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The Photo-Activated Antibacterial Action of Toluidine Blue O in a Collagen Matrix and in Carious Dentine

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Photo-activated disinfection · Toluidine Blue O

Abstract
The main aim of this study was to determine the susceptibility to photo-activated disinfection (PAD) of *Streptococcus mutans* when the organism was present in a collagen matrix – an environment similar to that which would exist within a carious tooth. In addition, the susceptibility to PAD of bacteria present in carious human teeth was also determined. Light was delivered to the collagen and teeth using a system comprising a 0.8-mm diameter isotropic tip emitting light at 633 ± 2 nm. A single concentration of TBO (10 μg/ml) was used with both collagen and dentine. Two contact times, 30 and 180 s, were evaluated in intact collagen and additionally, for 180 s only, in collagen partially disrupted by shredding. The effect of energy doses from 1.8 to 14.4 J on the kills attained was assessed by determining the number of surviving viable bacteria. In carious dentine, two contact times, 30 and 60 s and one energy dose, 4.8 J, were used. Antibacterial effects were less than those obtained using planktonic suspensions with a maximum mean log reduction of 1.4 in shredded collagen and dentine. Increasing contact time increased the antibacterial effectiveness in both substrates although this was not always of statistical significance. Shredding the collagen resulted in significantly increased bacterial kills compared to those obtained in intact collagen for the 30-second contact time. The collagen matrix appeared to be a suitable model for carious dentine with advantages of availability and reproducibility. The results of this study have shown that PAD can achieve appreciable kills of oral bacteria, including *S. mutans*, when the organisms are embedded in a collagen gel or are present in carious teeth.

A combination of toluidine blue O solution (TBO) and light at 633 ± 2 nm kills bacteria in a process which has been termed 'photo-activated disinfection'. A new light delivery system using this process achieved bacterial kills of the order of 10^9 cfu/ml (100% kill) of *Streptococcus mutans* (the main causative agent of dental caries) in planktonic suspension at energy doses of 1.8 J or greater [Williams et al., 2003]. In planktonic suspension the process has the greatest chance of success whereas it has been shown that bacteria in biofilms have reduced susceptibility to antimicrobial agents such as sodium hypochlorite [Spratt et al., 2001]. In carious tissue bacteria will be pro-

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tected by the dentinal structure which may also limit the penetration of TBO. Bacterial penetration of the dentine may also vary, depending on the species. It has been shown that *Streptococcus sanguis* can penetrate nearly 500 μm into dentine tubules whereas *Prevotella intermedia* remains in the outer 25 μm [Berkiten et al., 2000]. Once demineralisation of the dentine has been initiated other bacteria can migrate into the developing lesion. Generally, current treatments of caries attempt to remove all infected tissue but this cannot be guaranteed [van Strijp et al., 1994; Bjørndal et al., 1997]. However Kidd et al. [1996] have proposed that the removal of only the softened and wet dentine is necessary, the effective sealing of the cavity with a restorative being sufficient to achieve 'healing of the lesion' as the bacteria become quiescent. While this is not in dispute, the difficulties in (a) determining the amount of tissue removal necessary clinically and (b) the inadequacies of most restorative materials currently available in effectively achieving a long-term seal means that an effective means of disinfecting both the infected and affected tissue is highly desirable before completion of treatment.

If bacteria in infected but only partly demineralised tissue could be killed, even more tissue could be retained. The need for the photo-activated disinfection process to move from demonstrable laboratory disinfection to clinical use has been cited [Burns et al., 1995], but this is possible only if the light can be produced within the carious lesion and reached by minimal access, as narrow as 1 mm in diameter. Such a system now exists and has been shown to work effectively in planktonic suspension [Williams et al., 2003]. The aim of this study was to use the same system to kill bacteria embedded in a collagen matrix and in naturally occurring carious dentine. In collagen a range of energy doses were evaluated but limits on the number of extracted teeth available restricted experiments to one energy dose. The results obtained in carious dentine were compared with those in collagen to assess the usefulness of collagen as a model system for infected dentine. Results in both substrates were compared with those previously reported in planktonic suspension.

