

Sensitivity of oral bacteria to 254 nm ultraviolet light

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Abstract

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Aim To explore the sensitivity of bacteria commonly found in root canals to 254 nm ultraviolet (UV) light, either as individual cells or as participants of a bacterial multilayer.

Methodology The sensitivity of oral bacteria, as individual cells, to UV light was tested by subjecting plates streaked with bacteria to 254 nm UV, at a fluence of 1–20 mJ cm⁻². An experimental model was designed to produce a bacterial multilayer and to study absorption of UV light by bacteria in an outer layer and its effect on the elimination of bacteria in the inner layer.

Results Direct exposure to relatively low doses of UV light (2–7 mJ cm⁻²) effectively eliminated all bacterial strains tested. Furthermore, an *Enterococcus faecalis* strain, partially resistant to a 24 h exposure to calcium hydroxide, was effectively eliminated within several seconds of exposure to UV light ($P < 0.001$). UV was absorbed by a multilayer of bacteria. When 4 bacterial cells μm^{-2} were present in the light path, the UV light dose had to be increased by a factor of $\times 10$ to achieve 100% elimination of the bacteria in an inner layer.

Conclusions The application of UV light to eliminate endodontic pathogens may be possible. Nevertheless, its absorbance by outer layers of bacteria should be considered and the UV light dose adapted accordingly.

Keywords: absorption, oral bacteria, ultraviolet.

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Introduction

Planktonic *Enterococcus faecalis* is killed *ex vivo* by sodium hypochlorite at an impressive rate. Total bacterial elimination occurs within 30 s when exposed to a 5.25% solution (Gomes *et al.* 2001). However, when applied in infected root canals, either under clinical conditions or *ex vivo*, sodium hypochlorite fails to thoroughly disinfect canals (Byström & Sundqvist 1985, Siqueira *et al.* 1997, Sjögren *et al.* 1997, Shuping *et al.* 2000, Shabahang & Torabinejad 2003). Thus, to attain this major goal of root canal treatment additional measures may be required, such as dressing with calcium hydroxide (Ca(OH)₂) medicaments

(Byström *et al.* 1985, Shuping *et al.* 2000, Trope & Bergenholtz 2000).

Calcium hydroxide medication is effective against most gram negative anaerobes, however, certain resistant bacteria, such as various *E. faecalis* strains may survive this protocol (Molander *et al.* 1998, Sundqvist *et al.* 1998, Distel *et al.* 2002, Chávez de Paz *et al.* 2003, Kvist *et al.* 2004). Furthermore, Chávez de Paz *et al.* (2003) recently reported that 54% of the root canals treated with Ca(OH)₂ or iodine–potassium iodine may harbour viable bacteria after prolonged application of these preparations, and in 13%, positive cultures were obtained even after three consecutive applications. Amongst the bacterial strains resistant to the above protocol, lactobacilli, non-mutans streptococci and enterococci were predominant.

Ultraviolet (UV) light, at a wave length of 254 nm, has been widely used for surface disinfection in laboratories and operating rooms, as well as for water

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and fruit disinfection (Mamane-Gravetz *et al.* 2005, Lagunas-Solar *et al.* 2006). It has two major potential benefits: (i) an instant action, avoiding the need for prolonged exposure time; and (ii) its bactericidal activity is based on targeting and causing damage to the DNA, thus it may be non or less selective than other bactericidal agents (Harm 1984, Durbeej & Eriksson 2003).

This preliminary study was designed to explore the sensitivity of bacteria commonly found in root canals to UV light, either as individual cells or as participants in a bacterial multilayer.

