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Effect of photo-activated disinfection on endodontic pathogens *ex vivo*

L. Bergmans[1], P. Moisiadis[1], B. Huybrechts[1], B. Van Meerbeek[1], M. Quirynen[2] & P. Lambrechts[1]

[1]BIOMAT Research Cluster, Catholic University of Leuven, Leuven, Belgium; and [2]Research Group for Microbial Adhesion, School of Dentistry, Oral Pathology and Maxillo-Facial Surgery, Catholic University of Leuven, Leuven, Belgium

Abstract

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Aim To test the hypothesis that photo-activated disinfection (PAD) has a bactericidal effect on pathogens inoculated in root canals, with emphasis on biofilm formation/destruction.

Methodology Root canals of extracted teeth (n = 38) were prepared (size 30, 0.10 taper), autoclaved, divided into three groups and two negative controls inoculated (*Streptococcus anginosus*, *Enterococcus faecalis or Fusobacterium nucleatum*) and treated (PAD, laser, dye or positive control) according to a cross-sectional design. Resultant colony-forming unit counts were associated with observations of cell structural changes using environmental scanning electron microscopy (ESEM) on inoculated dentinal surfaces (n = 22, two controls) before (1, 2 and 6 days of incubation) and after treatment with PAD.

Results Treatment of root canals with PAD (15 J) caused a significant reduction of the bacterial load, resulting in a 93.8% kill of *S. anginosus* ($P < 0.0001$), a 88.4% kill of *E. faecalis* ($P < 0.05$) and a 98.5% kill of *F. nucleatum* ($P < 0.0001$), but no sterilization. Laser alone had no significant effect on the load nor did the dye without laser. The ESEM experiment showed that individual cells or monolayers were easily eliminated with PAD. But when biofilms were present (2 and 6 days for *E. faecalis*, 6 days for *S. anginosus*), bacterial eradication was substantially reduced in deep layers.

Conclusions Photo-activated disinfection is not an alternative but a possible supplement to the existing protocols for root canal disinfection as the interaction between light (diode laser) and associated dye (TBO) provides a broad-spectrum effect. Some endodontic pathogens that grow as single-species biofilms, however, are difficult to eradicate.

Keywords: bacterial morphology, culturing, environmental scanning electron microscopy, laser, root canal infection.

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Introduction

The main treatment objective in teeth with apical periodontitis is the reduction or elimination of endodontic pathogens (Kakehashi *et al.* 1965). For that reason, the routine clinical procedure consists of mechanical instrumentation and chemical irrigation with sodium hypochlorite (NaOCl). In addition, the use of chlorhexidine (CHX) (Gomes *et al.* 2003), iodine potassium iodide (IKI) (Safavi *et al.* 1990, Baker *et al.* 2004), citric acid and/or ethylenediaminetetraacetic acid (EDTA) (Scelza *et al.* 2004) have been advocated to increase the disinfection potential. When irrigating, fine gauge needles can be used to facilitate deep penetration of the liquid but additional agitation using small size instruments, gutta-percha cones and/or ultrasonics is recommended (Huque *et al.* 1998, Lee *et al.* 2004b). The removal of smear layer from the canal wall, on the other hand, is common practice

Correspondence: Dr Lars Bergmans, Leuven BIOMAT Research Cluster, Department of Conservative Dentistry, School of Dentistry, Oral Pathology and Maxillo-Facial Surgery, Catholic University of Leuven, Kapucijnenvoer 7, B-3000 Leuven, Belgium (Tel.: 32 16 332466; fax: 32 16 332752; e-mail: lars.bergmans@med.kuleuven.be).

although there is no clear evidence that this procedure enhances disinfection or treatment outcome (Ørstavik & Haapasalo 1990).

Unfortunately, careful chemomechanical preparation in one visit cannot render all infected systems bacteria-free (Sjögren *et al.* 1997, Shuping *et al.* 2000). The greatest challenges are the morphological complexity of the root canal system, particularly of molars, and the ecological organization of the root canal microbiota into protected sessile biofilms (Nair 1987, Nair *et al.* 2005). Whilst microbial killing may continue after filling because of the antibacterial properties of sealer and/or gutta-percha (Saleh *et al.* 2004) or by blocking access to nutrients (Sundqvist & Figdor 1998), filling root canals having a positive culture will, in general, adversely affect treatment outcome (Sjögren *et al.* 1997).

Some investigators recommend the use of calcium hydroxide [$Ca(OH)_2$] as an intracanal dressing in a multiple-visit approach (Byström *et al.* 1985, Shuping *et al.* 2000). The environment within the system, however, is such that delivering the medicament and maintaining a high pH homogeneously is a challenge (Byström *et al.* 1985, Nerwich *et al.* 1993, Haapasalo *et al.* 2000). For these and other reasons, certain microbial species (e.g. *Enterococcus faecalis*) in a limited group of cases do survive and can be responsible for persistent infections (Ørstavik & Haapasalo 1990, Gomes *et al.* 1996, Sundqvist *et al.* 1998, Chavez De Paz *et al.* 2003).

Dealing with persistent microorganisms is a challenge, especially in a case with an existing large canal preparation with adequate shape for irrigants and medicaments (Coldero *et al.* 2002). Overall, the treatment of choice should be effective, reproducible and not result in further removal of tooth tissue. In this regard, a somewhat controversial way to manage endodontic pathogens is to use lasers. For instance, the efficacy of neodymium:yttrium–aluminum–garnet (Nd:YAG) lasers for photo-thermal disinfection has been investigated and their application was found to be safe and have potential (Klinke *et al.* 1997, Ramskold *et al.* 1997, Schoop *et al.* 2004, Bergmans *et al.* 2006). Unfortunately, Nd:YAG laser irradiation could not render all systems bacteria-free with the result that no superior effect when compared with NaOCl irrigation occurs (Hardee *et al.* 1994, Moshonov *et al.* 1995). As for more conventional therapy, endodontic pathogens that grow as a multilayered structure persist in being difficult to eradicate even when having direct exposure to the Nd:YAG laser beam (Bergmans *et al.* 2006).

