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Bactericidal effect of Nd:YAG laser irradiation on some endodontic pathogens *ex vivo*

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Abstract

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Aim To define the role of neodymium:yttrium–aluminum–garnet (Nd:YAG) lasers in root canal disinfection along with a minimally invasive treatment concept.

Methodology The hypothesis was tested *ex vivo* that Nd:YAG laser irradiation has a bactericidal effect on endodontic pathogens inoculated in root canals. Resultant colony-forming unit counts were associated with observations of bacterial cell structural changes using conventional scanning electron microscopy (CSEM) and environmental scanning electron microscopy (ESEM) on inoculated dentine surfaces, following indirect and direct Nd:YAG laser irradiation, respectively.

Results The Nd:YAG laser irradiation (1.5 W, 15 Hz, four times for 5 s) of *Enterococcus faecalis* inoculated canals resulted in a significant reduction ($P < 0.05$, Wilcoxon signed rank test) of the bacterial load, meaning a 99.7% kill, but no sterilization. The CSEM procedure verified that the extent of radiation damage was in line with the total amount of laser energy applied. After 2 h of incubation and three cycles of indirect laser treatment (i.e. through a 1-mm-thick dentine disc), no morphologically intact bacteria of *Actinomyces naeslundii* or *Streptococcus anginosus* were discernible. However, when micro-colonies of *S. anginosus* and specially biofilms of *E. faecalis* were present after 2 days, the *in situ* experiment using ESEM and direct laser treatment showed that bacterial eradication was reduced in deep layers.

Conclusions The Nd:YAG laser irradiation is not an alternative but a possible supplement to existing protocols for canal disinfection as the properties of laser light may allow a bactericidal effect beyond 1 mm of dentine. Endodontic pathogens that grow as biofilms, however, are difficult to eradicate even upon direct laser exposure.

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

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Introduction

Antisepsis is the essence of root canal treatment in teeth with apical periodontitis (Kakehashi *et al.* 1965). As a result, the use of mechanical instrumentation and sodium hypochlorite (NaOCl) is routine in clinical practice. When irrigating with NaOCl, fine gauge needles can be used to facilitate deep penetration but agitation using small size instruments or ultrasonics (Lee *et al.* 2004b) may increase the potential to kill microorganisms (Huque *et al.* 1998). Smear layer removal from the canal wall by citric acid or ethylenediaminetetraacetic acid (EDTA) has been recommended (Scelza *et al.* 2004), although there is no clear

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evidence that this procedure enhances disinfection or treatment outcome (Byström & Sundqvist 1985, Ørstavik & Haapasalo 1990).

Unfortunately, careful use of stainless steel hand and/or nickel–titanium rotary files with NaOCl in one visit cannot render all systems bacteria-free (Sjögren *et al.* 1997, Shuping *et al.* 2000, 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 advocate the use of calcium hydroxide [$Ca(OH)_2$] as an intracanal medicament 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 (Nerwich *et al.* 1993). Careful packing of the calcium hydroxide is essential because if not in direct contact with its target it is unreliable at best (Byström *et al.* 1985). In addition, root dentine appears to reduce the activity of $Ca(OH)_2$ by buffering the local pH (Haapasalo *et al.* 2000).

Despite the use of $Ca(OH)_2$, certain microbial species in a limited group of cases do survive and can be held responsible for persistent infections (Ørstavik & Haapasalo 1990, Gomes *et al.* 1996, Sundqvist *et al.* 1998, Chavez De Paz *et al.* 2003). *Enterococcus faecalis*, for instance, is able to withstand a pH of 11.1 due to a functioning proton-pump (Evans *et al.* 2002). In addition, pure cultures of this species can form protective biofilms in $Ca(OH)_2$-medicated canals (Distel *et al.* 2001) and have the ability to invade dentinal tubules (Love 2001, Peters *et al.* 2001). Under starved conditions, *E. faecalis* shows resistance to NaOCl (LaPlace *et al.* 1997), whilst the upregulation of stress-induced proteins has been shown to be important for cell survival (Hartke *et al.* 1998).