Materials and methods

Bacteria and growth conditions

Three strains of *Porphyromonas gingivalis* were used: *P. gingivalis* PK 1924, *P. gingivalis* 274 and *P. gingivalis* W50. These strains, as well as *Fusobacterium nucleatum* PK1594 were all originally clinical isolates, previously used in studies conducted in the same laboratory (Weiss *et al.* 2000, Metzger *et al.* 2001). *Enterococcus faecalis* strain (TA) was initially isolated and previously used by Weiss *et al.* (1996) and is kept as a frozen stock. *Streptococcus sanguis* N1 (Tel Aviv University frozen stock) was previously used in studies of infected dentinal tubules (Lin *et al.* 2003). *Lactobacillus brevis* ATCC 8287 was obtained from Hy Laboratories (Rehovot, Israel).

Enterococcus faecalis was grown aerobically at 37 °C, either in a brain–heart infusion (BHI) broth (Difco, Becton Dickinson, Sparks, MD, USA) or on a BHI agar (Hy Laboratories). *F. nucleatum* and *P. gingivalis* were grown anaerobically in a Coy chamber (85% nitrogen, 5% carbon dioxide and 10% hydrogen) either in Wilkins–Chalgren anaerobic broth (Oxoid, Basingstoke, UK), or on CDC anaerobic blood agar plates (Hy Laboratories). *S. sanguis* was grown anaerobically in a Coy chamber in either BHI broth or on BHI plates. *L. brevis* ATCC 8287 was grown aerobically at 28 °C in either APT broth or on APT agar plates (Hy Laboratories).

Each strain was grown in the proper broth and harvested at a late logarithmic to early stationary phase, washed and re-suspended in saline and the suspension adjusted to $OD_{640} = 1.0$. The suspensions were further diluted in saline and 10 µL aliquots, containing approximately 100 colony forming units (CFU) were streaked on agar plates and subjected to the tests. Each bacterial strain was grown in the above

conditions until clear colonies were detected. Experiments were conducted in quadruplicate.

Ultraviolet light source and measurements

A Bio-Link UV illumination apparatus (Vilber-Lourmat, Marne La Valee, France) was used as an UV light source. This instrument emits 254 nm UV light from $5 \times 8W$ tubes and may be calibrated to the desired energy dose by length of exposure. Spectral curves indicated 94% emission in a narrow spike at 254 nm. UV light exposures ranged from 1 to 70 $mJ\ cm^{-2}$. The actual energy flow of the UV light at the plane of the target was directly measured using a photometer (Ophir Optronics Inc., Jerusalem, Israel), calibrated to measure only at a wavelength of 254 nm; energy flow was expressed as $mW\ cm^{-2}$.

Bacterial sensitivity to ultraviolet light

To determine bacterial sensitivity to UV light, agar plates freshly streaked with bacterial suspensions were opened under the UV light source and the bacteria directly exposed to the light for varying durations, resulting in exposures ranging from 1 to 70 $mJ\ cm^{-2}$. The plates were incubated in the above mentioned conditions for each bacterium and CFU counted at the end of the incubation. This protocol did not result in any contamination, as plates were opened in a protected chamber of the Bio-Link apparatus. Plates identical to the above, which were also opened in the same conditions but not exposed to UV light, served as controls. The number of CFU per plate was adjusted so that control plates contained about 100 CFU per plate. Each plate was digitally photographed and colony counting was conducted on an enlarged digital image of the plate to which a digital grid was adapted. Elimination of viable bacteria was calculated from the ratio between the number of CFU in the UV irradiated plates and that of the control plates and expressed as per cent elimination.

Enterococcus faecalis sensitivity to calcium hydroxide versus ultraviolet light

Enterococcus faecalis TA was grown in BHI broth and harvested from an overnight culture (late logarithmic phase of growth), washed, re-suspended in saline and the bacterial suspension adjusted to $OD_{640} = 1.0$.

