micro-organisms were determined by colony counting, and the values were expressed as CFU per milligram of biofilm.

Microhardness assessment

At the end of each experimental period, the tooth slabs were longitudinally sectioned through the centre of the exposed enamel area. One of the halves was embedded in epoxy resin, and the cut surface was serially polished with aluminum oxide disks of 400, 600 and 1200 grain, and a 1-μm diamond paste. In all samples, three lanes of eight indentations each were made at depths of 10, 30, 50, 70, 90, 110, 200 and 300 μm from the outer enamel surface in the central region of the dental slab, using a Knoop diamond under a 25-*g* load for 5 s (Fig. 1). The distance between the lanes was 100 μm. The mean Knoop hardness number values at each distance from the surface were obtained and converted into volume per cent mineral (32). Volume per cent mineral was plotted against depth for each specimen, and the integrated mineral content of the lesion was calculated. A mean value of volume per cent mineral for depths greater than 100 μm was used as a measure of the integrated mineral content of sound inner enamel. To compute ΔZ parameters, the integrated mineral content of the lesion was subtracted from the value obtained for sound enamel (33).

Photosensitizer and light sources

Toluidine blue O was obtained from Sigma Chemicals (Poole, UK), dissolved in deionized water (0.1 mg ml^{-1}), and stored in the dark. The light source used was an LED (Laserbeam, Rio de Janeiro, Brazil) with a spectrum of emission ranging from 620 to 660 nm and a 638.8-nm predominant wavelength. The LED light source output power was 32 mW, and biofilms were exposed to an 85.7 J cm^{-2} energy density after 7 min of irradiation.

Photosensitization

Biofilms were grown, as described above, for 5 d and then washed twice in sterile saline solution in order to remove loosely bound material. Next, biofilms on enamel surfaces were sensitized with TBO solution for 5 min and were exposed to a red LED light. Four set conditions were tested, as follows: untreated biofilm controls (S-L-); biofilms treated with TBO, but not exposed to LED (S + L-); biofilms exposed to LED, but not treated with TBO (S-L +); and biofilms sensitized with TBO and exposed to LED light (S + L +). After photosensitization, the biofilms were collected and weighed, placed in 0.9% (w/v) NaCl (10 mg ml^{-1} final concentration of biofilm) and sonicated, as previously described, in order to disperse cells (31). Tenfold serial dilutions were carried out, and aliquots were plated onto BHI agar to determine the numbers of viable organisms, as described above.

Statistical analysis

Two-way analysis of variance (ANOVA) with interaction allowed the significance of the factors under study to be determined (CFU mg^{-1}, mineral loss, and polysaccharide concentration) and the interactions between them related to the three variables. The difference between treatments was assessed by the Tukey test. In order to assess the effect of photosensitization process on cariogenic biofilms, the dependent variables were sensitizer and LED light. The data were evaluated to check the equality of variances and normal distribution of errors, and then they were transformed to $\log_{10}$. To determine the significance of the irradiation alone, the presence of sensitizer alone and the combination of sensitizer and light, the data were analysed by a variance analysis (ANOVA) model using the factorial (2×2) design. The Tukey test was chosen to evaluate the significance of all pairwise comparisons. For both experiments, three repetitions were carried out with 10 enamel slabs in each group. The software SAS (version 8.02; SAS Institute Inc., Cary, NC, USA) was used with the significance limit set at 5%.

Results

The *P*-values for the factors under study, micro-organism, and time, were statistically significant for all analysed variables, showing that type of micro-organism and age of biofilm influenced the cariogenicity of the biofilms (Table 1). However, their interaction was statistically significant only for microbial counts (Table 1). Table 2 shows the results for CFU mg^{-1} of biofilm, mineral loss (ΔZ), and water-insoluble polysaccharide concentrations

Table 1

P-*values obtained for each response variable tested according to the factors under study*

Factors	CFU mg^{-1}	ΔZ	WIP
Micro-organism	< 0.0001	0.0007	0.0311
Time	< 0.0001	< 0.0001	< 0.0001
Micro-organism * Time	< 0.0001	0.1271	0.0731

P-values for the dependent variables microbiological counting [colony-forming units (CFU) mg^{-1}], mineral loss (ΔZ), and water-insoluble polysaccharide (WIP), according to the factors micro-organisms and age of biofilms.
Significance level, $P < 0.05$ (Tukey test).