Materials and Methods

A solution containing 40 μg/ml TBO in deionised water was used for the collagen experiments. This was prepared by serial dilution of a stock solution containing 2,012 μg/ml TBO (Sigma, Gillingham, UK). The final solution had a pH of 5.2 ± 0.3, measured using a calibrated pH meter (Orion Research Inc., Beverly, Mass., USA). The solution was stored in an amber glass bottle at room temperature for 4 weeks. Experiments using the collagen matrix involved a 4-fold dilution of the TBO. Thus, for experiments in carious tissue, the solution was diluted to 10 μg TBO/ml to allow comparison between the two parts of the study.

Studies Using a Collagen Matrix

S. mutans NTCT 10449 was used, maintained by sub-culturing on blood agar (Oxoid, Basingstoke, UK) every 7 days. For experimental purposes the organism was grown for 6 h at 37°C in tryptone soya agar broth (TSB, Oxoid). Rat tail collagen (type VII acid soluble) was dissolved in 8.7 *M* acetic acid to a concentration of 20 mg/ml. The solution was dialysed for 6 h with 0.1 *M* Tris buffer (pH 7.3). One hundred microlitres of collagen and 50 μl of bacterial suspension were micropipetted into wells of a microwell plate, mixed and allowed to set at 4°C for 16 h forming plugs of bacteria-laden gel. Either 50 μl of de-ionised water (control) or 50 μl of TBO solution (experiment) were injected into the centre of the plug. A drop of liquid sometimes appeared on the surface of the plug but no attempt was made to disperse this. The solution was left in contact with the plug for either 30 or 180 s. The laser (Denfotex Light Systems Ltd., Inverkeithing, UK) delivered light (633 ± 2 nm) at a pre-set power output via a fibre to a spherical tip of an 800-μm diameter. This tip was pushed into the entry hole left by the syringe needle and activated for L+ samples. For L– samples the tip was inserted but not switched on. The output, 60 or 80 mW, was checked using an Integrating Sphere Unit (Macam Photometrics, Livingstone, UK) before each experiment. Each microwell was surrounded with aluminium foil to shield other wells from irradiating light. In combination with three irradiation times, 30, 60 and 180 s, the energy doses supplied to the bacteria ranged from 1.8 to 14.4 J. Four treatments were used: (a) water, no light treatment (control; L–S–); (b) water, light of set power for pre-determined time (L+S–); (c) TBO solution, no light treatment (L–S+), and (d) TBO solution and light of set power for pre-determined time (L+S+).

Each plug was digested with 50 μl of 0.5 mg/ml type 1A collagenase (Sigma, Poole, UK) and incubated at room temperature for 3 h. This process has been shown to have no adverse effect on bacterial viability [Burns et al., 1995]. Serial 10-fold dilutions of the suspension were prepared in TSB and aliquots spread over TSA plates. Colonies were counted after 48 h anaerobic incubation at 37°C.

The collagen plug had a very firm consistency and the effect of disrupting the plug to give a more open structure was also investigated. Samples were prepared as above, but prior to adding TBO or water the plug was broken by sucking it into and out of an empty syringe needle 3 times. Only 180 s contact time was used with energy doses ranging from 2.4 to 14.4 J.

Four replicate measurements were made on each sample with 2 samples/treatment. The mean and standard deviation of numbers of *S. mutans* surviving each treatment were calculated. Log_{10} transformations of cfu counts were made to normalise the data before comparing mean values using Student's t test (level of significance set at $p < 0.05$).

Studies Using Carious Dentine

Teeth scheduled for extraction were used with the informed consent of patients. These teeth, by virtue of their highly carious condition, had lost much of their coronal tooth structure. This was an extreme test limited by the availability of ex vivo tissue. Initial measurements found *S. mutans* levels in such teeth were low, of the

