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ORIGINAL ARTICLE

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Bactericidal effects of different laser wavelengths on periodontopathic germs in photodynamic therapy

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Abstract This study was an attempt to clarify whether the bactericidal effects of photodynamic therapy (PDT) are wavelength or dose-dependent. We also attempted to create an optimised protocol for a light-based bactericidal modality to eliminate periodontal pathogens. Cultures of *Actinobacillus actinomycetemcomitans*, *Fusobacterium nucleatum*, *Porphromonas gingivalis*, *Prevotella intermedia*, and *Streptococcus sanguis*, were exposed to a He-Ne laser (632.8 nm) with a 30 mW power output, a 100 mW diode laser at 665 nm, or a 100 mW diode laser at 830 nm, in the presence or absence of methylene blue (MB) as a photosensitiser. A control group was also used with exposure to MB alone without laser exposure. The cultures were analysed by viable counts. The results indicated that exposure to the 100 mW laser light could eliminate up to 40% of bacteria on average. In particular, the most effective killing occurred with exposure to laser light in combination with the MB photosensitiser. The results of kinetic studies indicated that the best PDT response rate was achieved with a 60 s (energy density 21.2 J/cm^2) exposure to the 665 nm wavelength diode laser in the presence photosensitiser. In this condition, approximately 95% of *A. actinomycetemcomitans* and *F. nucleatum*, and 99–100% of the black-pigmented bacteria (*P. gingivalis* and *P. intermedia*) and *S. sanguis* were eliminated. These results showed that both wavelength and energy density are important factors, and that a low power laser of optimal wavelength and dosage, in combination with an appropriate photosensitiser, is a practical bactericidal modality. We concluded that using a diode laser of proper power and wavelength to deliver 60 s of irradiation could be a useful adjunct with mechanical debridement in the prevention of the re-colonisation of subgingival lesions by pathogenic microorganisms.

Keywords Bactericidal · Diode laser · Methylene blue · Oral bacteria · Photodynamic therapy

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Introduction

As microbial plaques have been proven to be the primary aetiological agent of inflammatory periodontal disease, the major purpose of periodontal therapy has been to eliminate all bacterial deposits on the tooth surface [1, 2]. Unfortunately, the efficacy of debridement has varied in different clinical cases [3]. The use of systemic antibiotics as an adjunct in the treatment of periodontal disease has been necessary. However, overuse of antibiotics has been a major culprit in the production of drug-resistant organisms [4]. Therefore, the application of an alternative method to eradicate bacteria from periodontal pockets is desirable. One such approach is photodynamic therapy. In 1904, Jodlbauer and von Tappeiner [5] first successfully demonstrated the photodynamic inactivation of bacteria by an exogenously applied photosensitiser. Recently, a series of studies have shown that it is possible to kill bacteria with a light source from a low power laser after the microorganisms have been sensitised with a low concentration of dye, such as methylene blue (MB) or toluidine blue O (TBO) [6–8]. The main factors in successful PDT include the optimisation of the type and dosage of the photosensitiser, the energy density of the lasers, and the dye–laser interval [9, 10]. But further investigation to determine the maximal absorption of light to different dyes, and the role of wavelength in PDT has been necessary.

In this study, we tried to understand whether the photosensitisation of microorganisms is dosage dependent. We also attempted to create a practical protocol for the use of PDT in clinical therapy.

Materials and methods

A 30 mW He-Ne laser of wavelength 632.8 nm (CAS HN-15-2, An-Hui, PROC), and two Al-Ge-As diode lasers (power output of 100 mW) with wavelengths of 665 nm (PIDC IRD-665, Taiwan, ROC), and 830 nm (power output of 100 mW, PIDC IRD-830, Taiwan, ROC), were used in this study. All the emitted radiation was confined to a collimated beam 6 mm in diameter via a quartz optic fibre.

