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PAPER

Light activated antimicrobial agents can inactivate oral malodour causing bacteria

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Abstract

Oral malodour is a common condition which affects a large proportion of the population, resulting in social, emotional and psychological stress. Certain oral bacteria form a coating called a biofilm on the tongue dorsum and degrade organic compounds releasing volatile sulfur compounds that are malodourous. Current chemical treatments for oral malodour such as mouthwashes containing chlorhexidine or essential oils, are not sufficiently effective at reducing the bacterial load on the tongue. One potential alternative to current chemical treatments for oral malodour is the use of light activated antimicrobial agents (LAAAs), which display no toxicity or antimicrobial activity in the dark, but when exposed to light of a specific wavelength produce reactive oxygen species which induce damage to target cells in a process known as photodynamic inactivation.

This study aimed to determine whether oral malodour causing bacteria were susceptible to lethal photosensitization. Five bacterial species that are causative agents of oral malodour were highly sensitive to lethal photosensitization and were efficiently killed by methylene blue in conjunction with 665 nm laser light. Between 4.5–5 $\log_{10}$ reductions in the number of viable bacteria were achieved with 20 μM methylene blue and 14.53 J cm^{-2} laser light for *Porphyromonas gingivalis*, *Prevotella intermedia*, *Peptostreptococcus anaerobius* and *Solobacterium moorei.* The number of viable cells fell below the limit of detection in the case of *Fusobacterium nucleatum*.

These findings demonstrate that methylene blue in combination with 665 nm laser light is effective at killing bacteria associated with oral malodour, suggesting photodynamic therapy could be a viable treatment option for oral malodour.

1. Introduction

Upon irradiation with light of a suitable wavelength, a photoactivated antimicrobial agent, known as a photosensitiser, is able to generate reactive oxygen species which can cause damage to target cells. This process is known as photodynamic inactivation. Photodynamic therapy has been used extensively in the treatment of skin conditions, cancer, opthalmology and more recently, in dentistry and oral infections (Diamond *et al* 1972, Johnsson *et al* 1987, Braun *et al* 2008, Trachtenberg 2008, Taylor and Gonzalez 2009, Garcez and Núñez 2015).

A photosensitizer in its ground singlet state has two electrons with opposite spins in a low energy molecular orbital. Once exposed to light of an appropriate wavelength, the photosensitizer absorbs a photon and one of the electrons transitions to a high energy excited singlet state. This energy can then be lost through fluorescence and internal heat conversion, or can undergo a conversion to an excited triplet state via intersystem crossing (Robertson *et al* 2009). This excited triplet state is longer lived than the excited singlet state (Juzeniene and Moan 2007), and can react in one of two ways to produce reactive oxygen species. These reactive oxygen species can then cause destruction to the target area (figure 1). The type I mechanism occurs via electron or hydrogen transfer directly from the photosensitiser upon direct reaction with a substrate, such as water, which results in the formation of the superoxide anion (O_2^-). These radicals can then react with oxygen to produce highly reactive species, such as the hydroxyl radical and hydrogen peroxide The type II mechanism involves the photosensitiser, in its excited triplet state, reacting with molecular oxygen to produce highly reactive singlet oxygen. These reactive oxygen species can then cause rapid cytotoxic

