Original Paper

Caries Research

Caries Res 2003;37:190–193
DOI: 10.1159/000070443

Received: June 21, 2002
Accepted after revision: January 22, 2003

The Effect of Variable Energy Input from a Novel Light Source on the Photoactivated Bactericidal Action of Toluidine Blue O on *Streptococcus mutans*

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Key Words
Bactericide · Energy dose · Novel light delivery · Photoactivated disinfection · *Streptococcus mutans*

Abstract
Although the combination of toluidine blue O (TBO) dye and laser light at a wavelength of 633 nm has a bactericidal effect, light from laboratory lasers can only be directed externally at a bacterial colony or suspension. In this study a novel delivery system guided the laser light to an 800-μm diameter spherical tip (an isotropic tip) from which light radiated producing a uniform sphere of light within the colony or suspension. The system was highly effective in killing TBO-treated *Streptococcus mutans* NCTC 10449 in stirred planktonic suspension, killing at least 10^9 cfu/ml. Antibacterial action increased as the delivered energy dose increased. Energy doses of 1.8 J or more produced 100% kills and log reductions of 8–10 cfu/ml. Neither TBO dye nor light alone had a significant antibacterial effect under the experimental conditions used. The existence of a threshold energy, i.e. a minimum energy required before bactericidal action occurred, could not be demonstrated.

Many dyes bind to cells and some which bind to bacteria are photoactivated by light of a suitable wavelength [Coyle, 1986] to produce short-lived species of oxygen and/or free radicals which can be toxic to bacteria [Ito and Kobayashi, 1977; Burns et al., 1993, 1994]. This may be termed photoactivated disinfection. Toluidine blue O (TBO), a thiazine dye of the quinone-imine family, used with a fixed output laboratory laser light source has been shown to kill several types of oral bacteria involved in dental caries [Burns et al., 1993, 1994]. The laser delivered light as a spot (diameter 1.3 mm) at a wavelength of 633 ± 2 nm and, while effective in irradiating bacteria cultured on a flat surface, was less suitable for use in a multilayer colony or volume of bacterial suspension.

A new type of laser light delivery system has a flexible fibre-optic cable conducting light to an isotropic tip, a sphere approximately 800 μm in diameter. Light is produced as a uniform sphere (essentially 4 π steradians). The power output of the new device was variable up to 80 mW, compared to the fixed 7.3 mW previously used. The maximum power density at the surface of the tip was 4 $W \cdot cm^{-2}$ compared to 0.6 $W \cdot cm^{-2}$ for the laboratory laser.

The tip is small enough to be used inside a minimal dental cavity, the requirement for which has been pre-

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viously highlighted [Burns et al., 1994] but, before use in this situation, we have assessed the effectiveness of the system against bacteria in planktonic suspension. The study had a number of objectives. Although primarily designed to find the relationship between irradiation energy and bacterial kill, it also aimed to establish whether there were preferred combinations of power and time, whether a minimum value of power, time or energy was needed for bactericidal action to occur and to compare the efficiency of the new system with the previous method.

Materials and Methods

Materials

Streptococcus mutans NCTC 10449 was maintained by subculture on tryptone soya agar every 7 days. For experimental use the subcultured organism was grown on tryptone soya broth overnight at 37°C in anaerobic conditions before dilution with sterile 0.85% saline solution. An absorption of approximately 0.1 at 560 nm gave suitable initial concentrations of 10^8–10^{10} colony-forming units (cfu) per millilitre.

Two sources of TBO were used, TBO-A (Aldrich, Gillingham, Dorset, UK) and TBO-B (Zila Biotech Inc., Phoenix, Ariz., USA), to see whether similar effects on *S. mutans* were seen. Fresh solutions containing 13 ± 3 mg/l of TBO-A were made in distilled water. TBO-B was diluted to the same concentration, checked by absorbance at 633 nm. Solution pH was adjusted to 5.25 ± 0.25 just prior to test. This pH was selected for a number of reasons. Acidity influences the quantum yield of singlet oxygen production such that the [$^1O_2$ yield] decreases by a factor of 3.4 for a pH change from 9 to 5 [Pottier et al., 1975]. Between pH 5 and pH 6 the quantum yield remains relatively constant. Finally, this pH is closer to that found in dental plaque during a cariogenic challenge [Kidd and Joyston-Bechal, 1987]. *S. mutans* showed decreasing levels of kill as pH changed from 7.4 to 4.5 [Burns, 1997]. The pH chosen provides the most severe test and is representative of clinical conditions.

