



Antibacterial action of photoactivated disinfection {PAD} used on endodontic bacteria in planktonic suspension and in artificial and human root canals

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Summary Objectives: To measure antibacterial action of photoactivated disinfection (PAD) on endodontic bacteria in planktonic suspension and root canals. **Methods:** Four bacteria, *Fusobacterium nucleatum*, *Peptostreptococcus micros*, *Prevotella intermedia* and *Streptococcus intermedius*, were tested in suspension. After mixing equal volumes of Tolonium chloride and bacterial suspension for 60 s, each 200 μ L of concentration ($> 10^6$ cfu mL⁻¹) was irradiated with light at 633 ± 2 nm. Each energy dose/Tolonium chloride concentration combination was tested eight times, with controls. Prepared root canals in Training Blocs and extracted human teeth were inoculated with *S. intermedius* followed by 10 mg L⁻¹ Tolonium chloride or saline. Bacteria in canals were sampled before and after light irradiation. Student *t*-test assessed significance of changes in viable bacteria produced by treatment of either light or Tolonium chloride alone and light/Tolonium chloride combinations.

Results: In suspension, reductions in bacteria were highly significant ($P < 0.01$) for light/Tolonium chloride combinations compared to light or Tolonium chloride alone. Maximum mean log reductions of 1.14 (*P. intermedia*), 2.48 (*P. micros*), 2.81 (*F. nucleatum*) and 6.73 (*S. intermedius*) were at 4.8 J/20 mg L⁻¹. Antibacterial action was increased by energy dose increase (not always significantly), but not by Tolonium chloride concentration. In control canals mean log reductions of 0.42 (Bloc) and 0.38 (teeth) from initial levels were not significant. PAD mean log reductions of 2.40 (Bloc) and 2.01 (teeth) were highly significant. Changes for PAD/energy dose combinations were not significant.

Conclusion: PAD killed endodontic bacteria at statistically significant levels compared to controls. Kills varied with bacterial species.

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Introduction

It is widely recognised that residual infection in root canals is a major reason for failure in endodontics.¹⁻³ Bacteria remaining within a root canal after endodontic treatment must be reduced to a minimum for successful treatment. The mode of action and efficacy of a wide variety of cleaning, antimicrobial and disinfecting agents such as sodium hypochlorite, chlorhexidine, EDTA, citric acid, hydrogen peroxide, halogens and ozone have been investigated⁴⁻¹¹ as have the effects of endodontic techniques.¹²

Most disinfectants with effective bactericidal activity are used at concentrations where toxicity is becoming a significant factor.¹³⁻¹⁵ This could cause adverse tissue reactions. Antibiotic use can allow resistant strains of bacteria to evolve and even when broad spectrum, are not effective against all bacterial strains.^{16,17}

Disinfecting solutions, ideally, should avoid such problems and photoactivated disinfection (PAD) appears to have this potential.^{18,19} PAD uses a combination of photosensitising dye, such as Tolonium chloride solution (TC) (synonym Toluidine Blue O), and light of a specific wavelength. This combination using light at 633 ± 2 nm has been shown to kill high numbers of bacteria in planktonic suspension, and in collagen and carious dentine,^{18,19} probably by disruption of the bacterial membrane by short range free radicals or reactive oxygen species.²⁰ TC is unchanged by the process, which ceases when irradiation stops.

The objective was to examine the application of PAD to endodontics. Initial experiments to measure PAD efficiency against a range of endodontic bacteria in planktonic suspension were carried out, irradiating with a spherical emitter tip which has been effectively used against *S. mutans*.¹⁸ The objective was to see whether these species varied in susceptibility. Results were compared to this previous study.

The antibacterial effect of PAD on *S. intermedius* in artificial root canals and in prepared root canals in extracted human teeth was then measured using a second light source, one which delivered the light to the inside of the root canal.

Materials and methods

Materials

Tolonium chloride (Zila Biotech Inc., Phoenix, AZ., USA) (TC) was dissolved in deionised water to

concentrations of 10.0 and 20.0 mg L⁻¹. Solution pH was adjusted to 5.25 ± 0.25 with sodium phosphate buffer to mimic acidity which might be found in endodontic lesions.

