An *in vitro* comparison of the bactericidal efficacy of lethal photosensitization or sodium hyphochlorite irrigation on *Streptococcus intermedius* biofilms in root canals

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Abstract

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Aim To compare the bacterial killing of *Streptococcus intermedius* biofilms in root canals using lethal photosensitization with various combinations of photosensitizer concentration and laser light dose or 3% sodium hypochlorite (NaOCl) irrigation.

Methodology Extracted teeth ($n = 35$) with single canals were selected and the canals prepared to apical size 25 with a 10% taper. The teeth were autoclaved and the canals inoculated with *Streptococcus intermedius* in brain heart infusion broth and were incubated for 48 h to allow a biofilm to form. The teeth were then subjected to 3% NaOCl irrigation ($n = 4$) or lethal photosensitization using combinations of a range of toluidine blue O (TBO) photosensitizer concentrations (12.5, 25, 50, 100 μgmL^{-1}) and light doses (60, 90, 120, 300, 600 s equivalent to energy doses of 2.1–21 J) using a 35-mW helium–neon low power laser targeted at the access cavity ($n = 4$ for each combination). Controls consisted of laser light only (TBO = 0 μgmL^{-1}) ($n = 4$), TBO only (light dose = 0 s) ($n = 4$), and no treatment (positive control $n = 17$). Following treatment the canal contents were sampled with sterile paper points, the sample was dispersed in transport medium, serially diluted and cultured on blood agar to determine the number of colony forming units (CFU).

Results The combination of 100 μgmL^{-1} TBO and 600 s (21 J) of laser energy achieved maximum reduction in recovered viable bacteria (5 $\log_{10}$ CFU). TBO at low concentrations (≤50 μgmL^{-1}) was not bactericidal but treatment with 100 μgmL^{-1} TBO alone reduced recovered viable bacteria by 3 $\log_{10}$ CFU. Laser light alone had limited bactericidal effect. No viable bacteria were recovered following treatment with 3% NaOCl.

Conclusions The combined use of a photosensitizing agent and a low power laser directed at the access cavity was bactericidal to *S. intermedius* biofilms in root canals but was unable to achieve total kill, unlike 3% NaOCl.

Keywords: biofilm, laser, photosensitizer, root canal, *S. intermedius*.

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Introduction

Microorganisms play a crucial role in the development of pulpal and periapical disease (Kakehashi *et al.* 1965). Infected root canals have a complex microbial flora consisting of cocci, rods, spirochaetes, filaments and fungi (Nair 1987) that are distributed along the root canal (Thilo *et al.* 1986) and may exist as loose collections in the moist canal lumen or as dense aggregates (biofilms) adhering to the dentine wall (Nair 1987). They may also penetrate the dentine to variable depths (Shovelton 1964, Ando & Hoshino 1990), up to 300 μm (Horiba *et al.* 1990) or more. Eliminating such a polymicrobial

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infection from the root canal system to allow healing of the associated periapical lesion is the ultimate goal of root canal treatment. Contemporary treatment procedures to eliminate the infection, include mechanical enlargement of the main canal, irrigation with an antibacterial agent, interappointment dressing of the canal with an antibacterial medicament and finally, obturation of the resulting dead space. A range of different techniques result in similar success rates (Smith *et al.* 1993) but 20–30% may fail and if retreatments are considered, the failure rate is even higher (Sjögren *et al.* 1990). This may be attributed to many factors, but a major hindrance is the complex anatomy of the root canal space (Haapasalo & Ørstavik 1987, Wada *et al.* 1998). Further disadvantages of conventional treatment procedures include their skill-dependent nature, long treatment time, possible weakening of teeth due to widening of the root canal and use of medicaments such as sodium hypochlorite (Sim *et al.* 2001) and calcium hydroxide (Grigoratos *et al.* 2001).