The bacterial strains used in the study were *Actinobacillus actinomycetemcomitans* (ATCC 29522), *Fusobacterium nucleatum* (ATCC 23726), *Porphromonas gingivalis* (ATCC 33277), *Pnevotella intermedia* (ATCC 25611), and *Streptococcus sanguis* (ATCC 10556). All the bacteria were maintained by weekly subculture on brucella blood agar plates (BBAP) (Oxoid, USA) enriched with haemin and menadione, and were incubated in an anaerobic workstation (Electrotek, West Yorks, UK) at 37 °C [11, 12]. After an overnight culture in brain heart infusion (BHI) broth (Oxoid, USA), each tested bacteria was quantified with a colorimeter (Vitek 52-1210 Hazelwood Mo. USA). The bacteria were then diluted in solution to an optical density of McFarland No. 1 at 405 nm (approximate numbers 3×10^8 cells). A 5 µl sample of adjusted bacterial suspension was put into the wells of a flat bottom 96-well microtitration plate (well diameter 6.2 mm) in triplicate. The plate was then placed on a microplate mixer (Tomy Seiko, Co. Tokyo, Japan) for even shaking prior to the following test.

Samples were distributed to four test groups:

1. Negative controls untreated by either lasers or photosensitisers.
2. Laser alone – bacterial suspensions were radiated with varying wavelengths of laser energy in the absence of any photosensitisers.
3. Photosensitisers alone – methylene blue (M9140, Sigma Ltd., Poole, UK) was added to each sample to a final concentration of 0.01% weight/volume.
4. Photosensitiser and laser – bacterial samples had MB added to a final concentration of 0.01% weight/ volume as in group 3, but were then subsequently treated with lasers of varying wavelength as in group 2.

The delivery of laser energy to each group follows: He-Ne laser 30 mW for 30 s (energy density 3.2 J/cm^2) or 60 s (energy density 6.4 J/cm^2), diode laser 100 mW for 30 s (energy density 10.6 J/cm^2) or 60 s (energy density 21.2 J/cm^2). The distance between the laser fibre and sample was 5 mm approximately. After irradiation, 195 µl of pre-reduced dispersing media [13] was added in each well, and 1 µl of each diluted suspension was cultured by pour plate method on the BBAP. After 48–96 h of anaerobic incubation at 37 °C, bacterial colonies were counted and converted into colony forming units (cfu).

Statistical analysis was performed. One-way analysis of variance was used to statistically analyse differences and using the Student–Newman–Keuls method performed multiple comparison procedures.

Results

The reduction of colony forming units in each of the test groups is tabulated for each of the three lasers used in this study. Table 1 shows the susceptibility of the various bacteria to the He-Ne laser. The effects of the 665-diode laser are shown in Table 2, and the effects of the 830-diode laser are in Table 3. A comparison between groups 1 and 3 reveals that treatment with photosensitiser in the absence of laser irradiation does not cause significant reduction ($p < 0.05$) in the viability of any of the tested bacterial cultures. This demonstrates that there is no direct toxicity with MB as a sensitiser at the concentration of 0.01% wt/vol. Similarly, irradiation with a He-Ne laser for up to 60 s had no significant effect on the viability of colony counts for any of the targeted bacteria in the absence of MB as photosensitiser (Table 1). In contrast, exposure to light from both diode lasers at an energy density of 10.6 J/cm^2 resulted in a significant decrease in viable colony counts (p value < 0.05). There was a significant decrease in counts when

Table 1 Susceptibility of oral bacteria to light from a HeNe 632.8 laser following treatment with MB