Dealing with persistent infections remains a challenge even though various strategies exist. One such procedure is the use of chlorhexidine (CHX) or iodine potassium iodide (IKI) alone or in combination with $Ca(OH)_2$ (Safavi *et al.* 1990, Haapasalo *et al.* 2000, Gomes *et al.* 2003, Baker *et al.* 2004, Siren *et al.* 2004). Alternatively, a larger preparation and 'deep shape' may facilitate flow of the irrigants (Albrecht *et al.* 2004, Lee *et al.* 2004a). In this respect, the diameter of the apical preparation appears to be less important for debris removal and bacterial removal when a certain taper is achieved (Card *et al.* 2002, Coldero *et al.* 2002). One may further assume that improved shaping facilitates packing of the medicament, especially in systems with complex anatomy. Overall, the treatment of choice should be effective, reproducible and minimally invasive.

A controversial way to manage pathogens is to use lasers. The efficacy of neodymium:yttrium–aluminum–garnet (Nd:YAG) lasers for photo-thermal disinfection has been investigated and laser application was found to be safe and have potential (Klinke *et al.* 1997, Ramskold *et al.* 1997, Moritz *et al.* 1999, Schoop *et al.* 2004). 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). Given the high costs of lasers, clarity should be provided before advocating their use as an alternative or even a supplement to existing protocols.

This investigation *ex vivo* was performed to better define the role of Nd:YAG lasers in minimally invasive root canal disinfection (i.e. without further dentine removal). The hypothesis was tested that Nd:YAG laser irradiation has a bactericidal effect on endodontic pathogens inoculated in root canals. Resultant colony-forming unit (CFU) counts were associated with observations of bacterial cell structural changes using conventional scanning electron microscopy (CSEM) and environmental scanning electron microscopy (ESEM) on inoculated dentine surfaces, following indirect and direct Nd:YAG laser irradiation, respectively. 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

Eight maxillary incisor teeth 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 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 up to a size 30, 0.10 taper instrument at 1 mm from the apical foramen. Throughout instrumentation,

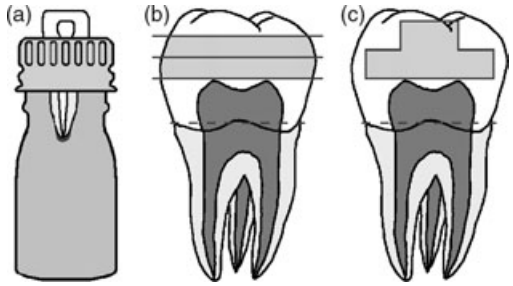

Figure 1 Sample preparation (a) for microbiological analysis: Prepared maxillary incisor teeth were mounted in bijou bottles; (b) for CSEM: Molar crowns were sectioned in the occlusal plane near the pulp expensions. Each crown was sectioned a second and third time coronally from the first cut, resulting in two dentine discs of 1mm thickness; (c) for ESEM: 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.

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 g L^{-1}, Oxoid Ltd, Basingstoke, UK) until the available root was surrounded (method according to Seal *et al.* 2002).

A bacterial suspension of *E. faecalis* (LMG 7937) from the Belgian Coordinated Collections of Microorganisms ($BCCM^{TM}$, Gent, Belgium) and standardized (4×10^8 CFU mL^{-1}) in BHI broth was inoculated into six of the prepared root canals using sterile syringes (Monoject). Next, the samples 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. The two remaining samples were uninfected (sterile BHI broth, negative controls).

After incubation, residual medium within the root canals was removed with sterile paper points and replaced by 0.9% sterile saline. Afterwards, the six infected teeth were randomly assigned to either a positive control (no laser treatment, $n = 3$) or a laser irradiation ($n = 3$) group.

In the experimental group, laser irradiation without water spray, air cooling or photosensitizing dye was performed in wet root canals with a Nd:YAG laser (Smarty A10; DEKA, Firenze, Italy) (wavelength of 1064 nm). Standardized settings were: power output (1.5 W), energy (100 mJ) and length (150 μs) of the pulse, and frequency (15 Hz). Light was transferred by means of a 300-μm-thin flexible fibre and the actual output was controlled with a power meter. The target beam was generated by a He/Ne laser (632.8 nm, 1 mW). Throughout laser treatment, the fibre tip was applied with a spiral movement starting 1 mm above the apex and then moving coronally, four times for 5 s, interleaved with 20-s recovery intervals. For the negative controls, the same procedure was performed 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 Reduced Transfer Fluid (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 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 *E. faecalis* were counted using a stereomicroscope at 16× magnification (Zeiss, Oberkochen, Germany) and recorded as number of CFU mL^{-1}.