Table 2

Response variables analysed in Streptococcus mutans, S. sobrinus and S. sanguinis biofilms at 3, 5 and 7 d of growth

Biofilm	Variables								
	10^6 CFU mg^{-1}			ΔZ (vol percentage min^{-1} μm^{-1})			WIP (mg g^{-1})		
	3 d	5 d	7 d	3 d	5 d	7 d	3 d	5 d	7 d
S. mutans	1.31 b A (0.51)	1.88 b B (1.55)	32.7 a A (25.4)	3399.3 b A (611.9)	4939.9 a A (461.7)	5686.6 a A (1173.6)	328.6 a A (73.4)	446.7 a A (157.1)	398.2 a A (195.6)
S. sobrinus	3.00 b A (0.94)	27.3 a A (20.0)	54.6 ab A (11.8)	2714.3 b AB (657.4)	4728.9 a AB (409.5)	5677.6 a A (681.7)	211.9 b A (22.7)	324.9 ab A (143.3)	398.1 a A (160.8)
S. sanguinis	0.18 b B (0.17)	1.63 a B (0.60)	3.75 a B (3.57)	2566.9 c B (709.3)	3917.7 b B (791.0)	5451.8 a A (555.2)	194.6 b A (54.4)	279.4 ab A (92.6)	459.9 a A (166.4)

Data are expressed as mean (standard deviation) of colony-forming units per milligram of biofilm (CFU mg^{-1}), mineral loss (ΔZ) and water-insoluble polysaccharide concentration (WIP) produced by the tested biofilms ($n = 3$). Statistical differences are indicated by different lower case letters in rows and by capital letters in columns (Tukey test, $P < 0.05$).

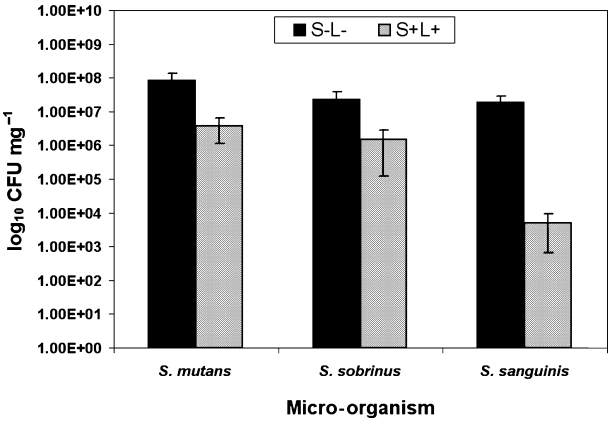

Fig. 2. Effects of toluidine blue O (TBO) and light-emission diode (LED) light (S + L +) on the viabilities of *Streptococcus mutans*, *S. sobrinus* and *S. sanguinus* biofilms compared with untreated controls (S-L-). Data represent the mean values ($n = 3$) and error bars represent standard deviations. CFU mg^{-1}, colony-forming units per milligram of biofilm.

produced by *S. mutans*, *S. sobrinus*, and *S. sanguinis* biofilms after 3, 5 or 7 d of growth. The extent of demineralization and the numbers of micro-organisms present increased according to the age of biofilms for the three bacteria tested. *S. mutans* and *S. sobrinus* biofilms were more cariogenic in the initial stages of demineralization. However, there were no differences between the extent of softened enamel for the three streptococcal species after 7 d of growth. A higher level of water-insoluble polysaccharide production was observed for the older biofilms.