A 100 µL bacterial suspension, was mixed with an equal volume of $Ca(OH)_2$ paste (Calciject, Centrix, Hofheim, Germany), which was diluted 1 : 2 in saline,

to allow volumetric measurement, and incubated aerobically at 37 °C for 24 h. The mixture was re-suspended and the Ca(OH)₂ allowed to settle for 10 min to a sediment layer. The bacterial suspension above the Ca(OH)₂ sediment was collected, washed twice with saline and serially diluted 1 : 10. A 10 µL sample, from each dilution was streaked on individual fields of BHI agar and incubated aerobically at 37 °C for 24 h. A dilution field containing >10 but <50 colonies was selected for counting and the CFU in the original sample calculated. Equivalent cultures that were incubated with saline but not Ca(OH)₂ served as control.

To test for UV sensitivity, a sample of the same original *E. faecalis* culture was used at time 0. It was serially diluted and streaked on BHI agar as above. The plates were then opened and exposed to UV light (5 mJ cm⁻², ~6 s with the above UV light source). Equivalent plates not exposed to UV light served as control. The plates were incubated and CFU counted as above. The experiment was conducted in quadruplicate.

A model for bacterial absorption of ultraviolet light

A model was constructed (Fig. 1) to study the effect of UV light absorption by bacteria in outer layer(s) on the survival of bacteria in an inner layer.

A circular hole with a 40 mm diameter was prepared in the cover of a disposable culture plate (90 mm,

Miniplast, Ein Shemer, Israel). A neutral-density glass filter (50 mm diameter, 36.5% passage at UVC, Oriol, Stratford, CT, USA) was permanently cemented on the upper side of the cover, using cyanoacrylate adhesive (Loctite Superglue-3, Henkel, Boulogne, France). A standard 50 µm spacer was produced by cementing three stripes of a 50 µm thick brass metal sheet on the periphery of the upper side of the filter. A second identical filter placed on top of these spacers allowed for a uniform space of predetermined thickness between the filters. Bacterial suspensions, 2×10^{10} – 8×10^{10} cells mL⁻¹, were used and filled the space between the two filters by capillary action.

Ultraviolet light passed through this combined structure, hitting freshly streaked bacteria on a culture plate placed beneath the cover. The distance between the filters and bacterial suspension concentration were used to determine the number of bacteria in the light path, which was adjusted to 1–4 bacterial cells µm⁻².

The absorption factor of the empty setup, including two filters and the gap filled with 0.9% sodium chloride solution (saline), was determined by using a photometer calibrated for UV light at 254 nm. This factor was then used to determine the UV light exposures and the actual absorption by bacterial present in the light path.

Two parameters were tested with this model: (i) the absorption of UV light by bacteria present between the filters; and (ii) the effect of this absorption on survival of bacteria on the culture plate below. The first was