To test reproducibility, the teeth of the experimental and positive control group were re-used being assigned to the other group (cross-sectional design). For this purpose, the content of each root canal and bottle was removed, and the systems were returned to their baseline state (irrigated with tap water for 2 min, reassembled as a tooth-lid-bottle entity and autoclaved). Statistical analysis was performed using the Wilcoxon signed rank test (nonparametric paired *t*-test).

Conventional scanning electron microscopy

Eight carious-free, molar teeth were stored (0.5% solution of chloramine in water) at 4 °C. At the time of use, crowns were sectioned in the occlusal plane near the pulp extensions using a slow-speed diamond saw (Isomet Saw; Buehler Ltd, Evanston, IL, USA) under water-cooling. Each crown was sectioned a second and third time coronally from the first cut, resulting in two dentine discs of 1 mm thickness (Fig. 1b). To ensure smear layer removal, the discs 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 stored (0.9% refreshed saline) at 4 °C until use. Before inoculation, the discs were autoclaved and placed in sterile bijou bottles with the flat pulpal-oriented side up.

The bijou bottles were filled with BHI broth inoculated with equal proportions of *Streptococcus anginosus* and *Actinomyces naeslundii* (clinical isolates from the University Hospital, Leuven, Belgium) (4×10^8 CFU mL^{-1}). A 20 μL test of this solution was spread on supplemented BA plates, incubated for 24 h, and served as viability control at the point of inoculation.

After 2 h of incubation, the discs were held upside down using sterilized forceps at the border to evaluate the effect of laser energy through tubular dentine (procedure described by Moritz *et al.* 2000), randomly assigned to four groups ($n = 4$ discs per group), and treated as follows:

- no treatment (positive control);
- one cycle of indirect laser treatment (1.5 W, 15 Hz short pulsed mode) for 5 s;
- two cycles with a recovery interval of 20 s;
- three cycles with recovery intervals of 20 s.

During Nd:YAG laser irradiation, the handpiece was held to form an angle of *c.* 10° between the fibre and the dentinal surface, whilst the tip was slightly touching (contact mode) the moistened (0.9% sterile saline) disc. The movement begun from one corner and continued zigzag across the surface to imitate the treatment of a canal. After treatment, the end of the fibre was removed in order to guarantee in all cases the best power output.

Following laser treatment, the discs were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at 4 °C for 12 h, and prepared for CSEM: dehydration in ascending series of low concentration aqueous ethanol, air drying following a bath of hexamethyldisilazan, and sputter-coating with gold (Sputtering device 07 120; Balzers Union, Liechtenstein). Observations were done using a Philips XL30 Fe-SEM (Philips Co., Eindhoven, The Netherlands) with different magnifications at 10 kV.

Environmental scanning electron microscopy

Another eight molar teeth 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 mm × 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) under water-cooling (Fig. 1c). In this manner, one could achieve an image of coronal dentine approximating the morphology of cervical radicular dentine (Carrigan *et al.* 1984), whilst the flat surface simplifies focusing with ESEM. To ensure smear layer removal, the specimens underwent the ultrasonic treatment. 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. Next, the bottles were filled with BHI broth inoculated with strains of *S. anginosus* (LMG 14502) (4/8 samples) or *E. faecalis* (LMG 7937) (BCCM^{TM}) (four remaining samples). 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 microorganisms at the point of inoculation.

After 2 days of incubation, one sample of each microbial strain was taken and 20 μL tests were spread on supplemented BA plates and anaerobically incubated for 7 days to confirm viability of the microorganisms in solution at the point of laser 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, 2.9–4.9 torr gas pressure, 80–85% relative humidity, 10 kV). Initially, four to six interesting spots on each sample were selected, scanned and saved in the stage memory. Next, the ESEM chamber door was opened and the entire wet (0.9% sterile saline) surface of the sample was directly laser treated in one of two different ways:

- Two cycles of laser treatment (15 W, 15 Hz short pulsed mode) for 5 s with a recovery interval of 20 s (half of the samples, $n = 2$ per group).

• Three cycles of laser treatment with recovery intervals of 20 s (remaining half).