Light at 633 ± 2 nm produced by a laser diode device was transmitted to the isotropic emitter (Carl Zeiss Meditech Ltd., Inverkeithing, Fife, UK) where the power output was measured on each occasion with an Optical Power Meter PM203/IS2 and Integrating Sphere (Macam Photometrics, Livingstone, UK). The suspension of *S. mutans* was exposed to a range of energy doses (J) produced by varying power (mW) and time (T).

Method

Each experiment was carried out in stirred wells in a microtitre plate (Sterilin, UK) adding 30 µl of well-shaken bacterial suspension and 30 µl of either TBO solution or 0.85% sterile saline. A pre-irradiation time of 60 s allowed for mixing and introduction of the isotropic emitter. Each well was shielded from the others with a wrapping of aluminium foil. Four treatments were used: (a) sterile saline, no light treatment (control; L–S–); (b) sterile saline, light of set energy for predetermined time (L+S–); (c) TBO solution, no light treatment (L–S+), and (d) TBO solution, light of set energy for predetermined time (L+S+).

Power levels from 20 to 80 mW and irradiation times of 5 to 60 s produced energy doses of 0.4–4.8 J. At lower energy levels different combinations of power and time were used to provide the same energy dose. In all experiments the isotropic sphere was placed centrally into the stirred suspension of *S. mutans* within the well but activated only for L+ conditions. Immediately after treatment 50 µl of suspension was removed from each well and mixed with 450 ml of tryptone soya broth. Serial dilutions were carried out and surviving bacteria (cfu/ml) enumerated by viable counting on tryptone soya agar plates. Four replicates were made for each test and a number of repeats made. The mean and standard deviation of the number of viable bacteria surviving each treatment were calculated. Mean values were compared using Student's t test (level of significance set at $p \leq 0.05$).

Results

The results of the number of *S. mutans* surviving treatment (cfu/ml) are given in table 1. With percentage kills ≥99% for L+S+ treatment for energy levels ≥0.6 J the effectiveness of treatment was measured by calculating log reduction in *S. mutans* concentration between pre- and post-treatment values. However, this requires a relatively constant initial bacterial concentration. If 10^8 cfu/ml were present initially, a treatment 100% successful would appear to be worse than a test where 10^9 cfu/ml bacteria were present initially where the kill was also 100%. Figure 1 shows log reduction for each treatment against energy dose. Although the L–S+ treatment did not include light treatment it is shown for comparative purposes. The effect of each treatment was as follows:

The Effect of TBO Dye Alone (L–S+)

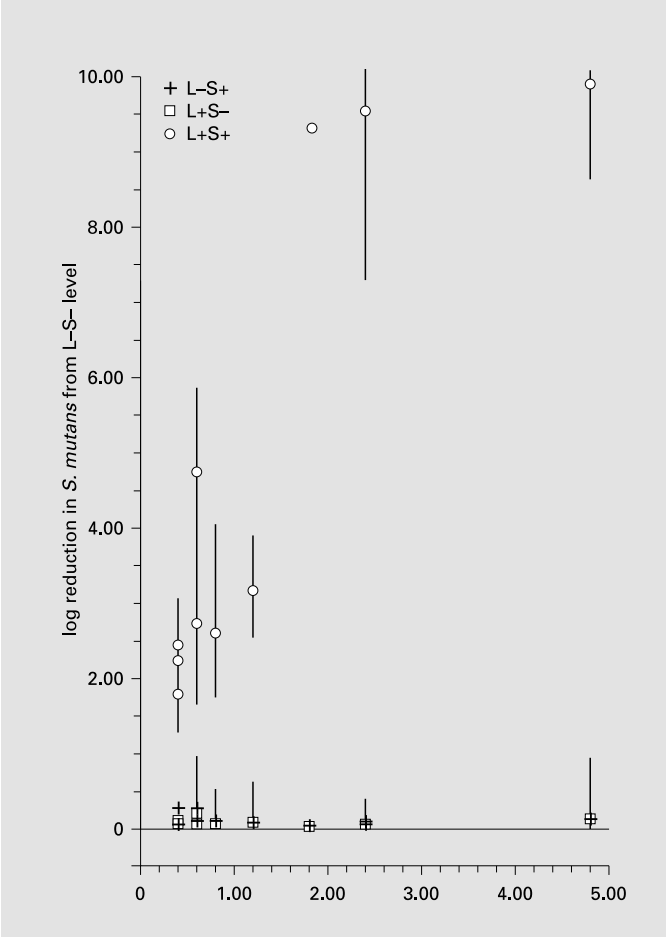
Overall mean log reduction was 0.08 (SD 0.11, n = 488) with a range of 0–0.90. The changes at each energy dose were not significant. There was no significant difference between results using TBO-A (mean 0.11, SD 0.11) and TBO-B (mean 0.07, SD 0.08).