A recent laser-assisted method to manage oral pathogens is to use photo-activated disinfection (PAD) (SaveDent; Denfotex Light Systems Ltd, Inverkeithing, UK). PAD refers to a process called 'lethal laser photosensitization' whereby laser radiation emitted from a low power (100 mW) laser device (i.e. a diode laser with a wavelength of 635 nm) activates a dye (i.e. tolonium chloride, TBO), which in turn exerts a lethal effect on particular cells such as bacteria. Neither the dye nor the reactive oxygen produced from it is toxic to the patient (Soukos *et al.* 1996, Walsh 2000). The clinical applications of PAD include disinfection of deep carious lesions (Walsh 2003, Williams *et al.* 2004) and periodontal pockets (Sarcar & Wilson 1993, Wilson 2004). How and to what extent PAD could improve root canal disinfection is, however, uncertain. Given the limited evidence on the effect of PAD in infected root canals (Seal *et al.* 2002, Lee *et al.* 2004a, Bonsor *et al.* 2006, Soukos *et al.* 2006), greater clarity should be provided before advocating this approach as an alternative or even a supplement to existing endodontic protocols.

This *ex vivo* investigation was performed to better define the role of PAD in minimally invasive root canal treatment (i.e. without further dentine removal). The hypothesis tested was that PAD has a bactericidal effect on endodontic pathogens inoculated in shaped root canals. Resultant colony-forming unit (CFU) counts were associated with the observations of bacterial cell structural changes using environmental scanning electron microscopy (ESEM) on inoculated dentinal surfaces before and after the treatment with PAD. With ESEM, samples can be imaged without prior dehydration and conductive coating, thus allowing real-time observation and testing of endodontic treatment modalities *in situ* (Bergmans *et al.* 2005).

Materials and methods

Microbiological analysis

A total of 38 single-rooted mandibular premolar teeth (gathered following an informed consent protocol approved by the Commission for Medical Ethics of the Catholic University of Leuven) were stored (0.5% solution of chloramine in water) at 4 °C. At the time of use, root surfaces were cleaned using an ultrasonic scaler (P5 Booster; Satelec, Merignac, France) and the treatment was initiated by creating access and by removing pulpal remnants. After pre-flaring with Gates Glidden burs sizes 1–4 (Dentsply Maillefer, Ballaigues,

Switzerland), each canal was instrumented using the GT rotary system (Dentsply Maillefer) in a crown-down sequence upto a size 30, 0.10 taper instrument at 1 mm from the apical foramen. Throughout the instrumentation, irrigation with a 2.5% NaOCl solution was performed using a 27-gauge needle (Monoject; Sherwood Medical, St Louis, MO, USA) and patency was assured by placing a size 08 k-file (Dentsply Maillefer) 1 mm beyond the apical constriction. Apical foramina were sealed with a restorative material (Z100; 3M ESPE, Seefeld, Germany) and root canals and outer surfaces were irrigated with 17% EDTA for 2 min, followed by tap water. The prepared teeth were mounted in bijou bottles (Fig. 1a) and the assembled entities were autoclaved (134 °C for 15 min) to obtain sterilized systems (procedure checked in sampling-based pilot study and by negative controls). The bijou bottles were then filled under strict asepsis with sterile Brain Heart Infusion (BHI) broth (37 gL^{-1}; Oxoid Ltd, Basingstoke, UK) until the available root was surrounded (method according to Seal *et al.* 2002).

Before inoculation, the samples were randomly divided into three groups (n = 12) and one negative control group (n = 2). The prepared root canals from the first group were inoculated with a bacterial suspension of *Streptococcus anginosus* (LMG 14502) from the Belgian Coordinated Collections of Microorganisms (BCCM^{TM}, Gent, Belgium) (standardized at 4×10^8 CFU mL^{-1} in BHI broth) using sterile syringes (Monoject). The prepared samples from the second and third group were inoculated with *E. faecalis* (LMG 7937) and *Fusobacterium nucleatum* (LMG 13131), respectively. The two remaining samples were left uninfected (sterile BHI broth, negative controls). All entities were incubated for 2 days (Concept 300 Anaerobic Workstation; Ruskin Technology, West Bradford, UK) under anaerobic conditions (80% N_2, 10% CO_2 and 10% H_2) at 37 °C in isolated boxes.

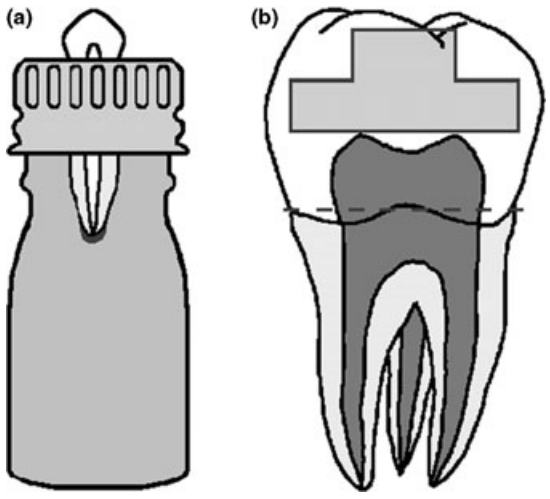

Figure 1 Sample preparation (a) for microbiological analysis : prepared mandibular premolar teeth were mounted in bijou bottles; (b) for environmental scanning electron microscopy: molar crowns were prepared in such a way that mounting them in a specimen stub was possible with a flat surface positioned horizontally. This surface was cut in the occlusal plane near the pulp extensions.

After incubation, residual medium within the root canals was removed with sterile paper points. All teeth were then randomly coded (Fig. 2) and treated according to the assigned code:

A. PAD (dye + laser)
B. laser control (reduced transport fluid (RTF) + laser)
C. dye control (dye + no laser)
D. positive control (RTF).