After Nd:YAG laser exposure, identical spots on the specimens were re-analysed with ESEM for morphologic 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 teeth that received no laser treatment (positive control) showed a median [interquartile range (IQR) = $x_{0.25}$–$x_{0.75}$) number of CFU mL^{-1} of 6.8×10^6 (3.0×10^6–6.9×10^6), whilst bacteria were found in all cases. Nd:YAG laser irradiation (1.5 W, 15 Hz, four times for 5 s) resulted in a significant ($P < 0.05$, Wilcoxon signed rank test) reduction of the *E. faecalis* bacterial load to a median number of CFU mL^{-1} of 2.3×10^4 (1.2×10^4–3.0×10^4), meaning a potential disinfection (99.7% kill) but no sterilization (Fig. 2). The negative controls yielded no cultivable cells.

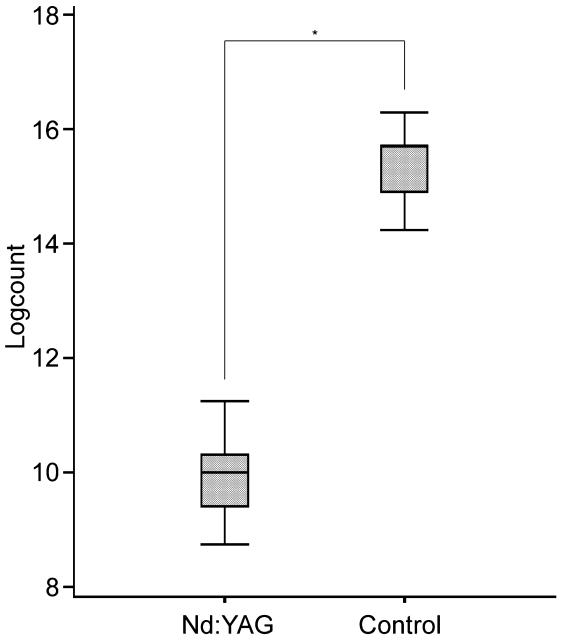

Figure 2 Microbiological analysis: Box plot representing the number of CFU mL-1 of *E. faecalis* present in the experimental (laser irradiation) and positive control group after log transformation. IQR contains 50% of the observed data. (Wilcoxon signed rank test, alpha = 0.05).

Conventional scanning electron microscopy

The shape of *A. naeslundii*, Gram-positive obligate anaerobe rods (*c.* 2 μm long), and *S. anginosus*, Gram-positive facultative cocci (*c.* 1 μm in diameter) occasionally forming a streptococcal chain, could be observed in detail (Figs 3A, a, b and d).

In general, the CSEM procedure provided evidence that the test organisms had different thresholds of sensitivity to radiation, whilst the extent of damage was in line with the total amount of laser energy (i.e. number of irradiation cycles) applied.

One cycle of treatment (1.5 W, 15 Hz, through 1-mm-thick dentine for 5 s) was well tolerated by both microorganisms. Even though some small alterations in morphology could be detected – many cells showed blebs on their surface, whilst others appeared shrivelled and perforated – most bacteria maintained their typical shape and size as in the control group (Fig. 3c,d).

After two cycles, clear signs of damage became visible for *A. naeslundii* which appeared to be more susceptible to radiation (Fig. 3e). Some cells showed protuberances and formation of nodular structures of variable size covering their bodies. Most cells, however, were completely disrupted and surrounded by scattered cell fragments. Coagulated, amorphous masses were seen, in which the shape of the bacteria merged with one another; the cell bodies seemed to have completely fused together. *Streptococcus anginosus* maintained its original shape; only some blebs and perforations could be observed.

After three cycles, the micrographs disclosed disintegrated cells and no intact bacteria were discernible (Fig. 3F,f). On the partially altered dentinal surface, coagulated amorphous masses could be found surrounded by debris consisting of scattered cell fragments of variable size.