order of 10^2 cfu/mg. Total bacterial counts however were high, 10^6–10^{11} cfu/mg. It was therefore decided to measure the total number of bacteria in the carious dentine before and after treatment rather than one species only. After extraction each tooth was placed in a sterile plastic vial (Sterilin, Stone, UK), immediately sealed and transported to the laboratory. All experiments were carried out within 1 h of extraction. The tooth was transferred from the vial onto a sterile pad. All softened carious tissue was removed with a 129/130 dental excavator (Prima, Byfleet, UK) and divided into two approximately equal portions. Each was placed in a sterile, weighed bijou vial which was re-weighed to determine the weight of carious tissue. Sample weights ranged from 2 to 10 mg for the 30 s contact time and from 2 to 24 mg for the 60 s contact time. The vial was wrapped in aluminium foil. Either 200 µl of sterile saline (control) or 200 µl of 10 µg/ml TBO solution (experiment) were added. The vial was shaken and left for 30 or 60 s, during which time the isotropic tip was placed into the liquid. At the end of the contact period samples requiring irradiation received an energy dose of 4.8 J. Before inserting the tip the power output was checked as described previously. Where light was not used (L–), the tip remained in the liquid for the same length of time, without activation. The tip was removed and 2 ml sterile fastidious anaerobic agar plus blood (FAB) and glass beads were added to each vial, the beads were used to disintegrate the tissue during vortexing. Each sample was serially diluted in FAB, plated onto agar and incubated for 3–5 days, then the number of colonies were counted. The numbers of total viable bacteria present in each sample before and after each treatment were determined (cfu/mg carious dentine). Eight teeth were used for each contact time, with four replicate measurements made on each portion of tissue. The mean numbers of bacteria and standard deviation were calculated. Log_{10} transformations of cfu counts were made to normalize the data before comparing mean values using Student's t test (level of significance set at $p < 0.05$).

Results

Levels of individual control samples (L–S–) of *S. mutans* in collagen ranged from 4×10^8 to 7×10^9 cfu/ml and were similar in both intact (IC) and shredded (SC) collagen. The mean number of bacteria surviving each treatment in collagen (cfu/ml), standard deviation and the calculated percent of kill are given in table 1.

In carious dentine pre-treatment levels of total bacteria ranged from 1×10^4 to 1×10^{11} cfu/mg. The mean pre- and post-treatment total bacterial counts in carious tissue (cfu/mg), the calculated percent kill and log reductions are given in table 2.

S. mutans in a Collagen Matrix – the Effect of TBO Alone (L–S+)

Antibacterial effects were slight and not statistically significant ($p > 0.05$) compared with their corresponding controls. Changes in contact time, energy dose and the state of the collagen had no significant effect. In IC, the mean log reduction was 0.07 (n = 48, s.d. = 0.12) for contact times of 30 s (30 s/IC) and 0.05 (n = 24, s.d. = 0.10) for contact times of 180 s (180 s/IC). For SC, (180 s/SC) the log reduction was 0.08 (n = 24, s.d. = 0.10).

S. mutans in a Collagen Matrix – the Effect of Light Alone (L+S–)

Comparing the mean log reduction at each energy dose for each test condition with that at 30 s/IC/1.8 J showed only the result at 30 s/IC/2.4 J to be significantly different ($p < 0.05$). No statistically significant changes resulted from longer contact times or shredding the collagen.

Pooling results at each energy dose produced the following mean log reductions: 0.06 (1.8 J, n = 6, s.d. = 0.03); 0.13 (2.4 J, n = 24, s.d. = 0.16); 0.05 (3.6 J, n = 6, s.d. = 0.30); 0.08 (4.8 J, n = 24, s.d. = 0.14); 0.15 (10.8 J, n = 6, s.d. = 0.15) and 0.12 (14.4 J, n = 24, s.d. = 0.22).

S. mutans in a Collagen Matrix – the Effect of Photo-Activated Disinfection (L+S+)

For each energy dose the reduction in *S. mutans* compared with the corresponding L–S–, L–S+ and L+S– sample was either significant or highly significant.

Bacterial kill in 30 s/IC samples increased slightly as the energy dose increased. However, the changes were not statistically significant from the mean log reduction of 0.55 (mean kill of 70%) at 1.8 J. For 180 s/IC samples the log reduction at 4.8 J was a significant improvement over that at 2.4 J (mean log reduction = 0.78, mean kill = 73%), unlike the change between 14.4 J and 4.8 J. For 180 s/SC neither mean log reduction at 4.8 and 14.4 J was significantly different from that of 1.01 at 2.4 J (mean kill 91%).

For 180 s/IC and 180 s/SC the change in mean log reduction was compared to that at 30 s/IC. The longer contact time produced significantly improvement only at 4.8 J, but shredding the collagen produced a significant improvement for all energy doses. Compared to 180 s/IC improved mean log reductions for 180 s/SC were not significant.