New approaches to eliminate the infection from root canal systems include the non-instrumented technique (Lussi *et al.* 1995) and use of laser technology (Kimura *et al.* 2000). A key problem in achieving total kills of bacteria in root canals is that the antimicrobial agent may not have access to the bacteria because of anatomical barriers. A common property of these new techniques is that anatomical complexities do not pose the same barriers. Laser light shone on the crown surface could potentially be redirected in multiple directions by virtue of its transmission through enamel prisms and dentinal tubules, which effectively act as fibre optic channels (Odor *et al.* 1996). High power lasers such as CO_2 (Zakariasen *et al.* 1986, Moshonov *et al.* 1995, Le Goff *et al.* 1999), Nd:YAG (Rooney *et al.* 1994, Hardee *et al.* 1994, Fegan & Steiman 1995, Gutknecht *et al.* 1996, Ramsköld *et al.* 1997, Berkiten *et al.* 2000), Nd:YAP (Blum *et al.* 1997), diode (Moritz *et al.* 1997) and Er:YAG (Mehl *et al.* 1999) are commonly used but not all deliver the optic fibres into the canal. The antibacterial effects of these lasers are a function of dose-dependent heat generation. The amount of heat delivered can vary but has the potential to char dentine, ankylose roots, melt cementum, cause root resorption and periradicular necrosis (Bahcall *et al.* 1992, Hardee *et al.* 1994, Ramsköld *et al.* 1997). These disadvantages may however, be overcome by sensitizing the microorganisms with a photosensitive agent capable of triggering the release of bactericidal radicals when stimulated by light of an appropriate wavelength. This technique called 'lethal photosensitization', has been laboratory tested on planktonic microorganisms associated with periodontal disease (Dobson & Wilson 1992, Wilson *et al.* 1992, 1993, Sarkar & Wilson 1993), root canal infection (Poh *et al.* 2000) and carious lesions (Burns *et al.* 1993, 1995) with encouraging results.

The concept of the root canal infection as a biofilm has not been given much consideration thus far. A biofilm is defined as an aggregation of bacteria associated with a surface, embedded in an extra-cellular matrix of polysaccharide. Biofilm bacteria differ greatly in phenotype when compared with their planktonic counterparts and are far less susceptible to antimicrobial killing (Millward & Wilson 1989, Nichols 1991, Wilson 1994). Laboratory models of single-species biofilms have shown that large numbers of viable bacteria remain following 15 min contact time with sodium hypochlorite or povidone iodine (Spratt *et al.* 2001). To date, studies on the lethal photosensitization of bacteria that cause root canal infection have been limited to laboratory studies of bacterial suspensions or biofilms grown on nitrocellulose discs (Dobson & Wilson 1992, Poh *et al.* 2000). The lethal photosensitization of biofilms has shown promise (Dobson & Wilson 1992), but has not been applied to biofilms covering a root canal surface *in situ*. It is postulated that the formation and diffusion of the reactive free radicals responsible for the antimicrobial effect may be able to penetrate the conventionally unreachable areas within the root canal system and achieve the necessary bacterial killing.

The aim of this study was to determine the effect of lethal photosensitization of *S. intermedius* biofilms in root canals using Toluidine Blue O (TBO) photosensitizer and a 35 mW helium–neon (He–Ne) laser and compare the antibacterial effect of lethal photosensitization with 3% NaOCl irrigation. *S intermedius* was chosen for the test biofilm because (i) it has the ability to adhere to dentine and is a probable player in primary colonization (Tarsi *et al.* 1998); (ii) it has been frequently isolated from root canal infections (Vigil *et al.* 1997, Sunde *et al.* 2000); and (iii) it has also been isolated from root canals following debridement procedures (Sundqvist *et al.* 1998), indicating its ability to resist treatment and survive as a single colonizer.

Materials and methods

A helium–neon gas laser with a measured power output of 35 mW (Spectra-Physics, Hemel Hempstead, UK) was used. The emitted radiation was collimated in a beam of 0.25 cm, with a wavelength of 632.8 nm. TBO photosensitizer (Sigma Ltd, Poole, UK) was prepared to various concentrations (0, 12.5, 25, 50 and 100 μgmL^{-1}), filter

sterilized (0.2 μm filter, Whatman International Ltd, Maidstone, UK) and stored in light excluded containers until required.

Thirty-five freshly extracted, intact, adult, human, single-rooted, mature teeth with a single canal were collected and stored in sterile saline. Calculus and stains were removed from the root surface using an ultrasonic scaler (Cavitron, Dentsply Ltd, Weybridge, UK). After accessing, the root canal was prepared to an apical size 25 using Flexofiles® (Maillefer Instruments SA, Ballaigues, Switzerland) with a 10% taper using Greater Taper™ hand files (Dentsply, Weybridge, UK) to a point 1 mm short of the apical foramen, which was subsequently sealed with composite restorative material (Durafill®, Heraeus Kulzer, Hanau, Germany). The root canal and outer surfaces were irrigated with 17% (v/v) Ethylenediaminetetra-acetic acid (EDTA) (Merck, Poole, UK) for 2 min, followed by tap water. The prepared tooth was mounted in the lid of a bijou bottle. The assembled tooth, lid and bottle were covered with aluminium foil and autoclaved at 121°C, for 15 min. The bijou bottle was then aseptically filled with sterile brain heart infusion broth (BHI) (Oxoid Ltd, Basingstoke, UK) so that the root was covered.