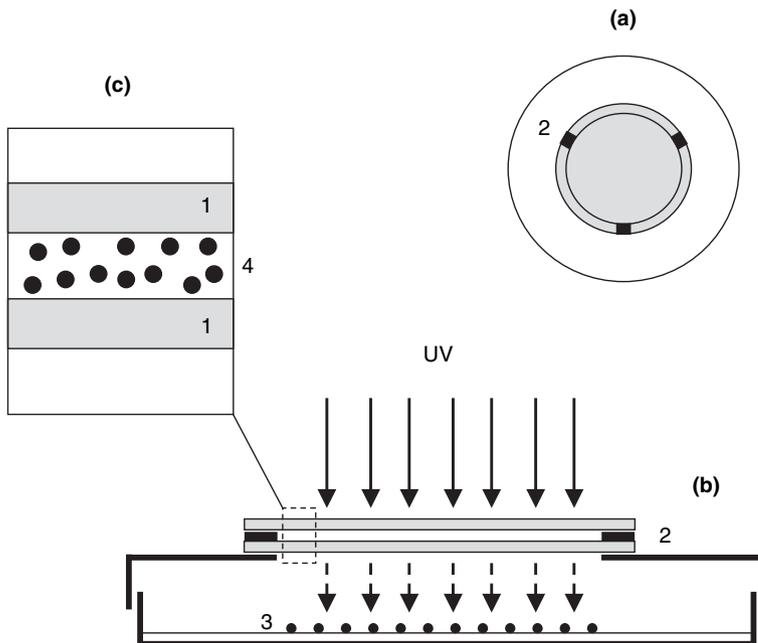


Figure 1 A model representing ultraviolet (UV) light absorption by bacteria in an outer layer, and its effect on bacterial elimination in an inner layer. (a) An overview of a petri dish cover with a filter adapted over a circular hole in its centre. (b) A cross-section of the assembled setup. (c) Enlarged cross-section of the two filters with bacterial suspension between them. (1) ND filters transmitting 254 nm UV light. (2) Metal sheet spacers, 50 µm thick. (3) Target bacteria on the agar surface. (4) Bacterial suspension between the filters.

undertaken using a photometer to directly measure the UV light that passed through the bacterial suspension and the second was completed by enumeration of CFU.

Results

Sensitivity of individual bacteria to ultraviolet light

All bacterial strains tested were sensitive to UV light at 254 nm, at a relatively low dose. At 6 and 7 mJ cm⁻², 99.9% elimination was obtained for *F. nucleatum* PK1594 and for *L. brevis* ATCC 8287, respectively (Fig. 2), whilst for *P. gingivalis* 1924, *E. faecalis* TA and *S. sanguis* N1 this goal was reached at 2 mJ cm⁻² (Fig. 2). Results within the same range were obtained with *P. gingivalis* 274 and with *P. gingivalis* W50: UV light at 6 mJ cm⁻² resulted in 99.9% elimination of both (data not presented).

Resistance of *Enterococcus faecalis* to calcium hydroxide versus ultraviolet light

Enterococcus faecalis TA was partially resistant to Ca(OH)₂. After 24 h of direct contact with Ca(OH)₂, bacterial counts were reduced by almost a factor of ×100. However, a high number of bacteria survived (Table 1). Exposure of the same bacteria to UV light at 5 mJ cm⁻² (~6 s) resulted in no bacterial survivors ($P < 0.001$, *t*-test).

Absorption of ultraviolet light by bacteria

Porphyromonas gingivalis W50 bacteria, which were present between the filters absorbed UV light. An UV energy flow of 0.155 mW cm⁻² was reduced to 0.04 mW cm⁻² when 1 bacterium μm⁻² was present in the light path, and further reduced to 0.011 mW cm⁻² with 2 bacteria μm⁻² in the light path (Fig. 3).

Bacterial elimination by ultraviolet light that passed through a bacterial suspension

When *Porphyromonas gingivalis* W50 was exposed to UV light with no bacteria in the light path, 100% elimination occurred at 7 mJ cm⁻² (Table 2). However,