For treatments A and B, laser irradiation was performed with the SaveDent system (Denfotex Light Systems Ltd). The power output was therefore standardized at 100 mW (semiconductor diode laser with a wavelength of 635 nm) whilst the light was transferred to a 300-μm-thin flexible tip of a specially designed handpiece for endodontics, which – according to the manufacturer – emits the radiation uniformly and peripherally. The PAD liquid that was part of the system consisted of a dye (i.e. toluidine blue, TBO) at a concentration of 12.7 mg mL^{-1} (pH 5). This product came in opaque syringes of 1 mL each and was protected from light and heat. For treatment A, the inoculated root canals were filled with toluidine blue to the level of the access cavity using a sterile endodontic needle (Monoject). The solution was then agitated for 60 s with a size 08 sterile k-file (Dentsply Maillefer) and laser light was applied for a specified time (150 s) according to the manufacturer's instructions. For treatment B, RTF was used instead of the dye and the laser was activated. For treatment C (and the negative controls), the same movement was performed with the fibre but without activating the laser.

After treatment, the liquid contents of the root canals of all groups were carefully absorbed with sterile paper points without intentionally touching the walls. The root canals were then filled with RTF and gently filed in a circumferential way using sterile, size 25 k-files (Dentsply Maillefer) to working length for 20 s. Next, the contents were again carefully absorbed with sterile paper points, transferred to 2 mL of RTF, and

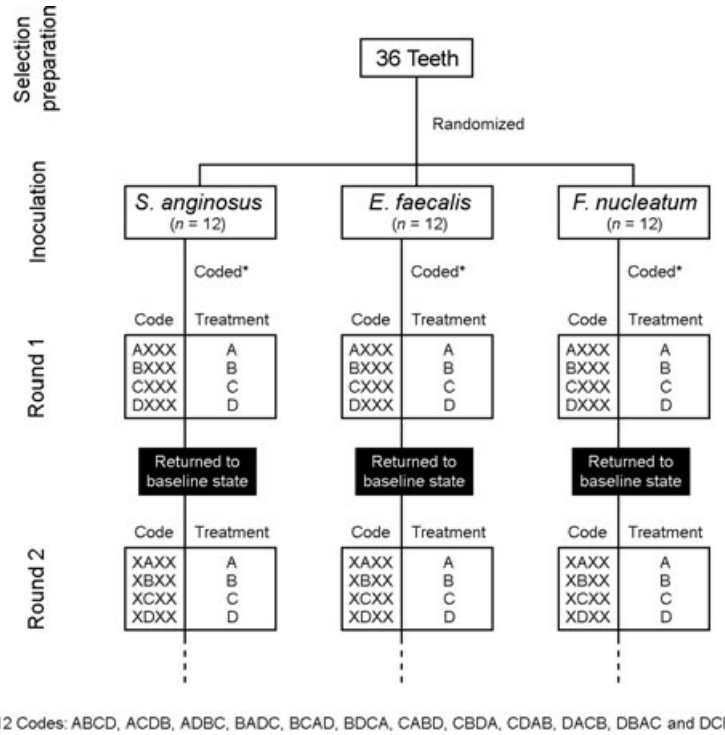

Figure 2 Flow diagram showing the sample progress through the cross-sectional-designed trial, except for the negative controls.

immediately processed. After vortexing for 30 s, the contents of the test tubes were serially diluted in log steps (20 μL in Eppendorf tube with 180 μL saline, five times, thus diluted to 10^{-5}) and duplicate samples of 50 μL were applied to Blood Agar (BA) plates (Blood Agar Base II; Oxoid Ltd, Basingstoke, UK), which were supplemented with haemin (5 mg mL^{-1}), menadione (1 mg mL^{-1}), 5% sterile horse blood and 0.8% (w/v) Bacto Agar (Difco Laboratories, Detroid, MI, USA), and incubated under anaerobic conditions at 37 °C. After 3–7 days, contamination by other species was ruled out and colonies of *S. anginosus, E. faecalis* and *F. nucleatum* were counted using a stereomicroscope at 16× magnification (Zeiss, Oberkochen, Germany) and recorded as number of CFU mL^{-1}.

To reduce the effect of root canal anatomy as a confounding variable, all teeth were re-used thrice (cross-sectional design; total of four rounds) being each time treated according to the respective letter within their code (Fig. 2). For this purpose, the content of each root canal and bottle was removed, and the systems were returned to their baseline state (irrigated with 17% EDTA and tap water for 2 min, reassembled as a tooth-lid-bottle unit and autoclaved).

The resultant CFU values were log transformed to obtain normal distributions and equal variances. The (two-way) analysis of variance (ANOVA) was then used – with species and treatment protocol as factors – to test for significant differences between means. When the overall *F*-test indicated a significant difference, the multiple comparison Tukey–Kramer procedure was taken to investigate which means differed from the others.

Environmental scanning electron microscopy

A total of 22 caries-free molar teeth (gathered following an informed consent protocol approved by the Commission for Medical Ethics of the Catholic University of Leuven) were stored (0.5% solution of chloramine in water) at 4 °C. At the time of use, the crowns of the teeth were isolated and prepared in such a way that mounting them in a specimen stub was possible with a flat surface (measuring 3 × 6 mm) positioned horizontally. This surface was cut in the occlusal plane near the pulp extensions using a slow-speed diamond saw (Isomet Saw, Buehler Ltd, Evanston, IL, USA) under water-cooling (Fig. 1b). In this manner, an image of coronal dentine approximating the morphology of cervical radicular dentine could be achieved (Carrigan *et al.* 1984), whilst the flat surface simplifies focusing with ESEM. To ensure smear layer removal, the

specimens were immersed in an ultrasonic bath with 2.5% NaOCl for 4 min followed by 17% EDTA for 4 min, after which three washes with saline for a period of 2 min each. The samples were kept (0.9% refreshed saline) at 4 °C until further use.