Figure 1 represents the data graphically, plotting the log reduction in *S. mutans* against the energy dose. Results from the L–S+ and L+S– treatments are also shown. Results from each L+S+ condition between 2.4 and 14.4 J were best approximated by a relationship of the type found by logarithmic regression where log reduction = a + b ln E, where E is the energy dose (J). For 30 s/IC a = 0.46, b = 0.13, $R^2 = 0.80$; for 180 s/IC a = 0.61, b = 0.25, $R^2 = 0.75$, and for 180 s/SC a = 0.83,

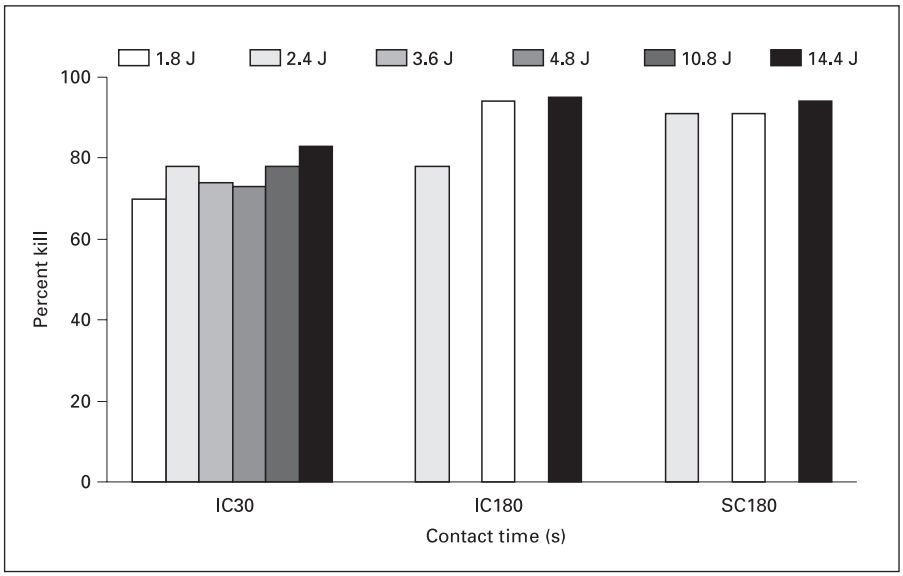

Fig. 1. The mean percentage kills from L– S– levels produced by each energy dose (J) for the L+S+ groups.

Table 1. Numbers of *S. mutans* present in a collagen matrix before and after various treatments, and calculated percent kill