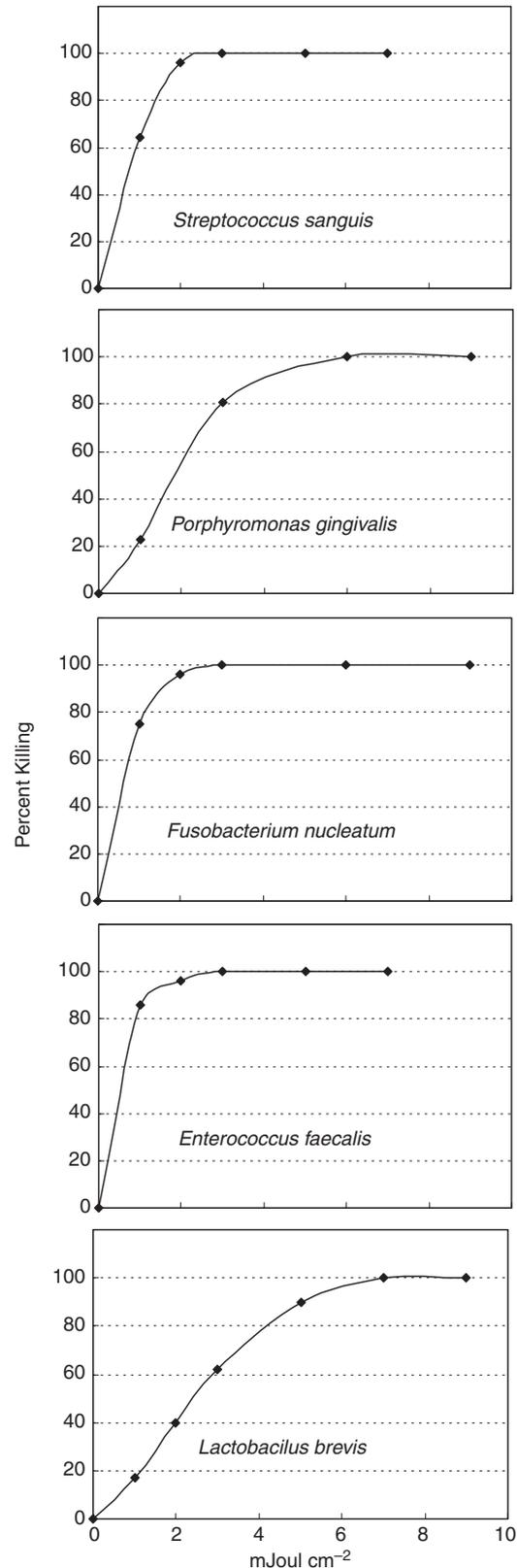
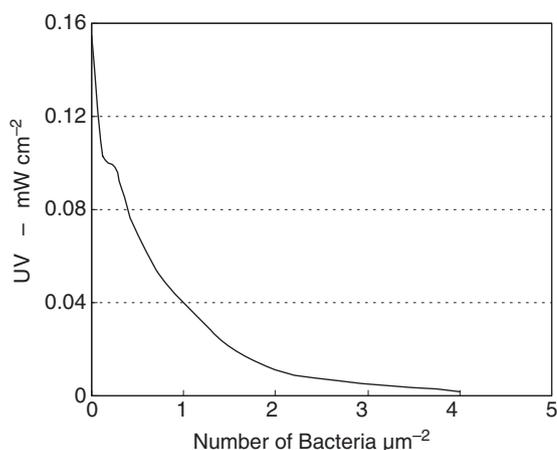


Figure 2 Elimination of oral bacteria by 254 nm ultraviolet light. Percentage of elimination of bacteria. Experiments were conducted in quadruplicate. Standard error of the mean within 10%.

Table 1 *Enterococcus faecalis* TA sensitivity to Ca(OH)₂ versus UV light

	Treated	Control
Ca(OH) ₂ , 24 h	3.5 × 10 ⁵ CFU	2.7 × 10 ⁷ CFU
UV, 5 mJ cm ⁻²	0 CFU	5.5 × 10 ⁸ CFU

Standard error of the mean within 10%. Ca(OH)₂, calcium hydroxide; CFU, colony forming units; TA, strain; UV, ultraviolet.

**Figure 3** Absorption of 254 nm ultraviolet light by bacteria.**Table 2** Effect of bacteria present in the light path on the elimination of bacteria in an inner layer by 254 nm ultraviolet light

Number of bacteria in light path	7 mJ cm ⁻²	35 mJ cm ⁻²	70 mJ cm ⁻²
0	100%	100%	100%
2 bacteria μm ⁻²	10%	100%	100%
4 bacteria μm ⁻²	0	25%	100%

Mean percentage of elimination of bacteria. Standard error of the mean within 10%.

the degree of elimination was significantly reduced when UV exposure was through a suspension of the same bacteria. With 2 and 4 bacteria μm⁻² in the light path, the dose had to be increased by 5 and 10, respectively, to obtain 100% elimination (Table 2).