Before inoculation, the samples were autoclaved and placed in sterile bijou bottles with the flat surface up. Then the bottles were filled with BHI broth inoculated with strains of *S. anginosus* (LMG 14502) (8/22 samples), *E. faecalis* (LMG 7937) (8/14 remaining samples), or a mixed culture of *E. faecalis* and *F. nucleatum* (LMG 13131) (BCCM^{TM}) (4/6 remaining samples) [*note:* Bergmans *et al.* (2005) demonstrated that pure cultures of *F. nucleatum* could not be imaged with ESEM; reason still unknown]. The control samples were incubated with *S. anginosus* and *E. faecalis*, respectively. The initial concentration was standardized at 4×10^8 CFU mL^{-1}. A 20-μL test of this solution was spread on supplemented BA plates and incubated for 24 h to confirm viability of the cells at the point of inoculation.

After 1, 2 and 6 days of incubation, one 20-μL aliquot of each microbial strain was aseptically removed from each tooth, spread on supplemented BA plates, and anaerobically incubated for 24 h to confirm viability of the microorganisms in solution at the point of PAD treatment. The ESEM samples were directly viewed in environmental 'wet' mode with a Philips XL30 ESEM-FeG (FEI/Philips Electron Optics, Eindhoven, The Netherlands) (working conditions: 4 °C, 2.9–5.9 torr gas pressure, 80–85% relative humidity, 5–10 kV). Initially, 4–6 interesting spots on each sample were selected, scanned and saved in the stage memory. Then, the entire surface of the sample was directly laser-treated in one of two different ways:

- The ESEM chamber door in *opened* position – application of the dye on the sample – 1 min break – laser treatment (endo handpiece, contact mode, 100 mW, 150 s, 15 J) (half of the samples of each group).
- The ESEM chamber door left in *closed* position – integrated and sealed microinjector was used to apply the dye on the sample under direct internal CCD observation – 1 min break – laser treatment (mounted caries handpiece, contact mode, 100 mW, 150 s, 15 J) – absorption of the dye by lowering the pressure inside the chamber and increasing the Peltier temperature (10 °C for 1–2 min) (other half of the samples).

The two remaining samples were used for laser control (RTF + laser) inside the chamber (first round), and then re-used for dye control (dye + no laser) (second round; after return to their baseline state).

After treatment, identical spots on the specimens were reanalysed with ESEM for morphological changes in bacterial configuration. At the end, dentinal surfaces were scrubbed with a sterile scalpel and resultant dentine chips were collected in an Eppendorf tube containing 2 mL of RTF. These samples were then vortexed for 30 s and plated (Spiral Systems Inc., Cincinnati, OH, USA) onto BA plates which were incubated anaerobically for 7 days to check for growth.

Results

Microbiological analysis

The positive control group (treatment D; baseline controls) showed a mean (±SD) number of CFU mL^{-1} of 1.6×10^6 ($\pm 1.3 \times 10^6$) for *S. anginosus*, 7.0×10^6 ($\pm 6.4 \times 10^6$) for *E. faecalis* and 9.7×10^5 ($\pm 1.2 \times 10^6$) for *F. nucleatum*, after 2 days, whilst bacteria were found in all cases. PAD (treatment A) resulted in a significant reduction of the bacterial load to a mean (±SD) number of CFU mL^{-1} of 1.0×10^5 ($\pm 8.3 \times 10^4$) ($P < 0.0001$), 8.1×10^5 ($\pm 7.9 \times 10^5$) ($P < 0.05$) and 1.5×10^4 ($\pm 1.4 \times 10^4$) ($P < 0.0001$), respectively, meaning a potential disinfection (93.8% kill, 88.4% kill and 98.5% kill, respectively) but no sterilization. Laser alone (treatment B) had no significant effect on the bacterial load [1.2×10^6 ($\pm 9.0 \times 10^5$) for *S. anginosus*, 3.1×10^6 ($\pm 2.9 \times 10^6$) for *E. faecalis* and 1.0×10^6 ($\pm 1.3 \times 10^6$) for *F. nucleatum*]. The same was true for using the dye without laser (treatment C) [bacterial load after treatment: 1.3×10^6 ($\pm 1.6 \times 10^6$), 2.8×10^6 ($\pm 2.7 \times 10^6$) and 8.7×10^5 ($\pm 1.1 \times 10^6$), respectively]. The results of the CFU mL^{-1} counts for all groups (different species) and treatment protocols (A–D) after log transformation are given in Fig. 3. The negative controls yielded no cultivable cells, ruling out contamination of the samples.

Environmental scanning electron microscopy

When examining bacterial growth on the dentinal surface at different intervals (1, 2 and 6 days of incubation), it became clear that *E. faecalis* grew faster than the other bacterial strains. At day 1, *S. anginosus* was present as single cells or microcolonies whereas *E. faecalis* had already formed a partial monolayer (Figs 4a and 5a,b). After 2 days of incubation, cells of *S. anginosus* were organized as a monolayer (note that

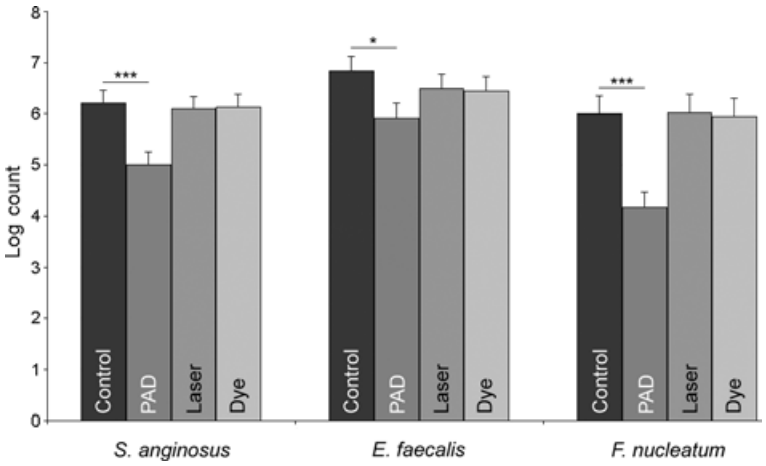

Figure 3 Microbiological analysis: chart representing the colony-forming unit mL^{-1} counts for all species and all treatment protocols after log transformation. (ANOVA, $^{*}P < 0.05$ and $^{***}P < 0.0001$).

parts of the dentinal surface were still visible) whereas *E. faecalis* covered most of the dentinal surface creating a biofilm – a matrix-embedded multileveled structure – that frequently hid the entrance of dentinal tubules (Figs 4b and 5c). The latter made the registration of the images taken before and after PAD treatment more difficult as the position of a tubular entrance as a reference was obscured. At day 6, confined biofilm formation was observed for *S. anginousus* (Fig. 4c). For the mixed culture, cells of *E. faecalis* were dominating over *F. nucleatum* from day 2 (Fig. 6a).