Environmental scanning electron microscopy

After 2 days of incubation, there was an apparent difference in growth between the two test strains. *Enterococcus faecalis* covered most of the dentinal surface creating a matrix-embedded multileveled structure that frequently hid the entrance of a dentinal tubule (Fig. 4c,d). This biofilm appeared, however, not

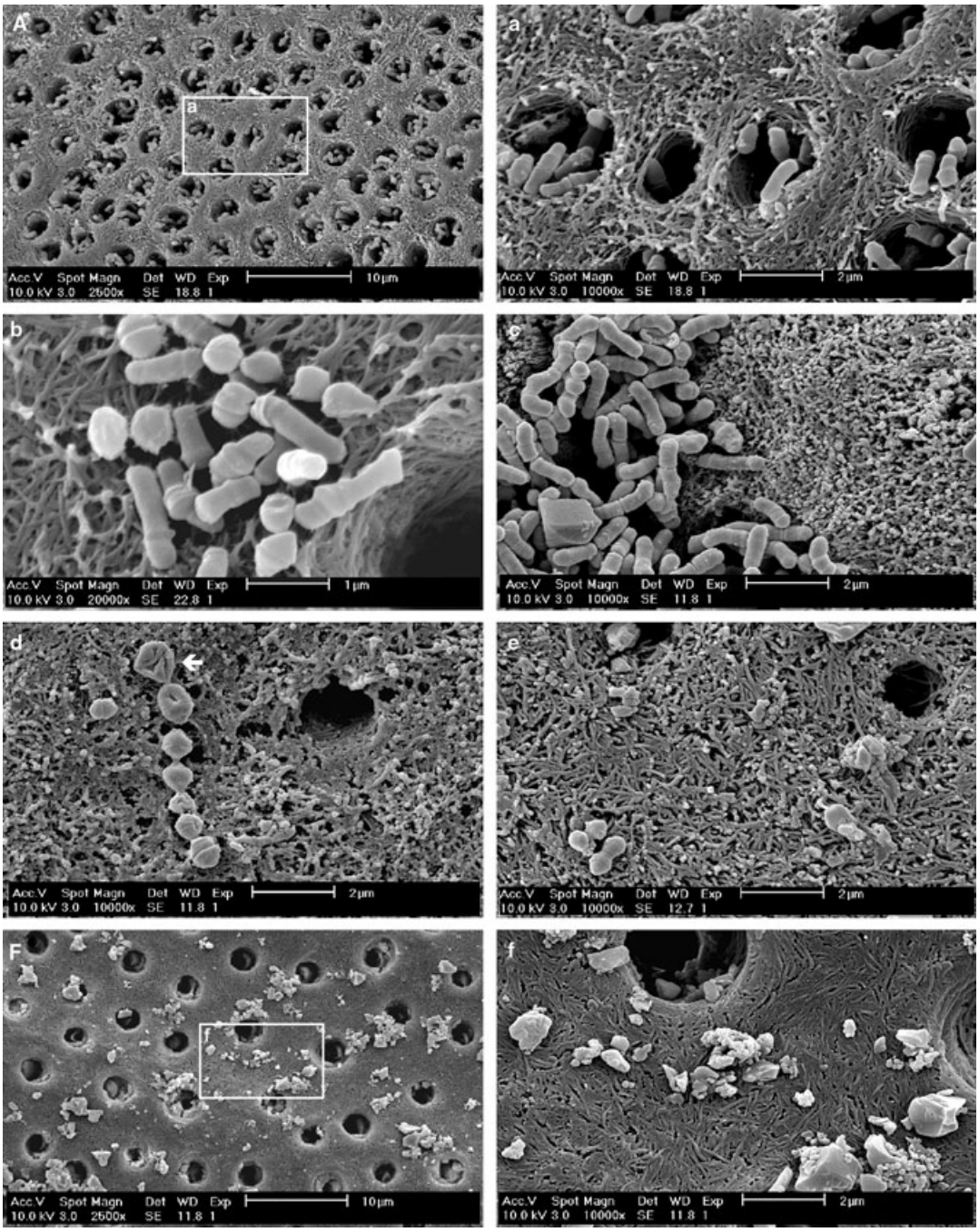

Figure 3 CSEM: (A) a mixed flora of *A. naeslundii* (rods 2 µm long) and *S. anginosus* (cocci 1 µm in diameter) of the control group; (a) detail of the previous image; (b) the same mixed flora at higher magnification (20,000x); (c) after one cycle of indirect laser treatment (1.5 W, 15 Hz, for 5 s): Small alterations in morphology, such as blebs on the surface, could be detected; (d) Streptococcal chain after one cycle of indirect laser treatment: Some cells appeared shrivelled and empty, displaying perforations of their cell wall (arrow); (e) after two cycles of treatment: *A. neaslundii* showed severe forms of damage including complete disruption and fusion of their cell bodies, while most cells of *S. anginosus* maintained their original shape; (F) after three cycles of indirect laser treatment: From both species, no intact bacteria were discernible. Note the partial melt of the dental substrate and the presence of coagulated cell masses of variable size; (f) detail of the previous image.