The antimicrobial effects of photosensitization on the viabilities of 5-d biofilms of *S. mutans*, *S. sobrinus*, and *S. sanguinis* are shown in Fig. 2. Neither irradiation of the biofilms in the absence of TBO (S-L +), nor incubation with TBO alone (S + L-), had significant effects on the viabilities of the streptococcal biofilms ($P < 0.05$). The 95% confidence intervals for all the controls for the three bacteria were $5.47–8.13 \times 10^7$ for *S. mutans*, $2.26–3.01 \times 10^7$ for *S. sobrinus*, and $1.08–1.82 \times 10^7$ for *S. sanguinis*. Significant decreases in bacterial viability were observed only when biofilms were exposed to both TBO and LED light (S + L +). Significant reductions in the median viable counts from 7.45×10^7 to 3.75×10^6 CFU mg^{-1} were observed for *S. mutans* biofilms, and from 3.01×10^7 to 1.48×10^6 CFU mg^{-1} for *S. sobrinus* biofilms after photosensitization (Fig. 2). *S. sanguinis* biofilms were more susceptible to photosensitization, with the viable counts reduced from 1.76×10^7 to 4.79×10^3 CFU mg^{-1} (Fig. 2). These values correspond to reductions in viability of $\approx$95% for *S. mutans* and *S. sobrinus* biofilms, and of 99.9% for *S. sanguinis* biofilms.

Discussion

Knowledge of biofilm composition and metabolism may lead to improvement of measures for preventing or

controlling dental caries development. However, investigation of plaque *in vivo* is difficult owing to the high degree of variability between individuals and because the site-specific microbial composition reflects the influences of a multitude of uncontrollable endogenous and exogenous dietary factors (34). Therefore, the first aim of this investigation was to standardize a simple model for culturing, *in vitro*, intact monospecies biofilms. The biofilms were formed at a solid–liquid interface using enamel slabs as a substrate and with the periodic replacement of nutrient growth medium. The biofilm model used has some inadequacies, such as the lack of continuous irrigation of the enamel slab with saliva substitutes. However, the nutrient media used can sustain the growth of a wide range of oral organisms, and the biofilm model tested maintained its cariogenic potential, with the number of micro-organisms, concentration of water-insoluble polysaccharide, and extent of mineral loss all increasing over time. Water-insoluble polysaccharide may be related to cariogenicity as, for many oral streptococci, extracellular glucans, produced in the presence of sucrose, promote adhesion and accumulation of cells to form oral biofilms (35). Research into dental plaque development and the aetiology of dental caries has established the central role of glucans in sucrose-dependent adhesion and there is a positive correlation between sucrose consumption and caries rates (36–38). For these reasons, many of the initial investigations into the virulence of the mutans group streptococci have focused on the synthesis of glucans by bacteria growing on smooth tooth surfaces. In addition, it is important to test the photosensitization process in biofilms containing high concentrations of extracellular polysaccharide as it may affect penetration of the photosensitiser, as well as reduce the quantity of light reaching the bacteria, thereby decreasing the effectiveness of the photosensitizing process (39). The results of the present study imply that all three streptococcal species tested in monoculture induced artificial enamel lesions to some extent, as described previously (28, 40, 41).

Although there is consensus that the use of human teeth is more relevant for demineralization studies, bovine teeth have also been used in *in vitro* microbial models (41). The advantage of using bovine instead of human teeth is that they are easier to obtain and to manipulate (25). Moreover, they have a relatively more uniform chemical composition, which allows for lower variations in experimental responses of the cariogenic treatments carried out on the substrate (25).