Light at 633 ± 2 nm, produced by a laser diode device, was guided to an emitter tip (Denfotex Technologies Ltd, Inverkeithing, Fife, UK). Prior to each test the power output at the tip was measured with an Optical Power Meter and Integrating Sphere (PM203/IS2, Macam Photometrics, Livingstone, UK). Light at the tip was distributed in two ways, depending on the test; either as a uniform sphere for the tip used in microwells for planktonic suspension (isotip), or along the length of the tip used in root canals (endotip). The isotip had a diameter of 800 μ m. The endotip was a flexible cylinder, 15 mm long and 400 μ m diameter emitting 70% of the light radially as a cylinder uniformly along the length and 30% of the total light intensity at the tip. This allowed light to be transmitted down to the apex of the tooth.

Planktonic suspension

In planktonic suspension the antibacterial properties of the technique were measured against four organisms known to occur commonly in root canals, *Streptococcus intermedius* (facultative anaerobe, Gram positive cocci), *Peptostreptococcus micros* (obligate anaerobe, Gram positive cocci), *Prevotella intermedia* (obligate anaerobe, black pigmented Gram negative rod) and *Fusobacterium nucleatum* (obligate anaerobe, Gram negative rod). Selection was based on a study of 51 bacterial species in root canals.²¹ *Streptococcus* spp were most abundant, present in 54.8% of root canals, with *Peptostreptococcus* and *Prevotella* species the most abundant anaerobes, present at 45.2 and 38.1%, respectively. The four selected also represented those found in the initial to final stages of root canal infection.²²

Each of the four pure cultures were clinical isolates, grown in fastidious anaerobic broth (FAB) (Oxoid Ltd Basingstoke, UK) at 37 °C in a CO₂ incubator (Heraeus, Germany), overnight for *S. intermedius* and 2 days for the other species. The culture was then vortexed at 3000 rpm for 10 minutes at room temperature before re-suspending the bacterial pellet in 0.85% sterile saline. Optical density measurements at 510 nm were used to adjust concentrations to 10⁶-10⁸ cfu mL⁻¹ prior to test.

Each experiment was carried out in a stirred well in a 96 well microtitre plate (Sterilin, UK). One hundred microliter of well shaken bacterial suspension and either 100 μ L of TC solution or 0.85% sterile

saline were placed in each well. Each well was shielded with a wrapping of aluminium foil. A pre-irradiation time (PIT) or dwell time of 60 seconds allowed solution mixing and introduction of the isotip. The tip was placed centrally into the stirred suspension in each well but switched on only when required. Four treatments were used for each experimental condition, each being carried out in one session:

- a. Sterile saline, no light treatment. (Control) (L-S-)
- b. Sterile saline, light of pre-set energy for predetermined time (L+S-)
- c. TC solution, no light treatment (L-S+)
- d. TC solution, light of pre-set energy for predetermined time (L+S+)

Energy doses of 2.4 and 4.8 J (80 mW for 30 or 60 s) were given to all species. When *P. intermedia* was found to be more resistant, an additional experiment using a higher dose of 7.2 J (80 mW for 90 s) was included. Immediately after treatment 100 μL of suspension was removed from each well and a series of 1-10 dilutions were prepared. Aliquots of each dilution were spread on the surface of fastidious anaerobic agar plates (FAA)(Oxoid Ltd Basingstoke, UK) and incubated anaerobically for at least one day for *S. intermedius* and 2 days for the other three bacteria. The numbers of bacteria surviving each treatment (cfu mL^{-1}) were counted on each of four replicate plates. Each treatment was then repeated. Log transformations of the individual results were performed to normalise the data prior to statistical comparison (Student's *t* test, level of significance set at 5%). The mean and standard deviation of the eight counts (cfu mL^{-1}) for each experiment were calculated.

Root canal preparation

Artificial root canals were prepared in Perspex Training Blocs (QED, UK). These were opened to Size 40, prepared with 2, 4 and 6° taper files (Maillefer™ Dentsply, Tulsa, USA) using hand instrumentation and sterile saline as irrigant. This allowed the flexible endotip (equivalent to an ISO 40 file along its full length) to fit to within 3 mm of the apex of the prepared root canal. Teeth, either single or double rooted and scheduled for extraction, were used after obtaining the informed consent of patients. One root canal in each tooth was prepared to the same dimensions as the training blocks using the same technique with walls left smooth and loose dentine debris removed

by irrigating with copious volumes of saline solution.