Collagen state	Contact time, s	Energy dose, J	Bacterial numbers, cfu/ml				Percent kill		
			L – S –	L – S+	L+S –	L+S+	L – S+	L+S –	L+S+
Intact	30	1.8	$2.71.10^9$ ($1.16.10^9$)	$3.00.10^9$ ($1.42.10^9$)	$2.54.10^9$ ($9.49.10^8$)	$8.20.10^8$ ($2.95.10^8$)	0	6	70
Intact	30	2.4	$2.05.10^9$ ($1.10.10^9$)	$2.77.10^9$ ($1.80.10^9$)	$1.47.10^9$ ($1.16.10^9$)	$4.56.10^8$ ($2.14.10^8$)	0	27	78
Intact	30	3.6	$2.48.10^9$ ($1.49.10^9$)	$3.31.10^9$ ($2.02.10^9$)	$3.49.10^9$ ($1.43.10^9$)	$6.45.10^8$ ($3.24.10^8$)	0	0	74
Intact	30	4.8	$1.80.10^9$ ($1.54.10^9$)	$1.78.10^9$ ($1.64.10^9$)	$1.64.10^9$ ($1.44.10^9$)	$4.80.10^8$ ($3.27.10^8$)	0	8	73
Intact	30	10.8	$2.62.10^9$ ($1.20.10^9$)	$3.63.10^9$ ($1.57.10^9$)	$3.10.10^9$ ($1.62.10^9$)	$5.76.10^8$ ($3.25.10^8$)	0	0	78
Intact	30	14.4	$1.81.10^9$ ($2.01.10^9$)	$2.49.10^9$ ($1.74.10^9$)	$2.11.10^9$ ($1.86.10^9$)	$3.08.10^8$ ($2.23.10^8$)	0	0	83
Intact	180	2.4	$2.78.10^9$ ($1.34.10^9$)	$3.75.10^9$ ($1.92.10^9$)	$3.80.10^9$ ($1.80.10^9$)	$6.19.10^8$ ($1.76.10^8$)	0	0	78
Shredded	180	2.4	$3.48.10^9$ ($1.29.10^9$)	$2.89.10^9$ ($1.26.10^9$)	$3.18.10^9$ ($1.79.10^9$)	$3.14.10^8$ ($1.53.10^8$)	17	9	91
Intact	180	4.8	$4.92.10^9$ ($2.08.10^9$)	$4.19.10^9$ ($1.85.10^9$)	$3.45.10^9$ ($1.34.10^9$)	$2.90.10^8$ ($1.23.10^8$)	15	30	94
Shredded	180	4.8	$5.10.10^9$ ($2.32.10^9$)	$4.45.10^9$ ($2.18.10^9$)	$1.50E.10^{10}$ ($1.47E.10^{10}$)	$4.55.10^8$ ($1.42.10^7$)	13	0	91
Intact	180	14.4	$4.21.10^9$ ($2.38.10^9$)	$4.71.10^9$ ($2.73.10^9$)	$4.30.10^9$ ($1.66.10^9$)	$2.50.10^8$ ($1.08.10^8$)	0	0	95
Shredded	180	14.4	$4.70.10^9$ ($3.7.10^9$)	$4.46.10^9$ ($2.16.10^9$)	$1.82.10^{10}$ ($1.76.10^{10}$)	$2.94.10^8$ ($7.29.10^7$)	5	0	94

Mean and standard deviation of eight measurements (cfu/ml). L – S – (control), L – S+ (dye without light), L+S – (light without dye), L+S+ (light/dye combination).

Table 2. Total bacterial count as cfu/mg in carious tissue, pre- and post-treatment using an energy dose of 4.8 J together with calculated percent kill and log reduction

	Contact time 30 s						Contact time 60s					
	pre-treatment cfu/mg	post-treatment cfu/mg	Number killed cfu/mg	% kill	log red	caries type	control cfu/mg	experimental cfu/mg	number killed cfu/mg	% kill	log red	caries type
	$1.26.10^{10}$ ($1.40.10^{9}$)	$2.43.10^{8}$ ($3.60.10^{7}$)	$1.24.10^{10}$	98	1.71	s	$4.67.10^{6}$ ($1.99.10^{6}$)	3.8010^{5} ($5.90.10^{4}$)	4.2910^{6}	92	1.09	s
	$3.35.10^{6}$ (600.10^{5})	$1.31.10^{6}$ ($4.10.10^{5}$)	$2.04.10^{6}$	61	0.41	h	$1.13.10^{10}$ ($1.40.10^{9}$)	$2.23.10^{10}$ ($1.40.10^{9}$)	0	10	0	s
	$4.48.10^{10}$ ($9.70.10^{9}$)	$4.28.10^{10}$ ($4.00.10^{8}$)	$2.00.10^{9}$	5	0.02	s	$1.12.10^{11}$ ($1.08.10^{11}$)	$1.92.10^{10}$ ($7.80.10^{9}$)	$9.28.10^{10}$	83	0.77	s
	$3.97.10^{10}$ ($6.20.10^{9}$)	$4.12.10^{8}$ ($4.70.10^{7}$)	$3.93.10^{10}$	99	1.98	s	$9.83.10^{10}$ ($1.08.10^{8}$)	$2.18.10^{8}$ ($8.80.10^{7}$)	$9.79.10^{10}$	100	2.65	s
	$1.08.10^{6}$ ($2.00.10^{5}$)	$3.42.10^{4}$ ($6.20.10^{3}$)	$1.05.10^{6}$	97	1.50	m	$4.53.10^{10}$ ($1.20.10^{10}$)	$2.13.10^{9}$ ($3.70.10^{8}$)	4.3210^{10}	60	1.33	m
	$1.57.10^{4}$ ($1.91.10^{4}$)	$1.35.10^{6}$ ($3.30.10^{5}$)	0	0	0	s	$1.97.10^{10}$ ($2.00.10^{9}$)	$2.94.10^{8}$ ($7.20.10^{7}$)	$1.68.10^{10}$	99	1.83	h
	$1.77.10^{11}$ $4.40.10^{9}$)	$1.08.10^{9}$ ($2.31.10^{8}$)	$1.76.10^{11}$	99	2.21	h	$8.28.10^{9}$ ($1.29.10^{9}$)	$1.79.10^{8}$ $7.40.10^{7}$)	$8.10.10^{9}$	99	1.67	s
	$9.25.10^{7}$ ($1.15.10^{7}$)	$6.42.10^{6}$ ($2.60.10^{5}$)	$8.61.10^{7}$	93	1.16	s	$2.53.10^{10}$ ($3.00.10^{9}$)	$2.17.10^{8}$ ($3.00.10^{7}$)	$2.51.10^{10}$	99	2.07	s
Mean					1.12 (0.88)						1.43 (0.77)	