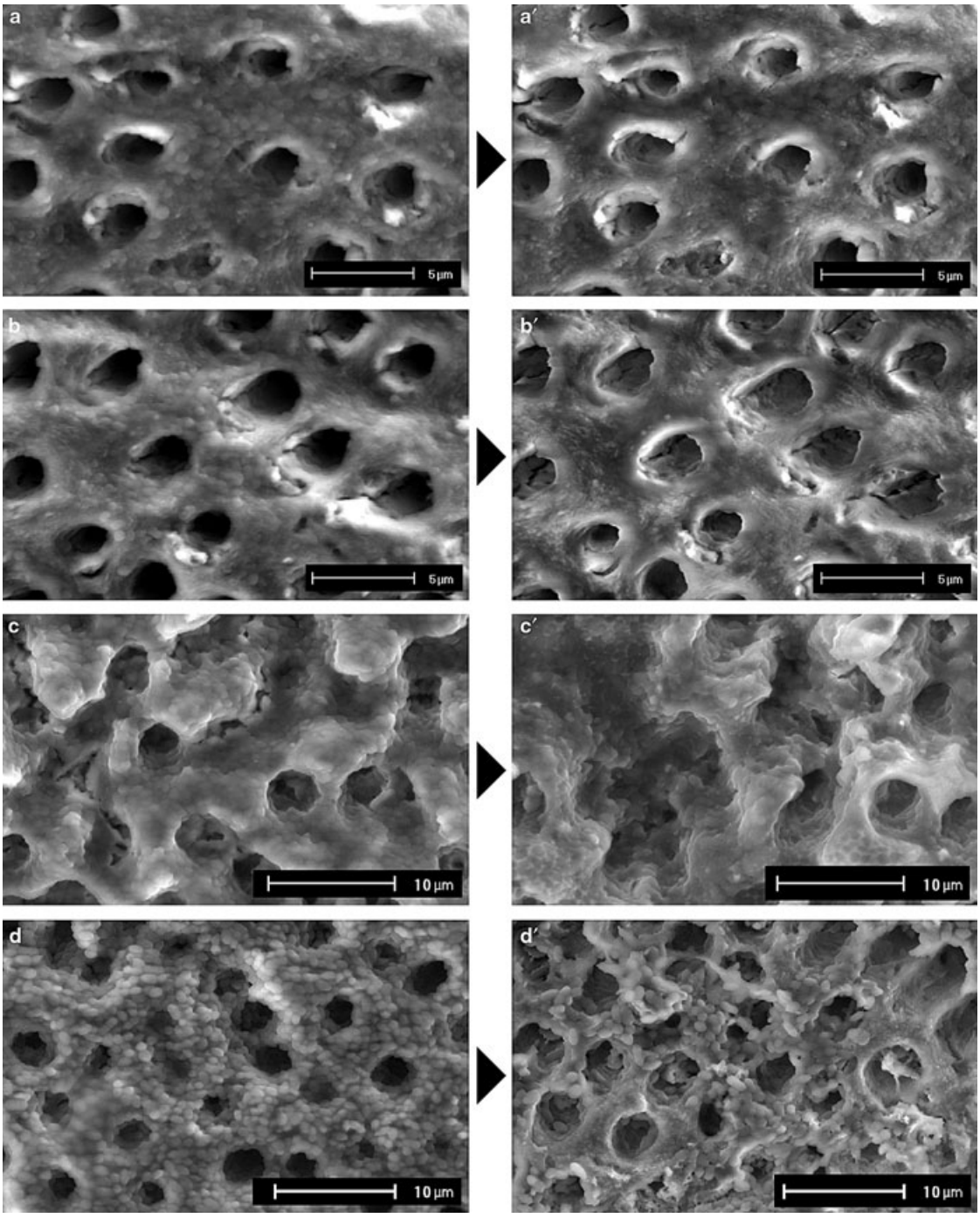

Figure 4 ESEM: (a,b) Micro-colonies and single cells of *S. anginosus* after 2 days of incubation (10,000x); (a′, b′) Re-analysis of spots (a) and (b) after laser treatment (1.5 W, 15 Hz, 20-s recovery interval). Two cycles (a-a′) eliminated most bacteria, but several apparently intact cells could be found. Even after three cycles (b-b′) some morphologically intact bacteria were observed; (c,d) After 2 days, cells of *E. faecalis* covered most of the dentinal surface creating a multileveled structure that frequently hid the entrance of a dentinal tubule (5,000x). Note the open formation due to the presence of various channels. (c′, d′) Re-anaysis of spots (c) and (d) after two and three cycles of laser treatment respectively: laser irradiation resulted in the destruction of superficial layers; more deeply, the number of remaining intact bacteria was uncountable.

as a solid structure but as an open formation due to the presence of various channels, which were expected to originate at the entries of the dentinal tubules. *Streptococcus anginosus* created micro-colonies in such a way that parts of the dentinal surface were still visible; confluent growth and biofilm formation were absent. This fact made the exact registration of the images taken before and after laser irradiation easier as the position of a tubular entrance could serve as reference.

Concerning the capacity of Nd:YAG to destroy bacteria, it was apparent that when individual cells or micro-colonies of *S. anginosus* were present, two cycles of laser irradiation (1.5 W, 15 Hz, directly for 5 s per cycle) eliminated most bacteria (Fig. 4a,a'). The dentinal surface appeared quite clean, whilst some cells remnants were visible. On closer inspection, however, several apparently intact bacteria could be found. After three cycles at the same setting, morphologically intact bacteria were just a rare observation (Fig. 4b,b'), but still their existence could not be ruled out.