Concerning the capacity of PAD to destroy single cells, microcolonies or monolayers obtained from a 1-day *(E. faecalis, S. anginosus)* or a 2-day (*S. anginosus)* culture, laser irradiation (100 mW, 150 s, 15 J) after application of the dye eliminated most bacteria (Figs 4a′,b′ and 5a′,b′). The dentinal surface appeared relatively clean, whilst some cell remnants were visible. Morphologically intact bacteria were a rare observation, but still their existence could not be ruled out. There was no difference between the results obtained in closed and opened position of the chamber door.

On the other hand, when a biofilm was present (2 and 6 days of incubation with *E. faecalis;* 6 days of incubation with *S. anginosus*), the effect of bacterial eradication appeared to be reduced substantially. Laser irradiation (100 mW, 150 s, 15 J) after application of the dye resulted in the destruction of superficial layers and a partial disruption of the biofilm (Figs 4c′ and 5c′). Layers that were situated deeply were revealed, whilst the number of remaining intact cells could not be counted. As the thickness of the biofilm was not constant over the surface, the observations at the diverse locations and on the different samples varied. For the mixed culture (*E. faecalis, F. nucleatum)*, however, PAD induced a distinct eradication of the infected site (Fig. 6a′). In general, the results obtained in closed and opened position were similar, whilst bacterial growth could be obtained on the BA plates after every distinct treatment.

Discussion

The three strains used in this study are referred to as predominant species in either primary pulp infection (Sundqvist 1994, Siqueira *et al.* 2002) or failed endodontic treatment (Sundqvist *et al.* 1998, Siqueira & Rocas 2004). In the present experiment, the quantitative microbiological as well as the visual morphological effects of PAD on artificially created endodontic biofilms, using these microorganisms, were assessed.

Single-rooted teeth were instrumented to create, after inoculation, a habitat for microbial growth. Biofilm development was linked with incubation time and confirmed by ESEM. Preparation of the canals upto a size 30, 0.10 taper instrument at working length was selected to obtain adequate size and taper and easy access for the PAD endotip (300-μm-thin tip, 500 μm at coated area) to the apical third. The final shape therefore allowed the precise manipulation of the tip and a correct sampling procedure. Samples were taken both passively by paper point imbibition and actively by circumferential filing. The latter was introduced to prevent false culture calculation through sampling planktonic bacteria in contact with the paper point whilst leaving the sheltered biofilm microbiota untouched (Molander *et al.* 1998, Nair *et al.* 2005, Wu *et al.* 2006).

After 2 days of incubation, the positive control group (treatment D) showed a mean (±SD) CFU mL^{-1} count