Root canal lengths ranged from 11 to 15 mm. Autoclaving, the normal method of sterilizing, would have distorted the plastic Training Blocs and since it was desirable to treat both types of root canal similarly, each root canal was cleaned initially and between each treatment by heating in water at 60 °C for at least 60 min, followed by ultrasonic cleaning. Alternative disinfection methods, using chemical reagents, where complete removal could not be guaranteed, were not used in case these chemicals affected the PAD process. Control treatments [C] were always carried out before experimental treatments [E] in the same root canal.

Photoactivated disinfection treatment

In root canals one bacterial strain, *S. intermedius*, was selected because it is known to resist treatment in root canals^{1,2} and adheres to dentine.²³ 15 μL of *S. intermedius* suspension were injected into the base of the root canal using an endodontic needle (Monoject™, Sherwood Medical Co, St Louis, MO, USA). The tooth was left at room temperature for 30 min in anaerobic, humid conditions for the bacteria to adjust to the new environment and early biofilm formation to occur. For *E* tests, 15 μL of TC solution were then injected, using a sterile endodontic needle. Bacteria surviving to this stage were sampled with a No. 20 sterile paper point, which removed approximately 4 μL of liquid. Three treatment regimes were investigated. Firstly, PITs of (a) 30 s or (b) 60 s was given during which time the endotip was inserted into the root canal and gently moved up and down to complete the mixing process before irradiation commenced. Thirdly, no PIT was given and irradiation took place during and after the 60 s mixing process. *C* tests used the same procedures with sterile saline replacing TC. Twenty-four experiments were carried out in Training Blocs (12C, 12E) and 30 in teeth (15C, 15E) for each of the three treatments. Each root canal was then re-sampled using a No. 20 sterile paper point. After serial dilution and culturing, as previously described, numbers of surviving bacteria were counted (four replicates). Log transformations of each result were performed to normalise the data prior to statistical comparison (Student's *t* test, level of significance set at 5%). The mean values and standard deviation were calculated.

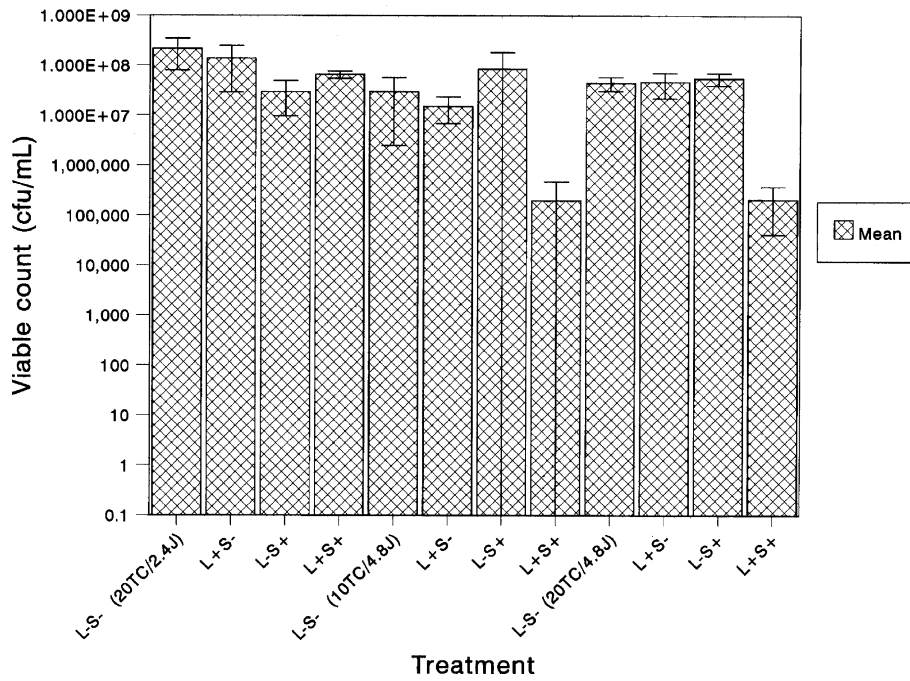


Figure 2 Viable *Peptostreptococcus micros* recovered in planktonic suspension in each treatment group. L–S+TC solution without light, L+S– sterile saline, light of pre-set energy dose, L+S+TC and light of pre-set energy dose. Standard deviation in parentheses, number in each group=8

1.36), 3.27 (SD 1.22) and 2.53 (SD 0.52), respectively, none being significantly different. There was, however, an increase in the number of high kills (> 99%) from 7 out of 12 (58%) to 10 (83%) (Table 3).