Discussion

Sterilization, or at least a thorough disinfection of the root canal, is a major goal of root canal treatment. Bactericidal solutions, such as sodium hypochlorite, are commonly used to supplement the mechanical preparation of the root canals, however, recent reports

indicate that viable bacteria remain in root canals following this procedure (Byström & Sundqvist 1985, Siqueira *et al.* 1997, Sjögren *et al.* 1997, Shuping *et al.* 2000, Shabahang & Torabinejad 2003). Therefore, intracanal dressing with Ca(OH)₂ for 1–2 weeks is commonly used in an attempt to further reduce the viable bacterial content of the canal prior to filling (Byström *et al.* 1985, Shuping *et al.* 2000, Trope & Bergenholtz 2000).

Completion of root canal treatment in one visit has gained in popularity in recent years (Whitten *et al.* 1996). Some reports support this approach by indicating that a healthy outcome may be achieved even when the canals are not sterile when filled (Weiger *et al.* 2000, Peters & Wesselink 2002). However, most studies indicate that remaining viable bacteria in the root canal are more likely to be associated with post-treatment disease (Sjögren *et al.* 1997, Trope & Bergenholtz 2000, Waltimo *et al.* 2005). Whilst this is a continuous controversy, both supporters of one-visit endodontics and those who object to its application in infected root canals, are likely to agree that simple and efficient methods, which would render infected root canals free of viable bacteria within a single visit, would be beneficial.

The current study was designed to explore the potential antibacterial effects of UV light against bacteria commonly found in infected root canals. This issue was addressed at three basic levels: (i) a preliminary exploration of the sensitivity of relevant bacteria to UV light; (ii) UV light compared with Ca(OH)₂ in a resistant strain of *E. faecalis*; and (iii) investigating the effect of bacterial presence in an outer layer on susceptibility of those in an inner layer to UV light.

Oral bacteria commonly found in infected root canals were selected, such as *P. gingivalis* and *F. nucleatum*, as well as representatives of bacterial groups recently reported to survive an intracanal application of Ca(OH)₂: non-mutans streptococci, lactobacilli and enterococci (Chávez de Paz *et al.* 2003). When these bacteria were directly exposed *ex vivo* to 254 nm UV light, effective elimination occurred at relatively low energy doses: 2–7 mJ cm⁻². These results are in agreement with studies that found high sensitivity to UV light in a wide range of bacteria (Bolton 1999).

Furthermore, even an *E. faecalis* TA, that was relatively resistant to Ca(OH)₂ and partially survived 24 h of direct contact with this agent, was easily eliminated by the UV light. The survival of *E. faecalis* TA, after 24 h exposure to Ca(OH)₂, is apparently in conflict with the findings recently reported by Abdulla

et al. (2005). They found a profound effect of this agent on *E. faecalis* within 60 min. This difference could be attributed either to strain differences or to the prolonged 24 h incubation used in the present study which may have allowed proliferation of the survivors. Further studies will be required to clarify this issue.

The findings reported in the present study indicate that UV light may be effective against bacteria. However, recently, heterogenic susceptibility amongst oral bacterial strains was found when visible blue light, at a spectral range of 380–520 nm, was used to eliminate bacteria (Soukos *et al.* 2005). Thus, further studies, with a wider range of bacterial species and strains, are needed to map the susceptibility of potential endopathogens to 254 nm UV.

Bacterial sensitivity to other antibacterial agents has been reported to be affected by the age and growth conditions of the bacterial culture (Portenier *et al.* 2005). Therefore, further experiments should also include bacteria derived from older cultures, in their late stationary phase, as well as 'starved' cultures. It should be verified whether the high sensitivity to UV, presently reported, is related to the relatively young age of the cultures used or is it an inherent phenomenon that affects bacteria from 'starved' cultures as well.