Bacteria strain	Viable count colony forming unit (cfu)					
	Group 1 (control)	Group 2 (lasing only)		Group 3 (dye only)[a]	Group 4 (dye + lasing)	
		30 s	60 s		30 s	60 s
Actinobacillus actinomycetemcomitans	136 ± 12[b]	131 ± 10 (96%)[c]	116 ± 5[*] (85%)	141 ± 10 (100%)	55 ± 5[*†‡] (40%)	17 ± 6[†*§] (13%)
Fusobacterium nucleatum	117 ± 9	151 ± 11 (119%)	113 ± 6 (97%)	112 ± 9 (96%)	36 ± 6[*†‡] (31%)	19 ± 3[*†§] (16%)
Porphyromonas gingivalis	129 ± 7	123 ± 10 (95%)	132 ± 8 (102%)	108 ± 12[*] (84%)	30 ± 5[*†‡] (23%)	16 ± 5[*†§] (12%)
Prevotella intermedia	105 ± 6	111 ± 8 (106%)	103 ± 7 (98%)	110 ± 7 (100%)	26 ± 8[*†‡] (25%)	13 ± 3[*†§] (12%)
Streptococcus sanguis	121 ± 5	100 ± 5[*] (83%)	103 ± 8[*] (85%)	116 ± 9 (96%)	23 ± 4[*†‡] (19%)	13 ± 4[*†§] (11%)

[a] The dye concentration (MB) is 0.01% wt/vol
[b] Mean cfu count ± SD
[c] Percentage of cfu
[*] Comparing to group 1, statistically significant with $p < 0.05$
[†] Comparing to group 3, statistically significant with $p < 0.05$
[‡] Comparing to group 2 lasing time 30 s, statistically significant with $p < 0.05$
[§] Comparing to group 2 lasing time 60 s, statistically significant with $p < 0.05$

Table 2 Susceptibility of oral bacteria to light from a Diode 665 laser following treatment with MB

Bacteria strain	Viable count colony forming unit (cfu)					
	Group 1 (control)	Group 2 (lasing only)		Group 3 (dye only)[a]	Group 4 (dye + lasing)	
		30 s	60 s		30 s	60 s
Actinobacillus actinomycetemcomitans	132 ± 12[b]	107 ± 8[*] (82%)[c]	88 ± 5[*] (67%)	137 ± 11 (100%)	32 ± 6[*†‡] (24%)	6 ± 3[*†§] (5%)
Fusobacterium nucleatum	106 ± 14	85 ± 11[*] (80%)	65 ± 9[*] (61%)	96 ± 6 (91%)	31 ± 5[*†‡] (29%)	4 ± 2[*†§] (4%)
Porphyromonas gingivalis	117 ± 8	75 ± 7[*] (64%)	67 ± 8[*] (57%)	111 ± 13 (95%)	19 ± 8[*†‡] (16%)	1 ± 0.3[*†§] (0.8%)
Prevotella intermedia	127 ± 14	93 ± 9[*] (73%)	81 ± 13[*] (64%)	118 ± 15 (93%)	15 ± 6[*†‡] (12%)	0[*†§] (0)
Streptococcus sanguis	93 ± 10	69 ± 7[*] (74%)	56 ± 4[*] (60%)	82 ± 8 (88%)	11 ± 7[*†‡] (12%)	2 ± 0.6[*†§] (2%)

[a] The dye concentration (MB) is 0.01% wt/vol
[b] Mean cfu count ± SD
[c] Percentage of cfu
[*] Comparing to group 1, statistically significant with $p < 0.05$
[†] Comparing to group 3, statistically significant with $p < 0.05$
[‡] Comparing to group 2 lasing time 30 s, statistically significant with $p < 0.05$
[§] Comparing to group 2 lasing time 60 s, statistically significant with $p < 0.05$

Table 3 Susceptibility of oral bacteria to light from a diode 830 laser following treatment with MB

Bacteria strain	Viable count colony forming unit (cfu)					
	Group 1 (control)	Group 2 (lasing only)		Group 3 (dye only)[a]	Group 4 (dye + lasing)	
		30 s	60 s		30 s	60 s
Actinobacillus actinomycetemcomitans	125 ± 11[b]	98 ± 11[*] (79%)[c]	65 ± 10[*] (52%)	128 ± 8 (100%)	86 ± 5[*†] (69%)	76 ± 5[*†] (61%)
Fusobacterium nucleatum	121 ± 9	99 ± 12[*] (82%)	67 ± 11[*] (55%)	117 ± 9 (97%)	93 ± 10[*†] (77%)	61 ± 4[*†] (50%)
Porphyromonas gingivalis	138 ± 8	88 ± 9[*] (64%)	81 ± 6[*] (59%)	126 ± 4 (91%)	91 ± 11[*†] (66%)	58 ± 6[*†§] (42%)
Prevotella intermedia	124 ± 10	82 ± 10[*] (66%)	72 ± 7[*] (58%)	113 ± 5 (91%)	77 ± 8[*†] (62%)	53 ± 8[*†§] (43%)
Streptococcus sanguis	106 ± 7	81 ± 13[*] (76%)	67 ± 5[*] (63%)	97 ± 10 (92%)	59 ± 7[*†‡] (56%)	47 ± 10[*†§] (44%)