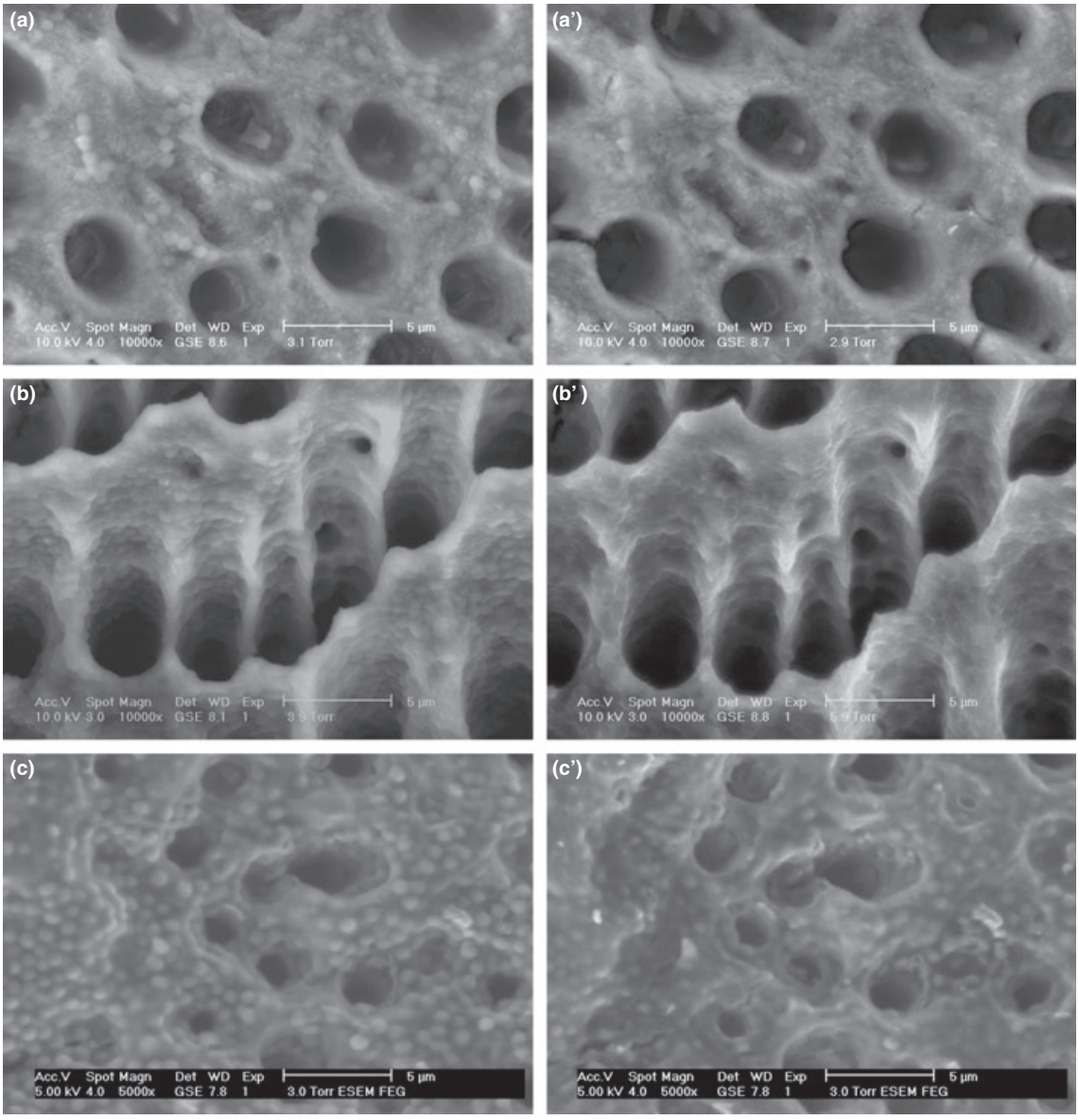

Figure 4 Environmental scanning electron microscopy: (a) *Streptococcus anginosus* after 1 day of incubation contaminated the dentinal surface as single cells or microcolonies (10 000×); (a′) re-analysis of spot (a) after photo-activated disinfection (PAD) (dye + laser) application. The dentinal surface appeared quite bacteria-free, whilst some cell remnants were visible (10 000×); (b) *S. anginosus* after 2 days of incubation. Bacterial cells were organized as a monolayer (10 000×); (b′) re-analysis of spot (b) after PAD application. Morphologically intact bacteria were just a rare observation, but still their existence could not be ruled out (10 000×); (c) After 6 days, cells of *S. anginosus* were organized as a confined multilayered biofilm (5000×); (c′) re-analysis of spot (c) after PAD application. Treatment resulted in the destruction of superficial layers and a partial disruption of the biofilm. The effect of bacterial eradication appeared to be strongly reduced (5000×).

of 9.7×10^5 ($\pm 1.2 \times 10^6$) for *F. nucleatum*, 1.6×10^6 ($\pm 1.3 \times 10^6$) for *S. anginosus* and 7.0×10^6 ($\pm 6.4 \times 10^6$) for *E. faecalis*. These quantitative results corresponded to the ESEM-based findings indicating a faster growth of *E. faecalis* on dentine when compared with other species. In the literature, doubling times of 65 min, 3.5 and 5 h are estimated for *E. faecalis*, *F. nucleatum* and *S. anginosus*, respectively (Beighton &