The bactericidal mechanism of UV light is based on DNA absorption of light, causing cross-linking between neighbouring pyrimidine nucleotide bases (thymine and cytosine) in the same DNA strand. This, in turn, impairs formation of hydrogen bonds to the purine bases on the opposite strand. DNA transcription and replication is thereby blocked, compromising cellular functions and eventually leading to cell death (Harm 1984, Durbeej & Eriksson 2003). To cause this damage, the UV light must first be absorbed by the bacterial DNA. UV light absorption by DNA peaks at a wavelength of 254 nm, which explains the excellent bactericidal effect of this specific wavelength (Bolton 1999).

During the first stage of the current study, each bacterium was individually and directly exposed to the UV light, with nothing to block the light path. However, in a root canal environment, it is more likely that bacteria will be found in a multilayer, ranging in thickness from several to numerous cells. As bacterial DNA absorbs UV light, it may block it from reaching the inner bacterial layers. Thus, bacteria in this inner layer may be protected and remain viable.

The UV bacterial absorption model used in the present study was specially designed to address this issue. Bacteria on the agar surface represented those in

the inner layer of a bacterial multilayer. Bacteria in the solution between the two filters represented an outer layer of bacteria, through which the UV light passed to reach those in the inner layer. Different concentrations of bacterial suspension allowed varying amounts of bacteria in the light path. This value was calculated and expressed as the number of bacteria per square μm^2 of the light path.

The use of this model established that bacteria in the light path absorbed the UV light and that absorption was dependent on the amount of bacteria present. Absorption by bacteria in the outer layer reduced the bactericidal potential of the UV light: to achieve 100% elimination, the UV dose had to be increased by a factor of $\times 5$ to $\times 10$ when 2 or 4 bacteria μm^{-2} were present in the light path, respectively.

It should be noted that the bacterial concentration used (2–4 bacteria μm^{-2}) may not fully block the light path. Given that the dimensions of each bacterial cell are 0.5 μm (diameter) \times 1 μm (length), its silhouette will be approximately 0.2 μm^2 when viewed axially and 0.45 μm^2 when viewed laterally. Free floating in a suspension it will have a calculated average silhouette of 0.325 μm^2 . Therefore, 2 bacteria μm^{-2} in the light path will not fully block free light passage and even 4 bacteria μm^{-2} will represent on the average no more than approximately one cell thickness. To represent thicker bacterial layers, in suspension, a layer thicker than the 50 μm used in the present study will be required.

The results of the present study are in agreement with those of Pozos *et al.* (2004), who studied UV disinfection of water distribution systems. They also concluded that bacterial survival was due to shielding by other bacteria, either in the suspension or in outer layers of bacterial clumps, rather than a resistance to the UV light.

This phenomenon was initially demonstrated in the present study using a single bacterial strain and should be extended to include a larger variety of bacterial strains as well as thicker spacers between the filters that will allow greater numbers of bacteria in the light path.

When attempting UV disinfection of a surface upon which individual bacteria may be present, such as carefully cleaned surfaces in operating rooms, a relatively low dose of UV light may be sufficient. Nevertheless, when targeting bacteria on heavily contaminated surfaces, such as the inner surface of the root canal, the initial step should be to reduce the amount of bacteria as much as possible by other means, e.g. instrumen-

tation and rinsing with sodium hypochlorite. Only then may an additional stage of exposing the surface to UV light be effective. It should also be noted that a UV dose, much higher than that required to disinfect operating room surfaces, may be required. It will have to compensate for absorption of the light by bacteria in the outer layers of a bacterial multilayer that may be present.

The current study may serve as a preliminary indication that application of UV light to improve disinfection of root canals may be possible. As the root canal environment is not similar to the surface of a culture plate, various technical challenges would need to be overcome to deliver a uniform UV illumination to the root canal walls. Furthermore, the safety of the surrounding host tissues should need to be addressed. Further studies to establish such a method and to examine its efficiency and safety are currently in progress.

Conclusions

An application of UV light to eliminate endodontic pathogens may be possible. Nevertheless, its absorbance by multilayers of bacteria should be considered and the UV light dose adapted accordingly.

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