On the other hand, when a biofilm was present (as in the event of inoculation with *E. faecalis*), the effect of bacterial eradication appeared to be strongly reduced. Two cycles of laser irradiation resulted in the destruction of superficial layers and a partial disruption of the three-dimensional biofilm (Fig. 4c,c'). Layers that were situated deeply were revealed, whilst the number of remaining intact cells could not be counted. One extra cycle caused destruction of more layers, but still a mass of morphologically intact bacteria could be seen covering the surface or hiding inside the dentinal tubules (Fig. 4d,d'). As the thickness of the biofilm was not constant over the surface, the observations at the diverse locations and on the different samples varied. Bacterial growth, however, could be obtained on the BA plates after every distinct treatment.

Discussion

Because bactericidal potential is developed through direct cell contact (Jones *et al.* 1991), the condition upon which chemical disinfection depends, is the extent to which substances spread into the system. Regarding penetration of dentinal tubules, it has been demonstrated that NaOCl (Berutti *et al.* 1997) and $Ca(OH)_2$ (Haapasalo & Ørstavik 1987) have a limited ability (about 130 μm) to penetrate and disinfect. Chlorhexidine and IKI are more effective in dentinal tubules than pure $Ca(OH)_2$ in a water vehicle, but no complete disinfection has been established (Safavi *et al.* 1990, Vahdaty *et al.* 1993). Although the relative importance of deep dentinal infection for the prognosis of treatment is not yet known (Oguntebi 1994), possibly all infected habitats within the system may constitute important reservoirs, from which root canal infection may re-occur after treatment (Nair *et al.* 2005).

Given the characteristics of laser light (i.e. monochromatic, coherent and directional) and the fact that direct contact between target and fibre tip is not required, emission of laser energy could represent a way to disinfect areas deep within the dentine. As a laser beam with a wavelength of 1064 nm can be directed trough an optical system and delivered by a thin flexible and durable glass fibre, Nd:YAG laser irradiation can be used after shaping to an apical preparation diameter of at least 200 μm (size 20). When laser energy is absorbed by the target, a reaction may occur depending on the total amount of energy applied, whilst the interaction type depends on power density (W cm^{-2}) and pulse duration. A photo-thermal interaction with bacteria will represent a bactericidal effect.