A root canal isolate identified as *Streptococcus intermedius* (by 16S rRNA gene sequence analysis) was used throughout the study. A frozen (–70°C, in BHI/glycerol, 10% v/v) stock ampoule was thawed and inoculated onto blood agar (BA) plates (Oxoid Ltd, Basingstoke, UK) containing 5% (v/v) defibrinated horse blood, incubated for 24 h at 37°C in a CO_2 incubator (B6420 Heraeus, Hanau, Germany). Cultures were then stored at 4°C until required and subcultured on a weekly basis. Bacterial cells were collected with a sterile swab and resuspended in 1 mL of sterile BHI broth to obtain an optical turbidity of 0.5 McFarland standard and incubated for 1 h at 37°C in the CO_2 incubator. The inoculum was syringed into the prepared root canal using a sterile endodontic irrigating syringe (Monoject™, Sherwood Medical Company, St. Louis, MO, USA) and incubated for 48 h under standard conditions as described previously.

Any residual medium in the root canals after the 48 h incubation was carefully removed with sterile paper points and the teeth were randomly selected for one of 31 treatment regimens. In order to reduce the effect of root canal anatomy as a variable, the 35 teeth were reautoclaved and reused up to a maximum of four times. After each test, the contents of the root canal and bijou bottle were emptied, the tooth irrigated with EDTA for 2 min, rinsed with water, reassembled in the bijou bottle and autoclaved before further use.

Test groups

Lethal photosensitization

Root canal biofilms were subjected to lethal photosensitization using 20 combinations of four TBO concentrations and five laser energy doses ($n = 4$ for each combination). The canals were filled to the level of the access cavity with TBO at various concentrations (12.5, 25, 50 or 100 $\mu g mL^{-1}$) and incubated for 30 s. Laser light was then applied for a specified time (60, 90, 120, 300 or 600 s equivalent to energy doses of 2.1, 3.2, 4.2, 10.5 or 21 J) at the orifice of the access cavity without introducing the laser optic fibres into the root canal.

Sodium hypochlorite irrigation

The canals ($n = 4$) were filled with NaOCl (3% v/v) for 5 min, removed with sterile paper points and fresh NaOCl solution was added for a further 5 min.

Control groups

Laser light only

The canals ($n = 4$ for each dose) were filled with reduced transport fluid (RTF) (Syed & Loesche 1972) for 30 s followed by application of various laser light doses (60, 90, 120, 300 or 600 s).

TBO only

The canals ($n = 4$ for each concentration) were filled with TBO at various concentrations (12.5, 25, 50 or 100 $\mu g mL^{-1}$) and incubated for 30 s.

No treatment

The canals ($n = 17$ included positive controls for several experimental runs) were filled with RTF and incubated for 30 s.

Post-treatment root canal sampling

Following all treatments, the liquid content of the root canal was carefully absorbed with paper points without intentionally touching the canal walls to avoid interference with the biofilm. The canal was refilled with RTF, circumferentially filed with a sterile, size 25 Flexofile® to the working length for 20 s. The resulting bacterial suspension was completely absorbed with paper points and transferred to

Table 1 Viable bacteria recovered after treatment of *S. intermedius* biofilm in root canals with various combinations of laser energy and TBO concentration

Laser parameters			TBO concentration (μgmL^{-1})				
Time (S)	Dose (J)	Density (J cm^{-2})	0	12.5	25	50	100
0	0	0	[d]1.6×10^8 (3.4×10^8) ($n = 17$)	[c]3.0×10^8 (4.7×10^8)	[c]1.8×10^8 (2.6×10^8)	[c]4.7×10^8 (5.8×10^8)	[c]1.1×10^5 (1.5×10^5)
60	2.1	42.9	[b]1.2×10^6 (1.6×10^6)	[a]1.3×10^8 (2.4×10^8)	[a]3.5×10^6 (3.6×10^6)	[a]1.2×10^7 (1.9×10^7)	[a]2×10^8 (4×10^8)
90	3.2	64.3	[b]2.0×10^8 (3.7×10^8)	[a]4.2×10^6 (7.9×10^6)	[a]1.1×10^8 (2.1×10^8)	[a]3.8×10^8 (5.8×10^8)	[a]3.2×10^8 (5.9×10^8)
120	4.2	85.7	[b]6.8×10^5 (7×10^5)	[a]3.9×10^8 (5.7×10^8)	[a]4.4×10^5 (5.1×10^8)	[a]2.5×10^8 (5×10^8)	[a]1.4×10^6 (2.5×10^6)
300	10.5	214.3	[b]1.1×10^7 (2×10^7)	[a]4.5×10^7 (8.8×10^7)	[a]4×10^7 (8×10^7)	[a]2.6×10^5 (4.7×10^5)	[a]4.1×10^5 (6.6×10^5)
600	21.0	428.6	[b]7.5×10^5 (3.3×10^5)	[a]2.1×10^5 (4.2×10^5)	[a]1.2×10^5 (2.4×10^5)	[a]1.2×10^4 (1.7×10^4)	[a]3.6×10^3 (2.5×10^3)