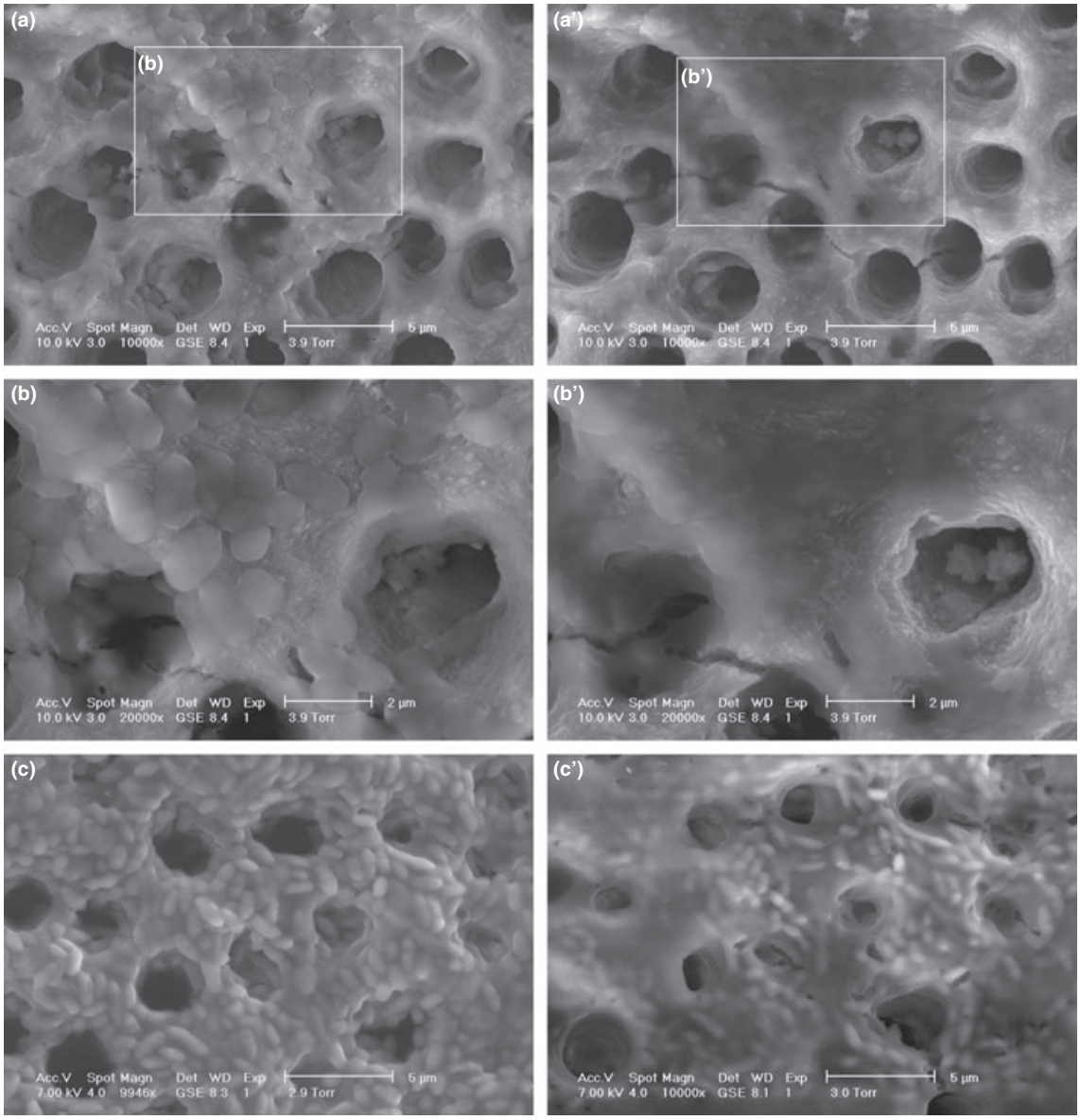

Figure 5 ESEM: (a) *Enterococcus faecalis* after 1 day of incubation. Microorganisms already formed a partial monolayer (10 000×); (a′) re-analysis of spot (a) after photo-activated disinfection (PAD) (dye + laser) application. Bacterial cell remnants were still visible, but morphologically intact bacteria were just a rare observation (10 000×); (b) detail of image (a). This higher magnification allowed a thorough inspection of the bacterial cell contours (20 000×); (b′) detail of image (a′). Re-analysis of spot (b) after PAD application showed a loss of the confined bacterial cell contour (20 000×); (c) after 2 days, cells of *E. faecalis* were already organized as a multilayered biofilm covering the entire dentinal surface (9946×); (c′) re-analysis of spot (c) after PAD application. The superficial layers of the Enterococcus biofilm were destructed, whilst its three-dimensional structure was still apparent (10 000×).

Hayday 1986, Rogers *et al.* 1991, Bogosian *et al.* 1998).

In the present study, a significant CFU count reduction was observed for group A (PAD) only. So, the combination of low-power laser light and the dye (TBO) was mandatory to obtain adequate disinfection. Similar findings have been reported by several authors in the past (Burns *et al.* 1993, O'Neill *et al.* 2002, Zanin

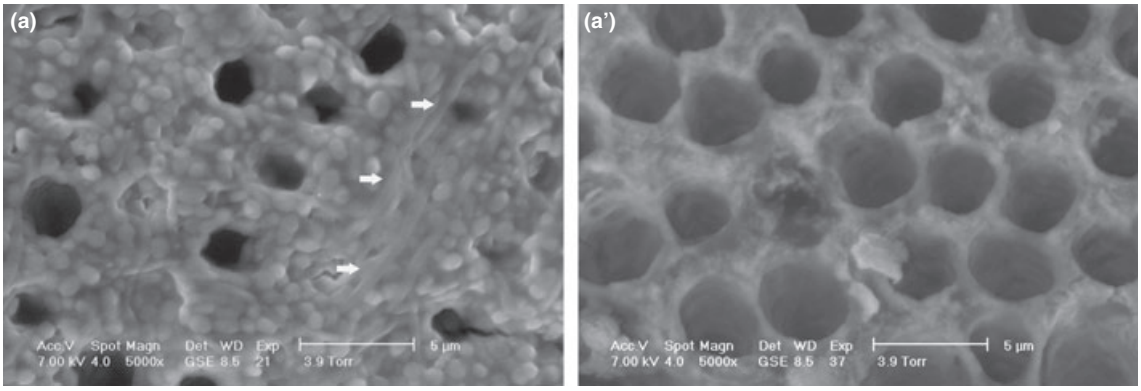

Figure 6 ESEM: (a) symbiosis of *Enterococcus faecalis* and *Fusobacterium nucleatum* after 2 days of incubation. The *E. faecalis* biofilm was clearly dominating over *F. nucleatum* (arrows) (5000×); (a′) re-analysis of spot (a) after photo-activated disinfection application. Surprisingly, an outspoken bacterial eradication could be seen (5000×).

et al. 2005, Williams *et al.* 2006). In the present study, a positive test group, including accepted practice of a NaOCl solution, did not exist because evaporation of NaOCl inside the ESEM chamber can damage the device. Special precautions adopted in a pilot study, however, made it possible to observe two samples that had been treated with a 2.5% NaOCl solution for 1 min outside the ESEM chamber (Fig. 7). Regarding microbial analysis, the bactericidal effect of a 3% NaOCl solution (applied for 10 min on a 2-day culture of *Streptococcus intermedius*) has been demonstrated by Seal *et al.* (2002) using the same model. They concluded that lethal photosensitization could not compete with NaOCl in achieving consistent 100% bacterial kills.

When one compared the kill percentages of the different species, *E. faecalis* (88.4% kill) was definitely more resistant to the current PAD regimen than *S. anginosus* (93.8% kill) or *F. nucleatum* (98.5% kill), thereby confirming *E. faecalis'* status as a highly resistant pathogen. Several factors related to both the individual cells and the biofilm could be held responsible for resistant behaviour, even though their particular contribution could not be clarified. Among them are: fast growth, the potential to colonize, dentinal tubule invasion, survival without dividing in diverse physical and poor nutritional conditions,physical properties of the biofilm structure and interaction between composing cells (e.g. the transfer of plasmids) (Nichols 1991, Distel *et al.* 2002). Whether or not *S. anginosus* or *F. nucleatum* would be more resistant to PAD if more time was given for growth and biofilm formation could not be evaluated in the present study as only an incubation period of 2 days was taken into account for the quantitative analysis. The ESEM observation, however, demonstrated biofilm formation for *S. anginosus* after 6 days. For that reason, the comparison between the kill ratio of a 2-day culture of *E. faecalis* and a 6-day culture of *S. anginosus* seems an interesting topic for further research.

Returning to the ESEM results, the bacterial cell structural changes for monocultures caused by PAD were more apparent when cells were grouped in a monolayer rather than a thick biofilm. In case of a thin layer, one could observe the destruction of most cells, with the exception of some small bacterial clusters that appeared to be untouched. In case of a multilayer with matrix-embedded cells, many apparently intact bacteria could be found underneath an electrolucent layer. This layer was assumed to be composed of cell remnants on a humid surface. Similar deformations could be found after the destruction of bigger colonies, where superficially located organisms were eliminated thereby covering and protecting the underlying species. In case of a mixed microbiota, however, bacterial eradication was surpisingly evident. One explanation could be the existence of more channels for dye penetration, but this assumption was not confirmed.