[a] The dye concentration (MB) is 0.01% wt/vol
[b] Mean cfu count ± SD
[c] Percentage of cfu
[*] Comparing to group 1, statistically significant with $p < 0.05$
[†] Comparing to group 3, statistically significant with $p < 0.05$
[‡] Comparing to group 2 lasing time 30 s, statistically significant with $p < 0.05$
[§] Comparing to group 2 lasing time 60 s, statistically significant with $p < 0.05$

the exposure time was increased from 30 to 60 s (p value < 0.05) but even then, about 45–50% of tested organisms survived by viable counts.

For all tested microorganisms at all time points, there was a significant decrease in the viable counts when treated with both sensitiser and laser (group 4) (p value < 0.05). Furthermore, there was a statistically significant difference in killing between the different lasers used. During a 30 s exposure of MB incorporated microorganisms with a He-Ne laser (energy density 3.2 J/cm^2), the death rates ranged from 55% to 81%. When the exposure time was increased from 30 to 60 s (energy density 6.4 J/cm^2) the bactericidal rate went up to 79% to 89% (Table 1). The 830-diode laser (Table 3) in this study was far less effective. Even when the photosensitised microorganisms were given a 60 s exposure, the average decrease in bacterial load was only around 40–55%.

From Table 2 it can be seen that irradiation of MB-treated bacteria with 665 diode laser was the most effective modality tested. During a 30 s (energy density 10.6 J/cm^2) exposure, the average bacterial death rates were 71–88%. When the exposure time was increased to 60 s (energy density 21.2 J/cm^2), the bactericidal rate went up to 99–100% for both black-pigmented anaerobes (*P. intermedia* and *P. gingivalis*) and *S. sanguis* (Table 2). For *A. actinomycetemcomitans* and *F. nucleatum*, this modality achieved a 95% and 96% kill rate, respectively.

Discussion

The results of this study show that exposure of bacterial cultures to laser light in the presence of MB as a photosensitiser results in a dose-dependent decrease in viability. The most-effective combination was that of MB with a 665-diode laser at 100 mW. This produced a 95–99% kill rate in the various species that were tested. To exclude the possibility that the absorption of laser energy in MB may raise the temperature to kill bacteria, a pretest was done to evaluate the thermal effects. The results showed that the temperature increased only 0.5–3.9 °C in all lasing group with or without MB (data not shown). It suggested that, as a photosensitiser, methylene blue did not convert laser energy into heat that may kill microorganisms under the test conditions.

In general, the ability of the laser light to kill the periodontal pathogens was species-dependent. It appears that *F. nucleatum* and *A. actinomycetemcomitans* are more resistant to killing than *P. gingivalis* and *P. intermedia*, at least under the conditions in this study. *S. sanguis* was the most susceptible strain; the cause of the differing susceptibilities between these species has yet to be elucidated.

In the beginning of the last century, it was recognized that microbes became susceptible to visible light mixed with a photosensitising compound [5]. More recently, the concept of selectively sensitising cells for targeted killing by safe doses of visible light has become the basis of a new therapeutic modality for the treatment of tumours: photodynamic therapy [6, 14]. During the last few years, there have been attempts to bring the concepts of PDT to the field of dentistry [9, 10], numerous research groups have verified the lethal effect of laser radiation on microorganisms associated with dental caries, periodontitis and periimplantitis [9, 15–17]; studies have shown light from both high-power and low-power lasers to be effective in killing oral pathogenic bacteria. However, the use of low-power light has advantages in that a bactericidal effect can be achieved without damaging host tissues [18], and in presenting little damage to the operator.