Previous studies have shown that photosensitization is capable of killing oral bacteria in planktonic cultures (18, 19). However, as clear evidence is now available that bacteria grown in biofilms are more resistant to antimicrobial agents (3,4), studies that focus on the effect of photosensitization in biofilms are required. In spite of the present study having used a LED light, its results corroborate those found in other studies that employed a wide variety of photosensitizers and conventional lasers on biofilms (12, 14, 15, 42) or plaque scrapings (17). As in related reports, the laser light and photosensitizer alone had no effect on the viabilities of the organisms in biofilms (43). Furthermore, this investigation found a significant reduction in the viable numbers of *S. mutans* and *S. sobrinus* biofilms after photosensitization, which is significant, as these species are amongst those reported to be most highly associated with caries in humans. However, under the tested parameters, these species were less susceptible to photosensitization than *S. sanguinis*. This might be explained by the great ability of mutans streptococci to produce polysaccharide from sucrose (44), which could then retard the diffusion of TBO into *S. mutans* and *S. sobrinus* biofilms, compared with *S. sanguinis* biofilms. The polysaccharide matrix acting as a diffusion barrier has been linked with a reduction of biofilm susceptibility to antimicrobial agents (45), especially when short-term antimicrobial treatments are used.

Dental caries may be a disease well suited to photosensitization therapy. Caries is often a localized infection, and so the sensitizer could be applied to the lesion by means of a syringe and the light could then be delivered via an optical fibre (46). If bacteria within carious lesions could be eradicated by photosensitization *in vivo*, there would be beneficial consequences for dental health. Infected or damaged dentine could be better preserved, thereby making patient treatment easier (for both dentist and patient) by enabling lesions to be restored with minimal tissue removal, and improving the long-term prognosis for the repaired tooth (47). In addition, the option of a LED light instead of a laser light has obvious financial advantages when compared with photosensitization using conventional lasers. Furthermore, the large band emission of an LED (620–660 nm) may be interesting, as there is light emission in the entire absorption spectrum of TBO (632 ± 8 nm), which may promote optimization of the photodynamic process.

In conclusion, the present study suggests that the biofilm model tested is effective for growing biofilms under high cariogenic challenge. The association of TBO and a red LED light at an energy density of 85.7 J cm^{-2} is effective in significantly reducing the viabilities of streptococcal biofilms grown *in vitro*. Although the results of this work are encouraging, further studies are needed to determine whether photosensitization of cariogenic biofilms is possible under conditions more closely resembling those encountered in the oral cavity, such as biofilms formed under a continuous supply of saliva substitutes, and biofilms grown *in situ*.

Acknowledgements – The authors thank Ms. Benedita Inês Franco Possignolo Rodrigues and Mr Paulo Cassieri Neto from CENA (Centro de Estudos Nucleares da Agricultura), for the gamma irradiation procedures. This research was supported by FAPESP process no. 02/00393–8.

References

1. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. *Science* 1999; **284**: 1318–1322.