The Effect of Light Alone (L+S–)

The effect of light alone gave an overall mean log reduction of 0.07 (s.d. 0.13, n = 488) and a range of 0.00 to 1.33. The log reduction between pre- and post-treatment values at each energy dose was not significant nor was there any significant change as the energy dose increased.

The Combination of Dye and Light (L+S+)

The log reductions at each energy dose were significantly greater ($p < 0.01$) than for L–S–, L–S+ and L+S– treatments. As the energy dose increased less bacteria survived. At 2.4 J bacteria survived in only 18% of samples,



in numbers from 50 to 300 cfu/ml. At 1.8 and 4.8 J all bacteria were killed. The log reduction in *S. mutans* was related to the applied energy (E) by the least-squares regression equation: log reduction = 0.56 + 3.89E (R^2 = 0.83). No threshold value of energy dose could be detected (fig. 1). The mean log reductions for TBO-A and TBO-B were 3.33 and 3.03 at 1.2 J energy dose and 9.58 and 9.72 at 2.4 J. The differences at the same energy dose were not significant.

The Effect of Power and Exposure Time Combinations (L+S+)

Combinations of high power/short time and low power/long time were used to give the same energy doses of 0.4 and 0.6 J (table 1). For 0.4 J energy dose, the differences between 80 mW × 5 s (mean 2.43) and 40 mW × 10 s (mean 1.77) and between 20 mW × 20 s (mean 2.22) and 40 mW × 10 s were highly significant. Between 80 mW × 5 s and 20 mW × 20 s the change was not significant. For 0.6 J the difference between 60 mW × 10 s and 10 mW for 60 s was significant.

Fig. 1. The mean and range of log reduction in *S. mutans* from L–S– levels produced by each energy dose (J) for the three treatment groups, L–S+, L+S–, and L+S+.

Table 1. Mean post-treatment bacterial counts (cfu/ml) and standard deviation in each of the three treatment groups (L–S+, L+S–, L+S+) after delivery of specific energy doses (J)

Power mW	Time s	Energy dose, J	Mean post-treatment numbers of viable *S. mutans,* cfu/ml (SD)			NS	NE
			L–S+	L+S–	L+S+		
20	20	0.4	$1.49 \cdot 10^9$ (0)	$2.33 \cdot 10^9$ ($2.90 \cdot 10^8$)	$1.96 \cdot 10^9$ ($9.76 \cdot 10^6$)	8	32
40	10	0.4	$4.92 \cdot 10^9$ ($2.03 \cdot 10^9$)	$6.26 \cdot 10^9$ ($3.65 \cdot 10^9$)	$8.65 \cdot 10^7$ ($1.27 \cdot 10^7$)	16	32
80	5	0.4	$2.00 \cdot 10^8$ ($4.04 \cdot 10^7$)	$2.11 \cdot 10^8$ ($6.29 \cdot 10^7$)	$1.28 \cdot 10^6$ ($1.18 \cdot 10^6$)	8	32
60	10	0.6	$4.32 \cdot 10^9$ ($2.58 \cdot 10^9$)	$4.24 \cdot 10^9$ ($2.69 \cdot 10^9$)	$3.67 \cdot 10^7$ ($3.37 \cdot 10^7$)	24	32
10	60	0.6	$1.22 \cdot 10^{10}$ ($2.83 \cdot 10^8$)	$8.21 \cdot 10^9$ ($8.61 \cdot 10^9$)	$5.20 \cdot 10^5$ (0)	8	32
80	10	0.8	$1.02 \cdot 10^{10}$ ($2.78 \cdot 10^9$)	$1.23 \cdot 10^{10}$ ($3.67 \cdot 10^9$)	$6.13 \cdot 10^7$ ($7.03 \cdot 10^7$)	24	24
40	30	1.2	$7.33 \cdot 10^9$ ($3.63 \cdot 10^9$)	$6.82 \cdot 10^9$ ($3.20 \cdot 10^9$)	$5.53 \cdot 10^6$ ($3.95 \cdot 10^6$)	32	32
60	30	1.8	$1.53 \cdot 10^9$ ($9.78 \cdot 10^8$)	$2.01 \cdot 10^9$ ($1.54 \cdot 10^8$)	0	32	32
80	30	2.4	$7.63 \cdot 10^9$ ($1.28 \cdot 10^9$)	$7.56 \cdot 10^9$ ($1.72 \cdot 10^9$)	$3.46 \cdot 10^1$ ($8.76 \cdot 10^1$)	64	64
80	60	4.8	$7.89 \cdot 10^9$ ($8.74 \cdot 10^9$)	$7.78 \cdot 10^9$ ($8.53 \cdot 10^9$)	0	32	32