In general, a prerequisite for effective lethal photosensitization is the deposition of a dye (i.e. photosensitiser) on the cell wall surface of an organism. As a result, for TBO, factors such as the diffusion/wetting capacity, the penetration depth in biofilms, the amount of adsorption to/absorption by the membranes, the time of contact, and the capacity to generate reactive oxygen may define its destructive potential. This could explain the difference in outcome for the PAD system when data from small bacterial colonies and biofilms

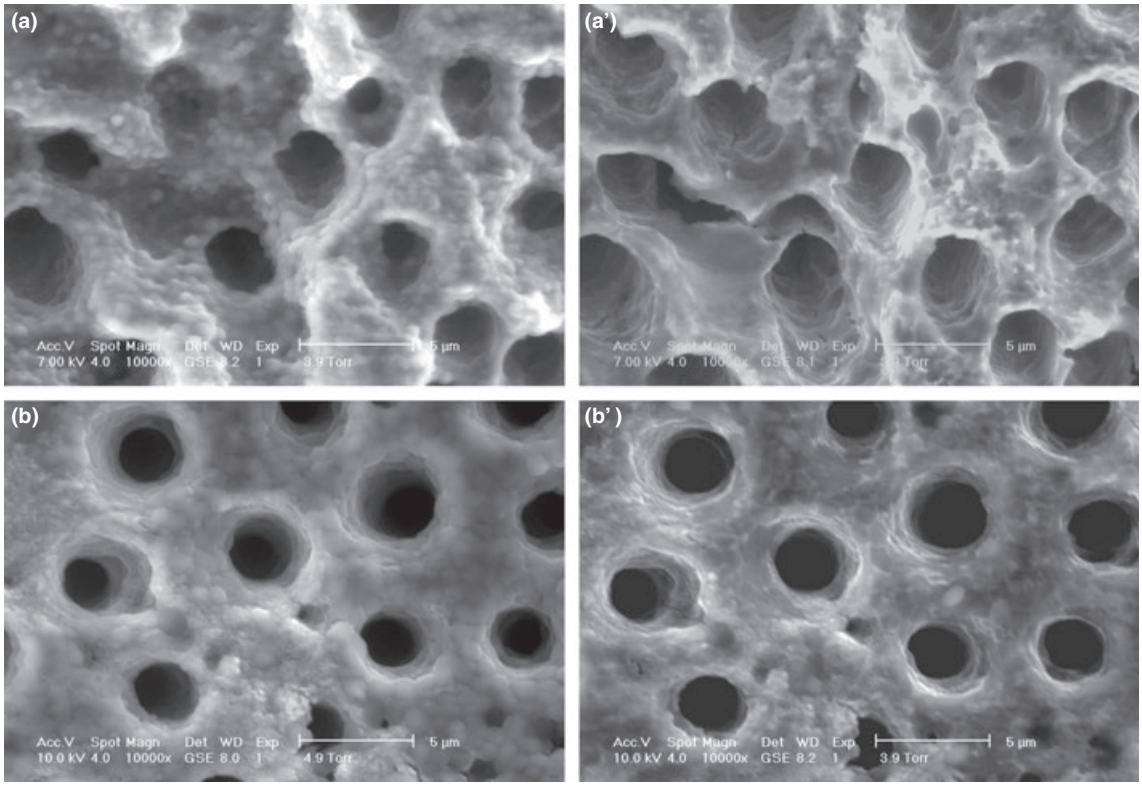

Figure 7 ESEM results from pilot study: (a) *Streptococcus anginosus* after 6 days of incubation. Bacterial cells were organized as a confined multilayered biofilm (10 000×); (a′) re-analysis of spot (a) after application of a 2.5% NaOCl solution for 1 min. Treatment resulted in the destruction of superficial layers and a partial disruption of the biofilm. (b) *Enterococcus faecalis* after 2 days of incubation. The cells were organized as a multilayered biofilm covering the dentinal surface (10 000×); (b′) re-analysis of spot (b) after application of a 2.5% NaOCl solution for 1 min. Superficial layers of the biofilm were destructed, whilst its three-dimensional structure was still apparent (10 000×). (Attention: the observation of NaOCl-treated samples with ESEM requires special precautions not to damage the device).

with various thicknesses were compared. In fact, from the ESEM data, it was assumed that either the TBO liquid did not penetrate the biofilm to full depth or that the laser light could not be efficiently distributed throughout the entire biofilm thickness. Regarding the latter, the absorption of too much energy by the superficial layers could have prevented or reduced the activation of the sensitiser in deep layers. It was noted, however, that the bright light could be seen through the whole root mass and, therefore, it seemed unlikely that the energy could not reach the deeply sheltered microorganisms in tubules and anastomoses. For that reason, it can be hypothesized that limited penetration depth of the TBO was the more likely factor.

Further assessment of the quantitative results of the study did support this hypothesis. Indeed, when more bacteria were found in the root canal, a relatively small reduction of them was found after treatment. The finding that individual cells and small groups of bacteria were easily destroyed by the combination of low-power laser light and a dye (TBO) was in total agreement with published results on the effectiveness of lethal photosensitization on bacterial planktonic suspensions (Williams *et al.* 2006). In accordance, several approaches exist to enhance the penetration potential of TBO. Amongst them are the assisted penetration using ultrasonics (Caron 2006) and TBO grafting/manipulation techniques (Jori *et al.* 2006).

In daily clinical practice, clinicians are challenged by biofilm-pathosis and one should be aware of the resistance of these microbiological communes. Application of the PAD concept for disinfection might be interesting and promising, but disruption of the biofilm prior to PAD and the adjunct use of a NaOCl-based conventional approach remain mandatory.

Conclusion

Photo-activated disinfection is not an alternative but a possible supplement to existing protocols for root canal disinfection as the interaction between laser light (diode laser, 635 nm) and the associated dye (tolonium chloride, TBO) allows a broad-spectrum bactericidal effect. Some endodontic pathogens that grow as a multilayered (biofilm) structure, however, remain more difficult to eradicate. To further optimize the potential of PAD, more research should be performed on the interaction between PAD, microbial specificity (especially in mixed microbiota) and biofilm disruption.

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