For Te canals no significant increase in bacterial kill was seen from increasing either the energy dose or the PIT. With all three energy doses 40-47% of samples had kills > 99%. The mean log reductions

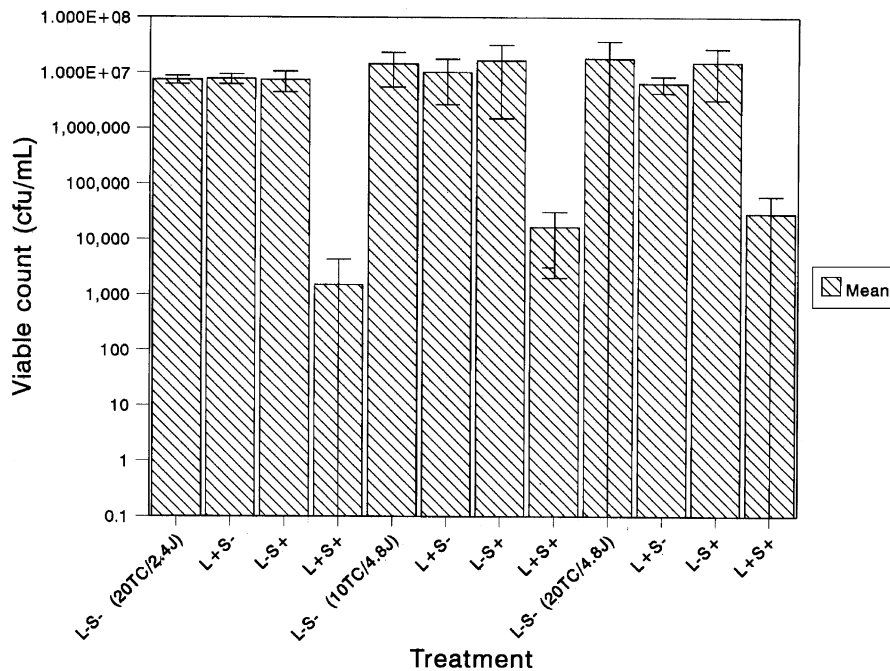


Figure 3 Viable *Fusobacterium nucleatum* recovered in planktonic suspension in each treatment group. L–S+TC solution without light, L+S– sterile saline, light of pre-set energy dose, L+S+TC and light of pre-set energy dose. Standard deviation in parentheses, number in each group=8.