Successful PDT always involves the optimisation of a large number of parameters. Obviously, selection of an effective photosensitiser is essential for the success of the technique. As well as being non-toxic to humans, the ideal photosensitiser needs to absorb a laser beam at the compatible wavelength and has to produce high excitation efficiency. Methylene blue, which belongs to the phenothiazinium family of dyes (which includes TBO), is a well-known photosensitiser. The LD_{50} of MB is 1180 mg/kg p. o. [19], it is safer than other photosensitising dyes. The studies reported by Millson et al. [20] have clearly demonstrated that the effect of increasing dose of low-power laser light on rat gastric mucosa. No damage was detectable to normal gastric epithelium with MB as photosensitiser when a light dose of 250 J/cm^2 was used; and MB has been proven to be an effective sensitiser in the photodynamic therapy of tumours in mice [21]. A considerable interest has been shown in the potential use of this dye for the photodynamic therapy of tumours and precancerous lesions in the oral mucous membrane [22]. The results of our study reveal that oral microorganisms incorporated with 0.01% wt/vol MB can be killed by short-term exposure to laser light. As the control group treated with MB did not reveal any demonstrable killing, it can be inferred that the bactericidal effect was produced by photodynamic therapy alone. We believe that the results of our study demonstrate that this approach could be used practically to kill oral bacteria.

It is obvious that the bactericidal effect was wavelength dependent, since the same power output diode laser with a monochromic infra red light of 830 nm wavelength could not kill the targeted organisms as effectively under the same conditions. These results suggest that the wavelength of the laser light source used in PDT is a crucial point for optimising the therapeutic effect and is an important factor in assessing the clinical applicability of this potential therapeutic approach. Recently, Wilson et al. [23] reported that TBO, having a maximized absorption at 633 nm, was the most effective photosensitiser they could find. It could sensitise *A. actinomycetemcomitans*, *F. nucleatum*, *P. gingivalis* and *S. sanguis* to killing by irradiation from a He-Ne laser (wavelength 632.8 nm). There are difficulties in obtaining a He-Ne laser with high-energy outputs – a single resonator with a 30 mW output is the highest He-Ne laser we could obtain. This makes it very difficult to achieve an optimal energy density capable of covering the entire surface of a target tooth. Currently, semiconductor diodes can produce higher power outputs in a continual wave mode, are extremely compact and reliable, and have had increased use in medical applications. With the appropriate delivery system, it is easy to deliver a high power density to an optimal area by diode laser.

From a practical point of view, the application of lethal photosensitisation to the elimination of microorganisms from a periodontal lesion would seem to be a relatively straightforward matter [14, 22]. This is especially so if one considers that the black-pigmented species, *P. intermedia* and *P. gingivalis*, both aggressive periodontal pathogens, are particularly susceptible to killing by photosensitisation. This might be due to the presence of protohaemin and protoporphyrin, two compounds present in these species that can absorb red light and therefore might enhance the killing effect [20]. Another advantage to PDT is that the photosensitiser can be applied topically into a periodontal pocket, hence avoiding overdoses. Also, as this is a local therapy, one can reduce the probability of side effects associated with the systemic administration of antimicrobial agents. Following application of a sensitiser, a laser beam could be delivered into the target area precisely via a fibre optic cable. Therefore, disturbances of the microflora at other sites in the oral cavity would not occur.

Although the results of this study have shown that oral pathogens can readily be killed by the appropriate

laser-dye treatment combination, further in vivo evaluation is necessary.