Mean and standard deviation of four replicate measurements. s = Soft carious tissue, m = medium hardness, h = hard, flaky tissue.

b = 0.23 and R^2 = 0.85. In the actual experiment L–S+ (E = 0) the mean log reduction ranged from 0.05 to 0.08, slightly lower than the values of the constant b.

Bacteria in Carious Dentine

Pre-treatment bacterial numbers were as follows. Of 16 samples, there was 1 of 10^4, 3 of 10^6, 1 of 10^7, 1 of 10^9, 8 of 10^{10} and 2 of 10^{11} cfu/mg total bacteria.

For a contact time of 30 s the percent kill of total bacteria ranged from 0 to 99.4%, with a mean of 69%. The mean log reduction was 1.12 (s.d. = 0.88). Increasing the contact time to 60 s resulted in 0–99.8% kill, a mean of 81% and a mean log reduction of 1.43 (s.d. = 0.77). This increase was not statistically significant.

Discussion

Bacteria in Collagen Matrix

In IC, TBO solution without light activation (L–S+) had little effect, indicating low toxicity of TBO alone when used at this concentration and applied for times typical of those used clinically. This was not unexpected and agrees with previous studies [Burns et al., 1993, 1994; Williams et al., 2003]. No significant changes were caused by shredding the collagen at any energy level.

In IC, light alone (L+S–) produced no noticeable increase in bacterial kill. No effect due to increasing energy dose was seen (table 1). This also agrees with previous studies [Burns et al., 1993, 1994; Williams et al., 2003] showing that for two laser systems, energy doses of up to 14.4 J in the absence of TBO are of low toxicity to *S. mutans*.

In all three collagen matrix experiments the combination (L+S+) produced significant bacterial kill and had a progressively greater effect as the energy dose increased.

Comparisons with a previous study using bacteria embedded in IC [Burns et al., 1995] where a mean log reduction of 0.30 was found at 0.44 J, increasing to 0.40 at 1.31 J, are limited by different experimental conditions. Log reductions in our study ranged from 0.52 (30 s/IC/1.8 J) to 1.23 (180 s/I/14.4J). The relationship between log reduction and energy found in the present study predicted that 0.44 J should produce log reductions of the

order of 0.35–0.50 (contact time 30 s, 180 s) and 0.40–0.68 at 1.31 J (contact time 30 s, 180 s). Improvements could be attributed to the higher energy density used in the present study and/or to irradiating from the interior of the plug rather than from the outside.

A reduced antibacterial effect was noted in a collagen matrix compared to planktonic suspension [Williams et al., 2003]. For an energy dose of 2.4 J a mean log reduction of 9.53 in planktonic suspension compares to 1.01 (for 180 s/SC) in the present study. It may be inferred that photo-activated disinfection is affected by physical constraints. Here, since the process seems time dependent, it is likely to be the rate at which TBO dye diffuses into the collagen plug. A plot of log reduction versus contact time, while evaluating only three sets of data, resulted in a linear trend for energy doses of 4.8 J and 14.4 J. This indicated that even after 3 h contact time the log reduction would (theoretically) only be of the order of 2.5. Experiments where the contact time was increased to 1 h failed because no bacteria survived, even in control samples. Shredding collagen increased antibacterial action as might be expected if diffusion is a limiting factor. However the term 'contact time' may be slightly misleading, since delivery of the maximum energy dose required 180 s. Thus energy doses of 14.4 J resulted in some bacteria being in contact with dye for times ranging from 30 to 210 s and 180 to 360 s, depending on the permeation of the dye into the plug. This is one limitation of the method, another being the welling up of the dye solution around the site of injection.