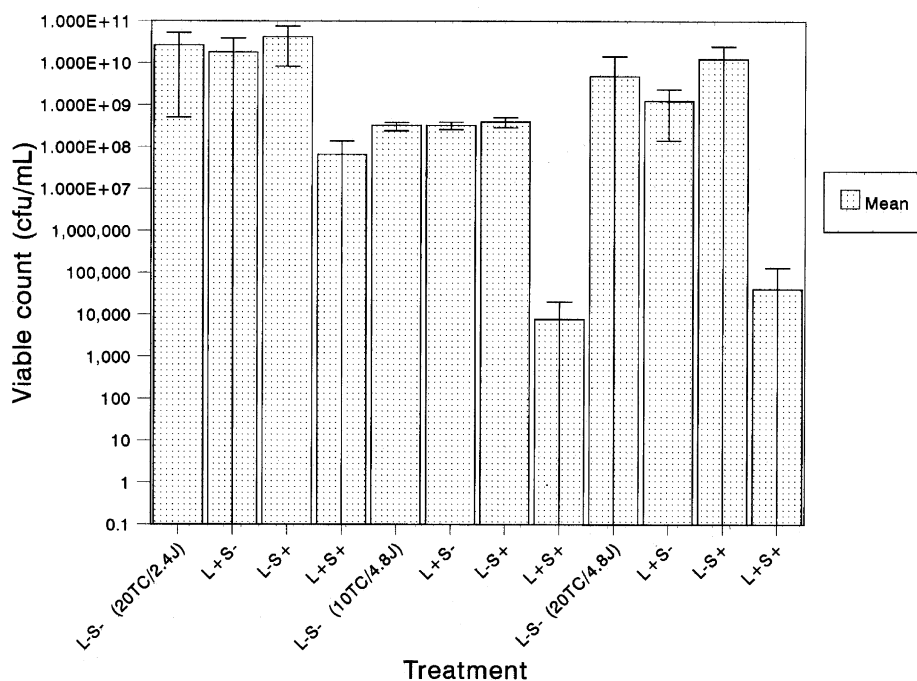


Figure 4 Viable *Streptococcus intermedius* recovered in planktonic suspension in each treatment group. L–S+TC solution without light, L+S– sterile saline, light of pre-set energy dose, L+S+TC and light of pre-set energy dose. Standard deviation in parentheses, number in each group=8.

for Te of 2.02 (SD 1.22) for PIT30s/13.2 J and 2.48 (SD 1.56) for PIT 0s/15.8 J were not significantly different to TB results under the same conditions. There was no observed residual staining of canals in teeth by TC solution.

Discussion

Planktonic suspension

The lack of significant decreases in bacterial numbers using L–S+ or L+S– treatment are similar to previous studies.^{18,24} When concentrations of Toluidine Blue O (Sigma, UK) similar to

those in the present study were used in root canals, minimal changes in viable bacteria for TC alone were also reported.²⁵ However, this study reported greater effects from the laser light alone compared to the present study, possibly because the root canal could be illuminated only from the outside and required substantially longer irradiation times.

L+S+ treatments produced significant decreases for all four bacteria. The results for each combination of TC concentration and energy dose indicate a ranking order for bacterial susceptibility to PAD with *S. intermedius* being most susceptible and *P. intermedia* most resistant. Although *F. nucleatum* had the highest level of kill of the four bacteria at 20 mg L⁻¹/2.4 J, this result seems high compared to

Table 1 Levels of significance between kills of bacterial species at each energy dose.

| Bacteria | Energy dose (J) | <i>P. intermedia</i> | <i>P. micros</i> | <i>F. nucleatum</i> | <i>S. intermedius</i> |
|----------------------|-----------------|----------------------|------------------|---------------------|-----------------------|
| <i>P. intermedia</i> | 2.4 | | S | HS | HS |
| | 4.8 | | S | HS | HS |
| | 7.2 | | HS | HS | HS |
| <i>P. micros</i> | 2.4 | | | HS | HS |
| | 4.8 | | | S | NS |
| | 7.2 | | | N | HS |
| <i>F. nucleatum</i> | 2.4 | | | | HS |
| | 4.8 | | | | HS |
| | 7.2 | | | | HS |

Statistical significance: NS, $P > 0.05$; S, $P < 0.05 > 0.01$; HS, $P < 0.01$.

Table 2 Bacterial kill in root canals using *S. intermedius*.

| Energy dose (J) | PIT (s) | Mean pre test (cfu mL ⁻¹), C | Mean pre test (cfu mL ⁻¹), E | Mean post test (cfu mL ⁻¹), C | Mean post test (cfu mL ⁻¹), E |
|-----------------|---------|--------------------------------------------------|--------------------------------------------------|--------------------------------------------------|--------------------------------------------------|
| <i>TB</i> | | | | | |
| 9.6 | 60 | 6.45 × 10 ⁴ (7.31 × 10 ⁴) | 7.90 × 10 ⁴ (1.10 × 10 ⁴) | 3.50 × 10 ⁴ (5.63 × 10 ⁴) | 4.07 × 10 ⁴ (8.75 × 10 ³) |
| 13.2 | 30 | 8.47 × 10 ⁴ (9.87 × 10 ⁴) | 1.08 × 10 ⁵ (8.63 × 10 ⁴) | 3.13 × 10 ⁴ (2.29 × 10 ⁴) | 4.29 × 10 ² (8.17 × 10 ²) |
| 15.8 | 0 | 1.44 × 10 ⁵ (7.28 × 10 ⁴) | 1.70 × 10 ⁵ (7.51 × 10 ⁴) | 5.47 × 10 ⁶ (1.20 × 10 ⁶) | 8.48 × 10 ² (8.23 × 10 ²) |
| <i>Te</i> | | | | | |
| 13.2 | 30 | 1.73 × 10 ⁵ (1.87 × 10 ⁵) | 3.08 × 10 ⁵ (3.35 × 10 ⁵) | 1.14 × 10 ⁵ (1.31 × 10 ⁵) | 1.15 × 10 ⁵ (1.59 × 10 ⁴) |
| 13.2 | 60 | 1.01 × 10 ⁵ (8.98 × 10 ⁴) | 2.10 × 10 ⁵ (2.99 × 10 ⁵) | 8.01 × 10 ⁴ (1.03 × 10 ⁵) | 4.69 × 10 ³ (6.97 × 10 ³) |
| 15.8 | 0 | 3.99 × 10 ⁵ (2.91 × 10 ⁵) | 5.71 × 10 ⁵ (5.51 × 10 ⁵) | 1.64 × 10 ⁵ (1.50 × 10 ⁵) | 7.63 × 10 ³ (9.16 × 10 ³) |