2. Marsh PD, Bradshaw DJ. Physiological approaches to the control of oral biofilms. *Adv Dent Res* 1997; **11**: 176–185.
3. Fletcher M. The physiological activity of bacteria attached to solid surfaces. *Adv Microb Physiol* 1991; **32**: 53–85.
4. Davey ME, O'Toole GA. Microbial biofilms: from ecology to molecular genetics. *Microbiol Mol Biol Rev* 2000; **64**: 847–867.
5. Bowden GH. Controlled environment model for accumulation of biofilms of oral bacteria. *Methods Enzymol* 1999; **310**: 216–224.
6. Mah TF, O'Toole GA. Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol* 2001; **9**: 34–39.
7. Kristoffersson K, Gröndahl H-G, Bratthall D. The more *Streptococcus mutans*, the more caries on approximal surfaces. *J Dent Res* 1985; **64**: 58–61.
8. Sbordone L, Bortolaia C. Oral microbial biofilms and plaque-related diseases: microbial communities and their role in the shift from oral health to disease. *Clin Oral Invest* 2003; **7**: 181–188.
9. Hamblin MR, Hasan T. Photodynamic therapy: a new antimicrobial approach to infectious disease? *Photochem Photobiol Sci* 2004; **3**: 436–450.
10. Malik Z, Hanania J, Nitzan Y. Bactericidal effects of photoactivated porphyrins – an alternative approach to antimicrobial drugs. *J Photochem Photobiol B* 1990; **5**: 281–293.
11. Minnock A, Vernon DI, Schofield J, Griffiths J, Parish JH, Brown ST. Photoinactivation of bacteria. Use of a cationic water-soluble zinc phthalocyanine to photoinactivate both gram-negative and gram-positive bacteria. *J Photochem Photobiol B* 1996; **32**: 159–164.
12. Wilson M, Burns T, Pratten J. Killing of *Streptococcus sanguis* in biofilms using a light-activated antimicrobial agent. *J Antimicrob Chemother* 1996; **37**: 377–381.
13. Komerik N, Wilson M. Factors influencing the susceptibility of Gram-negative bacteria to toluidine blue O-mediated lethal photosensitization. *J Appl Microbiol* 2002; **92**: 618–623.
14. O'Neill JF, Hope CK, Wilson M. Oral bacteria in multispecies biofilms can be killed by red light in the presence of toluidine blue. *Lasers Surg Med* 2002; **31**: 86–90.
15. Wood S, Nattress B, Kirkham J, Shore R, Brookes S, Griffiths J, Robinson C. An in vitro study of the use of photodynamic therapy for the treatment of natural oral plaque biofilms formed in vivo. *J Photochem Photobiol B* 1999; **50**: 1–7.
16. Burns T, Wilson M, Pearson GJ. Killing of cariogenic bacteria by light from a gallium aluminum arsenide diode laser. *J Dent* 1994; **22**: 273–278.
17. Wilson M, Burns T, Pratten J, Pearson GJ. Bacteria in supragingival plaque samples can be killed by low-power laser light in the presence of a photosensitizer. *J Appl Bacteriol* 1995; **78**: 569–574.
18. Zanin ICJ, Brugnera A Jr, Goncalves RB. *In vitro* study of bactericidal effect of low level laser therapy in the presence of photosensitizer on cariogenic bacteria. *Lasers Dentistry III* 2002; **2**: 154–161.
19. Williams JA, Pearson GJ, Colles MJ, Wilson M. The effect of variable energy input from a novel light source on the photoactivated bactericidal action of toluidine blue O on Streptococcus Mutans. *Caries Res* 2003; **37**: 190–193.
20. Wainwright M. Photodynamic antimicrobial chemotherapy (PACT). *J Antimicrob Chemother* 1998; **42**: 13–28.
21. Burns T, Wilson M, Pearson GJ. Effect of dentine and collagen on the lethal photosensitization of *Streptococcus mutans*. *Caries Res* 1995; **29**: 192–197.
22. Watson PS, Pontefract HA, Devine DA, Shore RC, Natress BR, Kirkham J, Robinson C. Penetration of fluoride into natural plaque biofilms. *J Dent Res* 2005; **84**: 451–455.
23. Soukos NS, Wilson M, Burns T, Speight PM. Photodynamic effects of toluidine blue on human oral keratinocytes and fibroblasts and *Streptococcus sanguis* evaluated *in vitro*. *Lasers Surg Med* 1996; **18**: 253–259.
24. Amaechi BT, Higham SM, Edgar WM. Factors affecting the development of carious lesions in bovine teeth *in vitro*. *Arch Oral Biol* 1998; **43**: 619–628.