Mean CFU per canal and standard deviation in parentheses; ($n = 4$ except as shown).
[a]Test groups subjected to lethal photosensitization.
[b]Control groups subjected to laser light only (TBO = 0 μgmL^{-1}).
[c]Control groups subjected to TBO only (laser = 0 s)
[d]Control group subjected to no treatment.

1 mL of RTF. After vortexing for 30 s, a 7-fold (neat to 10^{-7}) serial dilution was prepared and 25 µL of each was inoculated onto a blood agar plate and incubated for 24 h as described previously. Colony forming units (CFU) recovered from each treated root canal were calculated.

Results

The teeth ($n = 17$) with *S. intermedius* biofilms receiving no experimental treatment served as baseline controls, the mean number of viable bacteria recovered from them was 1.62×10^8 CFU/canal (Table 1).

The individual and combined effect of TBO and laser light on the viability of *S. intermedius* biofilms are also presented in Table 1. 'TBO only' treatment and 'laser light only' treatment, both had mild bactericidal effects. A maximum of 3 $\log_{10}$ viable bacterial reduction was observed with TBO (100 μgmL^{-1}) or laser light (120 s [4.2 J] and 600 s [21 J]).

The combined effect of TBO and laser was bactericidal but could not achieve 100% bacterial kill. A maximum reduction of 5 $\log_{10}$ of viable bacteria was achieved with 100 µg mL^{-1} TBO and 600 s (21 J) laser dose compared to baseline control.

In stark contrast, no viable bacteria could be recovered from any teeth with *S. intermedius* biofilms treated with 3% NaOCl for 10 min. Even the most effective combination of TBO and laser dose applied at the access cavity could not compete in effectiveness with 3% NaOCl.

Discussion

The *S. intermedius* strain used in the study was isolated from an infected root canal in our laboratory. *S. intermedius* is a Gram positive, facultative anaerobe and has previously been identified in coronal and apical parts of infected root canals (Baumgartner & Falkler 1991, Sundqvist 1992), deep layers of root dentine (Ando & Hoshino 1990), acute apical abscesses (Lewis *et al.* 1986), and root-filled canals with persistent infections (Sundqvist *et al.* 1998). In addition, the choice was also influenced by the ease of growth and laboratory manipulation of this strain.

Previous studies on effectiveness of lethal photosensitization have focused mainly on bacterial suspensions (Wilson *et al.* 1992, 1993). Their effect on bacterial biofilms has rarely been investigated and most of these have been performed on single-species biofilms grown on nitrocellulose discs (Dobson & Wilson 1992, Poh *et al.* 2000). The laboratory extracted tooth model, with a *S. intermedius* biofilm coating the root canal used in this study, simulated the *in vivo* situation a little more closely. However, the single-species biofilm used is not representative of the polymicrobial infection encountered in the root canal (Nair 1987, Molven *et al.* 1991). Unpublished work in our laboratory attempting to create a simple polymicrobial biofilm has proved unpredictable and certainly not reproducible thus far. It was therefore decided to select one of the commonly isolated species likely to

adhere to dentine and representative of those species more resistant to treatment. A series of pilot studies were carried out to establish the protocol for this model, including confirmation of the biofilm by SEM. The canals of all teeth were prepared to an apical size 25 with a 10% taper in order to allow accurate sampling and quantification of bactericidal effects, as well as to achieve a degree of standardization. The introduction of fibre optics into the canal was considered but not adopted in the first instance, as application at the access cavity is clinically straightforward, and experimentally avoids displacement of TBO and mechanical interference (not the subject of test) of the biofilm. Having shown the effectiveness of lethal photosensitization of a nitrocellulose disc biofilm model (Poh *et al.* 2000), the aim of the present study was to test the hypothesis that the structure of teeth would aid light transmission (Odor *et al.* 1996) and allow the reproduction of previous success in nitrocellulose-borne biofilms, in the current tooth model.