C, control canal; E, experimental canal; PIT, pre-irradiation time (between adding TC solution and light activation). TB, Training Blocs; Te, Teeth; N (TB), 12; N (Te), 15; SD in parentheses.

other results obtained for this species and might be an aberrant. In this group of eight results, two were low (log reductions of 3.02, 3.30) and six were high (6.72-6.92), and possibly some exposure of bacteria to oxygen had occurred in the latter case. A previous study¹⁸ measured the effect on *S. mutans* in planktonic suspension using the same system but in a smaller volumes (50 µL). This found 4.8 J to give a mean log reduction of 10 compared to 6-7 found using *S. intermedius*, the most vulnerable of the four, in the present study. Possibly *S. intermedius* is more resistant to PAD but the change in volume may have had an effect. Differences are unlikely to be due to TC concentration, since changes from 10 to 20 mg L⁻¹ (spanning the concentration used previously) produced no significant change in kill in *S. intermedius*.

Other studies have found bacteria to vary in response to antibacterial treatments. Measuring zones of inhibition¹⁵ found three anaerobes (*P. micros*, *P. intermedia*, *Porphyronoma gingivalis*) to vary in response to sodium hypochlorite solution (0.5-5.25% NaOCl). *P. micros* was most resistant at 0.5% and *P. intermedia* most resistant at 5.25% NaOCl. A study of four black pigmented Gram negative anaerobes indicated that *P. intermedia* was more affected by chlorhexidine and 0.5%NaOCl than by 2.5% and 4% NaOCl.⁹ It is therefore not unusual to find that bacteria vary in response to antibacterial measures and this appears to be the case for PAD. PAD is believed to act by altering membrane fluidity²⁰ and it is the two

Gram-negative species which appear to be more resistant to PAD. Others studies have also found this effect.^{26,27}

Changing the dose from 2.4 to 4.8 J, increased bacterial kill for the four species tested but was significant (highly so) only for *S. intermedius*. Increases from 4.8 to 7.2 J also produced a significant increase for *P. intermedia*. A possible explanation is that a minimum energy dose is required before changes in energy become significant. Bacterial resistance could be overcome by increasing the energy dose. Assuming bacteria used in the present study follow the linear response to energy dose shown by *S. mutans*,¹⁸ estimations of the energy dose required to kill 10⁶ cfu mL⁻¹ bacteria range from 4 J (*S. intermedius*), 13 J (*P. micros*) and 19 J (*P. intermedia*). This is achievable in clinically acceptable times (<4 min). At each energy dose, increasing TC concentration from 10 to 20 mg L⁻¹ increased bacterial kill for *F. nucleatum* and decreased kill *S. intermedius* and *P. micros*. None were statistically significant. At 4.8 J, 10 mg L⁻¹ TC produced a significant increase compared to 20 mg mL⁻¹ for *P. intermedia*. Therefore, indications are, that within the limits of this study, bacterial kill is less dependent on concentration than on energy dose.