In the present study, the role of Nd:YAG lasers in root canal disinfection was defined using three analytical approaches with proper specimen selection and preparation criteria. For bacteriological testing, maxillary incisor teeth were chosen as they have lesser areas inaccessible to sampling (Siqueira & Lopes 1999). The potential of laser energy to act deep within dentinal tubules was investigated by a second approach, by which the beam was emitted over a distance (i.e. indirectly through a 1-mm-thick dentine disc), whilst observing the opposite site using CSEM. A third approach was the use of ESEM (Danilatos 1993). The inconvenience of losing resolution is thereby outweighed by the ability to explore the effect on bacterial appearance of endodontic treatment modalities using the *in situ* testing environment (Bergmans *et al.* 2005). In this study, four to six spots were observed on a flat surface, direct laser irradiation was performed with the ESEM chamber door in open position (real atmospheric conditions), and the spots were relocated and re-analysed using the stage memory option.

Enterococcus faecalis, *S. anginosus* and *A. naeslundii* were selected as test organisms as they are common isolates (Sundqvist 1994) and easy cultivable. *Enterococcus faecalis* was of particular clinical relevance because this species has frequently been associated with therapy-resistant infections (Sundqvist *et al.* 1998). Incubations were performed anaerobically to determine strict and/or facultative anaerobic growth, whilst time of incubation varied between 2 h and

2 days depending on the approach. Within this period, bacteria of *A. naeslundii* and *E. faecalis* were able to superficially penetrate dentinal tubules.

The Nd:YAG laser irradiation was performed at operational settings advocated by the literature. One or more 5-s cycles (1.5 W power output, 100 mJ pulse energy, 150 μs pulse length, and a frequency of 15 Hz) were used with 20-s recovery intervals. According to Moritz *et al.* (2000), this protocol results in a safe application *in vivo*. Samples were used in wet condition and without photosensitizing dye to avoid ablation and degeneration of the dentine; an effect that would not fit in a minimally invasive approach.

As the number of CFU mL^{-1} represented a close estimate of viable bacteria inside the root canal system, our results indicated that Nd:YAG laser irradiation (four times for 5 s) without further dentine removal caused a 99.7% kill of *E. faecalis* (thus no sterilization). This finding was in close agreement with Moritz *et al.* (1999) (99.2%), despite the fact that in their study one lasing cycle comprised five irradiations (5 s each) instead of one.

Conventional scanning electron microscopy observation of structural changes was a way to support the quantitative data. Not all of the observed forms of damage might be immediately lethal, but likely most of them severely weaken the microorganisms as a whole and limit their survivability in the inhospitable environment of a root filled tooth (Moritz *et al.* 2000). In general, CSEM verified that the extent of radiation damage was in line with the total amount of laser energy applied. Whilst more difficult to determine, *A. naeslundii* bacteria seemed more sensitive to radiation damage than their *S. anginosus* counterparts. Similar morphologic evidence and a more pronounced difference using Gram-positive as well as Gram-negative species has been shown by Moritz *et al.* (2000). In the present study, Nd:YAG was effective having three cycles of indirect laser treatment through a 1-mm-thick dentine disc, which corresponds to a dentinal tubule depth of 1000 μm from the canal lumen. These results confirm previous findings by Klinke *et al.* (1997) and Schoop *et al.* (2004).

Using ESEM, it became clear that *E. faecalis* had grown in sessile biofilms, in which it was embedded in an extracellular matrix material (Nair 1987, Costerton *et al.* 2003). Three cycles of Nd:YAG laser irradiation (total energy of 22.5 J) in contact mode just partially affected biofilms; an observation that complemented their low susceptibility to antimicrobial agents, which was demonstrated previously (Gilbert *et al.* 1997). Mature biofilms can be notoriously resistant for reasons that have yet to be adequately explained. In the case of laser treatment, it can be hypothesized that the given energy was not sufficiently distributed in depth. From the results of this study, some adjustments to the adopted protocol were proposed to increase the bactericidal effect when dealing with protective biofilm formation: (1) copious irrigation with NaOCl prior to laser irradiation; (2) a higher number of lasing cycles, and (3) the use of specially designed fibre tips that maximize the lateral distribution of laser energy.

Conclusion

Nd:YAG laser irradiation is not an alternative but a possible supplement to protocols for disinfection as the unique properties of laser light may allow a bactericidal effect beyond the reach of traditional preparation. Endodontic pathogens that grow as a multilayered structure, however, persist in being difficult to eradicate even when having direct exposure to the laser beam. There is a need for research on biofilms created in root canal systems and the way of treatment to eliminate them along with the ideal of a minimally invasive treatment concept.

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