In this study, irradiation with He–Ne laser alone had no consistent bactericidal effect on *S. intermedius* biofilm. These data were in agreement with others (Wilson *et al.* 1992, 1993, Dobson & Wilson 1992, Sarkar & Wilson 1993, Burns *et al.* 1993) who investigated the effects of He–Ne laser on bacterial suspensions. TBO with an absorption maxima (632.2 nm) closest to the wavelength of the radiation emitted by the He–Ne laser (632.8 nm) has been shown to be an effective photosensitizer for He–Ne laser mediated killing (Wilson *et al.* 1992, Dobson & Wilson 1992). It was noted that TBO gave a bluish tinge to teeth after treatment but the discolouration could be easily removed with EDTA irrigation. The results of the present study have shown that the combined effect of TBO and He–Ne laser was bactericidal to the *S. intermedius* biofilm but not effective enough to achieve sterility even with a combination of high TBO concentration (100 μgml^{-1}) and laser dose (600 s, 428.6 J cm^{-2}). Dobson & Wilson (1992) reported successful kills of periodonto-pathogenic species biofilms (*Streptococcus sanguis, Actinobacillus actinomycetemcomitans, Fusobacterium nucleatum and Porphyromonas gingivalis*) using low concentrations of TBO (50 μgmL^{-1}) and low doses of He–Ne laser light (30 s, 16.5 J cm^{-2}). The difference in results could be explained by variations in the sensitivities of different bacterial species or indeed different properties of biofilms grown on agar plates compared to those on root canal walls. Low concentrations of TBO (<50 μgmL^{-1}) had no bactericidal effect on *S. intermedius* biofilms, but when they were incubated with 100 μgmL^{-1} TBO for 30 s, a 3 $\log_{10}$ reduction in viable bacteria was observed. Previous reports have shown that some planktonically grown periodontal pathogen suspensions were sensitive to 25 and 50 μgmL^{-1} of TBO (Wilson *et al.* 1993, Sarkar & Wilson 1993, Burns *et al.* 1993) but *Actinomyces viscosus* and *Streptococcus mutans* were significantly more resistant (Burns *et al.* 1993). Lethal photosensitization using a low power laser in an infected tooth model in the present study produced similar results to that of irradiation with higher power lasers (Nd:YAG, Nd:YAP, diode, CO_2, Er:YAG) which have also achieved various degrees of bacterial killing but not to the extent of achieving sterility (Hardee *et al.* 1994, Fegan & Steiman 1995, Goodis *et al.* 1995, Gutknecht *et al.* 1996, Blum *et al.* 1997, Moritz *et al.* 1997, Ramsköld *et al.* 1997, Le Goff *et al.* 1999, Mehl *et al.* 1999). The inconsistent trends evident in bacterial killing with increasing TBO concentration and laser light dose could be attributed to the variation in root canal morphology. The anatomy could impact on this mode of treatment at two levels. First, the manner of interaction of the bacteria with individual root canal systems could result in variable biofilm properties. Secondly, the anticipated circumvention of anatomical complexities by light transmission appears to be confounded; whether this is a function of actual hindrance of light transmission, lack of penetration of TBO or lack of generation and dispersal of free radicals is unclear. Lethal photosensitization could not compete with NaOCl (3% v/v) in achieving consistent 100% bacterial kills in this study. This is however, different from treatment of polymicrobial infections, where NaOCl may not be so effective (Byström & Sundqvist 1981, 1983). The results of this study reinforce the use of NaOCl irrigation in root canal treatment because of its bactericidal effect, as well as its ability to denature bacterial toxins (Safavi & Nichols 1994) and dissolve organic tissues (Baumgartner & Cuenin 1992).

A major advantage of lethal photosensitization in treating root canal infections is the absence of thermal side-effects in the tissues surrounding the roots, as associated with the use of high power lasers. The anticipated benefits of 'access' of laser light and photosensitizer were more limited than hypothesized. Further studies are necessary to determine the penetration of photosensitizers into the complex root canal anatomy and the range of activity of 'free radicals'. Moreover, refinement of the laser delivery system by introduction of the laser beam into the root canal and/or increased energy delivery may be needed to achieve a better antimicrobial effect. Overall the lethal photosensitization of single-species biofilms grown in a tooth model was interestingly effective considering delivery of the light dose at the access cavity, but ultimately not comparable to the conventional use of

3% NaOCl. The method may have potential, either as a modification of the current application or used as an adjunct to conventional approaches.

Conclusions

Within the limitations of this study, it was concluded that the combined use of a photosensitizing agent and a low power laser is bactericidal to *S. intermedius* biofilms in root canals but is not as effective as irrigation with 3% NaOCl.

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