Root canals

S. intermedius was present in numbers similar to those found in heavily infected root canals.⁶ Adding

Table 3 Proportion of results in low (95%), medium (95-99%) and high (>99% categories)

| Energy dose (J) | TB <95% | TB 95-99% | TB >99% | Teeth <95% | Teeth 95-99% | Teeth >99% |
|-----------------|---------|-----------|---------|------------|--------------|------------|
| 9.6 | 25 | 17 | 58 | | | |
| 13.2 | | | | 47 | 13 | 40 |
| 13.2 | 0 | 25 | 75 | 40 | 13 | 47 |
| 15.8 | 0 | 20 | 80 | 20 | 33 | 47 |

sterile saline [C] reduced bacterial numbers to some extent, with a mean log reduction of 0.4. A study combining irrigating solutions and instrumentation¹⁰ found saline to give a mean log reduction of 2.3 but using instrumentation can also affect bacterial survival¹² and may account for the higher figure.

PAD was less effective in root canals than in suspension, an effect similar to that found when comparing bacterial survival in collagen gels and dentine to planktonic suspension.¹⁹ Although some adhesion of bacteria to dentine might be expected in this time it was unlikely that a confluent biofilm would be formed. Even so, substrates appear to have an important effect on the PAD process. For TB root canals changing experimental conditions did not produce statistically significant differences although there was an indication that increasing energy dose increased the kill. The greatest mean log reduction compared to C, 2.82, was produced using PIT 60 s/150 s irradiation. Since this was higher than that produced by PIT 0 s/180 s it seemed to indicate PIT was important. However Te root canals showed the reverse, the latter condition being most effective. Increasing PIT from 30 to 60 s had no significant effect.

A previous study combining sodium hypochlorite (NaOCl)¹⁰ solutions and instrumentation found mean log reductions of 1.5–2.9 for NaOCl (1.5.25%) compared to saline controls. Thus it appears that PAD could be as effective as NaOCl. However in another study, when Toluidine Blue O solutions of similar concentration to the present study were used²⁵ a mean log reduction of only 2.88 at 21.0 J was found, with the light alone giving 2.32. This was said to be much less effective than 2 × 5 min applications of 3% NaOCl which, assuming that initial bacterial concentrations were similar, gave a mean log reduction of the order of 6. Here the higher kill using light alone may be due to the use of a low power HeNe laser, with the root canals illuminated from outside the root canal for extended periods of time. No attempt was made to ensure light was delivered to the site of bacterial contamination, which could also reduce bacterial action. The lower kill with Toluidine Blue O may also be due to the lower power density, 0.7 W cm⁻², compared to 4 W cm⁻² in the present study.

Higher levels of kill due to PAD in TB (Table 2) are probably due to an environment which is less hospitable to bacteria than are teeth. Since sampling was from the root canals only and it was unlikely that a confluent biofilm would have developed it is not surprising that differences between TB and teeth were not significant.

The less hospitable nature of TB may be indicated by the results (Table 3), where the proportions of results in the low, medium and high brackets are shown. In TB results >99% increase as the energy dose increases; those in teeth remain at 47% although the proportion <95% decrease at each stage. However the results may also be influenced by the initial numbers of bacteria, these being higher in teeth than in TB.

Similar numbers of bacteria were killed in both types of root canals; in teeth the mean numbers killed being 3.0 × 10⁵, 2.05 × 10⁵ and 5.63 × 10⁵ cfu mL⁻¹ compared to 7.9 × 10⁵, 1.1 × 10⁵ and 1.7 × 10⁵ in TB. Although the study did not allow time for a biofilm to develop, it is arguable that times used are similar to those used clinically in endodontic treatment. Only organisms residing deep in dentinal tubules might be untouched and retain a biofilm which confers survival benefits.²⁸ Model biofilm systems using multispecies are being developed for cariology problems²⁹ and could be of use in further evaluation of the PAD technique.

It was concluded that PAD using a specially designed light emitter was effective in killing bacteria in root canals. The effectiveness increased as the energy dose increased but TC concentration was a less important factor. Bacterial kills decreased in moving from planktonic suspension to artificial root canals to natural root canals, probably because of a more benign environment for the organisms. In natural root canals PAD killed > 10⁵ cfu mL⁻¹ bacteria. With continued reports of the failure of sodium hypochlorite solutions failing to remove infection in root canals even after three successive treatments³⁰ and with microbial regrowth occurring 7 days after NaOCl treatment,³¹ any additional treatment possibilities may be useful.

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