References

1. Slot J (1979) Subgingival microflora and periodontal disease. J Clin Periodontal 6:351–356
2. Haper OR, Dymock D, Booth V, Weightman AJ, Wade WG (1999) Detection of unculturable bacteria in periodontal health and disease by PCR. J Clin Microbiol 37:1469–1473
3. Wikström M, Renver S, Johnssont, Dahlén G (1993) Microbial association in periodontitis sites before and after treatment. Oral Microbial Immunol 8:213–218
4. Kleinfelder JW, Muller RF, Lange DE (1999) Antibiotic susceptibility of putative periodontal pathogenes in advanced periodontitis patients. J Clin Periodontol 26:347–351
5. Jodlbauer A, von Tappeiner H (1904) Über die Wirkung photodynamischer (fluoreszierender) Stoffe auf Bakterien. Münch Med Wochenschr 51:1096–1097
6. Karrer S, Szeimies RM, Ernst S, Abels C, Bäumler W, Landthaler M (1999) Photodynamic inactivation of staphylococci with 5-aminolaevulinic acid or photofrin. Lasers Med Sci 14:54–61
7. Jackson Z, Meghji S, MacRobert A, Henderson B, Wilson M (1999) Killing of the yeast and hyphal forms of *Candida albicans* using a light-activated antimicrobial agent. Lasers Med Sci 14:150–157
8. Wilson M, Dobson J, Harvey W (1992) Sensitization of oral bacteria to killing by low-power laser radiation. Curr Microbiol 25:77–81
9. Sarkar S, Wilson M (1993) Lethal photosensitization of bacteria in subgingival plaque from patients with chronic periodontitis. J Periodont Res 28:204–210
10. Spikes JD, Jori G (1987) Photodynamic therapy of tumors and other diseases using porphyrins. Lasers Med Sci 2:3–15
11. Slots J, Taubman MA (1992) Contemporary oral microbiology and immunology. St Louis:Mosby-year Book Inc, 275–282
12. Chan Y, Chien R (1994) Identification and analyses of periodontal pathogens in Taiwan by microbiological tests. Chin J Microbiol Immunol 27:59–69
13. Moller AJR (1966) Microbiological examination of root canals and periapical tissues of human teeth. Odontol Tidskr 74:371–380
14. Ackroyd R, Brown NJ, Davis MF, Stephenson TJ, Stoddard CJ, Reed MWR (1999) Aminolaevulinic acid-induced photodynamic therapy in the treatment of dysplastic Barret's oesophagus and adenocarcinoma. Lasers Med Sci 14:278–285
15. Bach G, Neckel C, Mall C, Krekeler G (2000) Conventional versus laser-assisted therapy of periimplantitis:a five year comparative study. Implant Dent 9:247–251
16. Dortbudak O, Haas R, Bernhart T, Mailath-Pokorny G (2001) Lethal photosensitization for decontamination of implant surface in the treatment of peri-implantitis. Clin Oral Implant Res 12(2):104–108
17. Kato T, Kusakari H, Hoshino E (1998) Bactericidal efficacy of carbon dioxide laser against bacteria-contaminated titanium implant and subsequent cellular adhesion to irraditated area. 23:299–309
18. Wilson M (1994) Bactericidal effect of laser light and its potential use in the treatment of plaque-related diseases. Int Dent J 44:181–189
19. Merck Kga A. Merck Chemical Reagents Index. Frankfurt, Germany. 1999/2000:877
20. Millson CE, Thurrell W, Buonaccorsi G, Wilson M, Macrobert AJ, Bown SG (1997) The effect of low-power laser light at different doses on gastric mucosa sensitized with methylene blue, haematoporphyrin derivative or toluidine blue. Lasers Med Sci 12:145–150
21. Williams JL, Stamp J, Devonshire R, Fowler GJS (1989) Methylene blue and the photodynamic therapy of superficial bladder cancer. J Photochem Photobiol B:Biol 4:229–232
22. His RA, Rosenthal DI, Glatstein E (1999) Photodynamic therapy in the treatment of cancer:current state of the art. Drugs 57(5):725–734
23. Wilson M, Dobson J, Sarker S (1993) Sensitization of periodontopathogenic bacteria to killing by light from a low-power laser. Oral Microbiol Immunol 8:182–187