25. Mellberg JR. Hard-tissue substrates for evaluation of cariogenic and anti-cariogenic activity in situ. *J Dent Res* 1992; **71**: 913–919.
26. Edmunds DH, Whittaker DK, Green RM. Suitability of human, bovine, equine and ovine tooth enamel for studies of artificial bacterial carious lesions. *Caries Res* 1988; **22**: 327–336.
27. Amaechi BT, Higham SM, Edgar WM. Efficacy of sterilization methods and their effect on enamel demineralization. *Caries Res* 1998; **32**: 441–446.
28. Gilmour ASM, Edmunds DH, Newcombe RG. Prevalence and depth of artificial caries-like lesions adjacent to cavities prepared in roots and restored with a glass ionomer or a dentin-bonded composite material. *J Dent Res* 1997; **76**: 1854–1861.
29. Hayacibara MF, Rosa OP, Koo H, Torres SA, Costa B, Cury JA. Effects of fluoride and aluminum from ionomeric materials on *S. mutans* biofilm. *J Dent Res* 2003; **82**: 267–271.
30. Dubois M, Gilles K, Hamilton JK, Rebers PA, Smith F. A colorimetric method for the determination of sugars. *Nature* 1951; **28**: 167.
31. Rosalen PL, Bowen WH, Pearson SK. Effect of copper co-crystallized with sugar on caries development in desalivated rats. *Caries Res* 1996; **30**: 367–372.
32. Featherstone JDB, Ten Cate JM, Shariati M, Arends J. Comparison of artificial caries-like lesions by quantitative microradiography and microhardness profiles. *Caries Res* 1983; **17**: 385–391.
33. Featherstone JDB, Shariati M, Brugler S, Fu J, White DJ. Effect of an anticalculus dentifrice on lesion progression under pH cycling conditions *in vitro*. *Caries Res* 1988; **22**: 337–341.
34. Thurnheer T, Gmur R, Guggenheim B. Multiplex FISH analysis of a six-species bacterial biofilm. *J Microbiol Methods* 2004; **56**: 37–47.
35. Gibbons RJ, Van Houte J. On the formation of dental plaques. *J Periodontol* 1973; **44**: 347–360.
36. Hamada S, Slade HD. Biology, immunology, and cariogenicity of *Streptococcus mutans*. *Microbiol Rev* 1980; **44**: 331–384.
37. Loesche WJ. Role of *Streptococcus mutans* in human dental decay. *Microbiol Rev* 1986; **50**: 353–380.
38. Nobre Dos Santos M, Melo Dos Santos L, Francisco SB, Cury JA. Relationship among dental plaque composition, daily sugar exposure and caries in the primary dentition. *Caries Res* 2002; **36**: 347–352.
39. Gad F, Zahra T, Hasan T, Hamblin MR. Effects of growth phase and extracellular slime on photodynamic inactivation of Gram-positive pathogenic bacteria. *Antimicrob Agents Chemother* 2004; **48**: 2173–2178.
40. Gilmour ASM, Edmunds DH. The polarized light microscope appearance of caries-like lesions adjacent to restored cavities in the crowns and roots of extracted human teeth. *J Oral Rehab* 1998; **25**: 929–939.
41. Shu M, Wong L, Miller JH, Sissons CH. Development of multi-species consortia biofilms of oral bacteria as an enamel and root caries model system. *Arch Oral Biol* 2000; **45**: 27–40.
42. Soukos NS, Mulholland SE, Socransky SS, Doukas AG. Photodestruction of human dental plaque bacteria: enhancement of the photodynamic effect by photomechanical waves in an oral biofilm model. *Lasers Surg Med* 2003; **33**: 161–168.
43. Zanin ICJ, Gonçalves RB, Brugnera A Jr, Hope CK, Pratten J. Susceptibility of *Streptococcus mutans* biofilms to photodynamic therapy: an *in vitro* study. *J Antimicrob Chemother* 2005; **56**: 324–330.
44. Banas JA, Vickerman MM. Glucan-binding proteins of the oral streptococci. *Crit Rev Oral Biol Med* 2003; **14**: 89–99.
45. Gordon CA, Hodges NA, Marriott C. Use of slime dispersants to promote antibiotic penetration through the extracellular polysaccharide of mucoid *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 1991; **35**: 1258–1260.
46. Wilson M. Photolysis of oral bacteria and its potential use in the treatment of caries and periodontal disease. *J Appl Bacteriol* 1993; **75**: 299–306.
47. Wilson M. Lethal photosensitization of oral bacteria and its potential application in the photodynamic therapy of oral infections. *Photochem Photobiol* 2004; **3**: 412–418.