NS = Number of tests in each subgroup; NE = number of tests at each energy dose.

Discussion

The Effect of TBO Alone (L–S+)

The mean log reduction of 0.08 was similar to the figure of 0.17 found previously [Burns et al., 1993]. The solution alone, irrespective of the source, had only minimal effects on the viability of bacterial cells, at the concentration and in the timescale used. Other constituents of TBO, which may differ slightly depending on the source, had no effect under the experimental conditions used.

The Effect of Using the Light Alone (L+S–)

With changes in viable bacteria numbers remaining small as the energy dose increased it is unlikely that significant quantities of heat or other harmful radiation were generated by the isotropic sphere, in spite of the increased energy density.

The Effect of the Light/TBO Combination (L+S+)

The combination of TBO solution and light was highly effective, killing large numbers of *S. mutans* and confirming other studies. Variations in the initial bacteria levels may have a minor influence on the results. The $\log_{10}$ reduction increased linearly with the energy dose up to 2.4 J. It was calculated that exposure to 0.44 J would produce a log reduction in *S. mutans* of 2.3 using the new isotropic probe in comparison with a log reduction of 0.45 calculated from a previous study [Burns et al., 1993], indicating that the isotropic emitter used in the present study was more effective. Probable reasons are that the emitter is small enough to be placed directly into the bacterial suspension and the energy density is greater at the tip surface. It was calculated from the emitter geometry that 80 mW power produces an energy density of 100 $J \cdot cm^{-2}$ on the emitter surface. However, this reduces as the distance from the surface of the emitter increases. In a stirred suspension each bacterium experiences a varying energy dose during the irradiation time. Taking 60-µl volume of bacterial suspension as a sphere surrounding the emitter tip, then the radius of the liquid sphere would be 2.43 mm and the surface area 0.69 cm^2. Even without attenuation of light by passage through TBO solution the energy density at the edge of this volume would be reduced from 100 $J \cdot cm^{-2}$ to approximately 2.9 $J \cdot cm^{-2}$. Therefore any measurement of lethal dose must be an estimate. In this study it was of the order of 1×10^{-6} mJ/cell. At the lowest energy, 0.4 J, where some 10^9 *S. mutans* were killed, the lethal dose was 4×10^{-7} mJ/cell. Differences between these figures and the 1.2×10^{-4} mJ/cell found previously [Burns et al., 1993] again point to the increased effectiveness of the new emitter.

Burns et al. [1993] concluded that a threshold energy of 8.4 $J \cdot cm^{-2}$ was required before any bacterial killing would occur. The present study concluded that the case for the existence of a threshold energy was unproven. It was considered that although the power component of the energy dose might have an influence the energy dose was the most important factor in killing bacteria.

In conclusion, this system killed up to 10^9 cfu/ml *S. mutans* in planktonic suspension using irradiated TBO solution with antibacterial action directly proportional to energy dose rather than power output. Energy doses of 1.8 J or more killed 100% of the bacteria present. The new system was 100–1,000 times more effective than those previously used. Bacteria could be killed to significant levels within 30 s. Neither light nor TBO solution alone had any significant antibacterial action.

Acknowledgements

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

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