Studies comparing susceptibility of bacteria present in biofilms compared to planktonic suspension also report reduced kills [Wilson, 1996; Shani et al., 2000]. In the latter case, using amine fluorides on *S. sobrinus* on saliva-coated hydroxyapatite beads, the log reduction was close to 1.4, a similar value to those found in our study. However, one advantage of the photo-activated process compared to conventional antimicrobial agents is that no bacterial resistance should develop.

Further work would be needed to ascertain whether shredding the collagen increased variability [$(\text{s.d.})^2$/mean $\times$ 100%], as indicated by this study. For 180 s, contact time and energy doses of 2.4, 4.8 and 14.4 J, the variability for IC was 2% in all three cases, whereas for SC it ranged from 14 to 33%.

It was concluded that the longer contact time was more effective and that optimisation of contact between bacteria and dye was an important parameter. Under the best conditions 95% kills were obtained.

Bacteria in Carious Tissue

Carious tissue samples were variable in character. Some were soft, easily cut and apportioned; others were hard, with caries removable only as flakes of tissue. Of the 16 specimens 11 were soft, 2 were hard and 3 medium (between the two extremes). With a limited number of samples, no correlation could be seen between the type of caries and initial bacterial counts, although one study [Kidd et al., 1993] found soft, wet caries contained significantly greater numbers of bacteria (1.4×10^4/sample) than hard, dry, dentine (7×10^1/sample) in a group of 37 teeth. The present study found higher levels of bacteria than another [Bjørndal et al., 1997] where there was a median of 1.0×10^4 cfu/ml (range of 1.2×10^4 to 9.5×10^5cfu/ml) in a group of 19 teeth. These higher levels, 10^4–10^{11} cfu/ml, may be attributed to retention of all extracted tissue for the experiment and to the extent of the disease.

No kills were achieved in 2 cases where the contact time was 30 s and in 1 case where this was 60 s. All 3 were soft specimens but other soft caries samples produced log reductions of up to 2.65, weight not appearing to be a factor although soft caries produced the larger samples. Possibly this was due to incomplete dye penetration. Microscopic examination of the rate of penetration of TBO solution into hard flakes of caries, where it had been assumed that penetration would be lowest, indicated about 300-μm penetration of dye after 30 s and about 400-μm penetration after 60 s. In specimens of dimensions greater than this dye penetration may be incomplete in the time allowed. Clinically, where the surface of a carious tooth is normally dried, dye uptake may be more rapid and complete but this would need to be established by further studies.

For the same energy dose, photo-activated disinfection was less effective in carious tissue than in planktonic suspension but was similar to, and slightly better than, that achieved using an SC matrix. Mean log reductions were 1.10 (180 s/IC), 1.25 (180 s/SC), 1.12 (30 s/caries) and 1.43 (60 s/caries). The results in caries showed a larger range than those in collagen, probably reflecting the variable nature of the dentine. Another possibility is that, unlike the collagen experiment, caries samples were irradiated from the outside and therefore each point on and within the dentine experienced a varying energy dose. Reduced effectiveness is not unexpected given the physical and biochemical hindrances and reinforces the advisability of performing tests in an appropriate medium. Although the effect was reduced it was noted that in half the samples, bacteria in excess of 10^{10} cfu/mg were killed.

Both the collagen and caries substrate experiments indicate that contact between bacteria and TBO solution is a critical factor and sufficient time must be allowed for the solution to permeate the structure in which the bacteria are situated. The photo-activated disinfection technique used on bacteria in both collagen and carious dentine showed it to be capable of killing at least 10^9 cfu/ml or cfu/mg bacteria (99% kill) in substrates heavily loaded with bacteria.

The SC matrix was found to be a suitable substitute for carious dentine, with the advantage of availability and reproducibility.

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Bacteriological procedures were carried out by H. Conway-Wallace and Z. Bhunnoo.

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