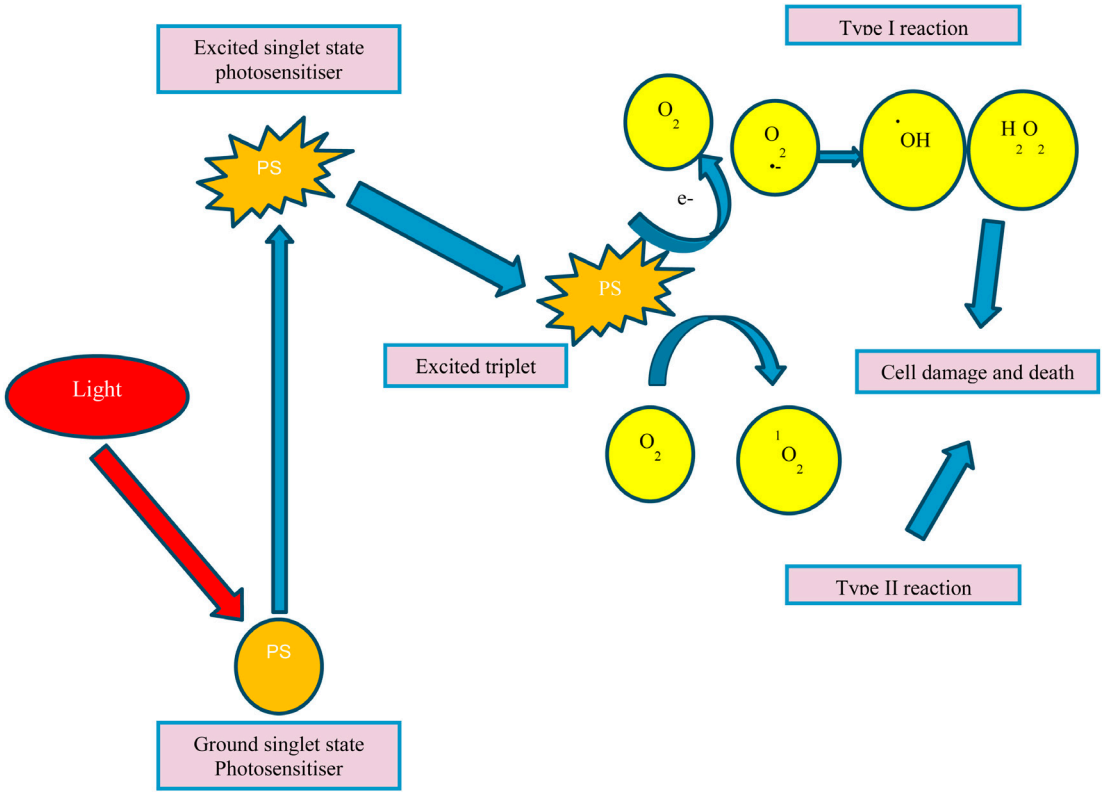

Figure 1. Overview of the mechanisms of photodynamic inactivation. The photosensitizer (PS) in its ground singlet state is exposed to light of a suitable wavelength and absorbs a photon. An electron is then excited to an excited singlet state and can then undergo intersystem crossing to an excited triplet state. The excited triplet state can then react in one of two ways; via the Type I or Type II mechanism to induce cell damage and death.

effects to the target area and result in target cell death via damage to bacterial cell walls and cytoplasmic membranes. This in turn results in the leakage of cellular contents, damage to DNA, such as breaks in single or double stranded DNA and possible inactivation of membrane transport systems (Raghavendra *et al* 2009, Rajesh *et al* 2011, Huang *et al* 2012).

Photodynamic therapy is a promising alternative antimicrobial therapy for dental and oral conditions, due to the ease of accessibility for both photosensitizer and light application, and successful results have been seen in the treatment of dental caries (Nagata *et al* 2012), peri-implantitis (Bassetti *et al* 2013), and periodontitis (Betsy *et al* 2014).

Halitosis, also known as oral malodour, is estimated to affect approximately 8–50% of the adult population, who perceive that they suffer from oral malodour as a chronic problem (Porter and Scully 2006), with 85–90% of cases of malodour being of intra-oral origin (Quirynen *et al* 2009). It is thought that the dorso-posterior region of the tongue is the main source of malodor of an oral origin due to the unique morphology and the location, which makes the area hard to cleanse. Bacteria located on the tongue dorsum, mainly Gram-negative anaerobic bacteria, are able to putrefy organic compounds found in postnasal drip and in the tongue coating, and produce volatile sulfur compounds (VSCs), which are characteristic of oral malodor, as by products (Kazor *et al* 2003, Bollen and Beikler 2012). Oral malodour causing bacteria are able to degrade sulfur containing amino acids such as methionine and cysteine, and non-sulfur containing amino acids such as lysine, arginine and tryptophan, and bio-transform them into VSCs such as hydrogen sulphide, methylmercaptan and dimethyl sulphide (Greenman 1999).

The bacteria which have been most commonly associated with oral malodor in the past are Gram-negative pathogens and include *Fusobacterium nucleatum*, *Porphryomonas gingivalis*, *Prevotella intermedia*, *Treponema denticola* and *Tannerella forsynthesis* (Morita and Wang 2001, Porter and Scully 2006). These bacteria reside in the periodontal pockets and on the posterior of the tongue and mouth, thereby avoiding removal and cleansing (Krespi *et al* 2006). Gram-positive bacteria have also been implicated in halitosis. The Gram-positive, anaerobic coccus *Peptostreptococcus anaerobius* has been found to produce hydrogen sulphide from L-cysteine (Persson 1990), and has been isolated on the tongue dorsum of people suffering from halitosis (Tyrell *et al* 2003). *Solobacterium moorei*, a Gram-positive bacterium which has only been found to be present in the oral cavities of halitosis sufferers, has also been implicated in oral malodour, suggesting that there are distinct bacteria associated with halitosis (Haraszthy *et al* 2007). A study carried out by Tanabe and Grenier characterising the capacity of *S. moorei* to produce volatile sulphur compounds found that the bacterium was able to produce high levels of hydrogen

sulphide from cysteine. This is thought to be catalysed by the enzyme cysteine desulfhydrase, the gene of which has been identified in the genome of *S. moorei* (Tanabe and Grenier 2012).

A preliminary study trialing photodynamic therapy of halitosis suggests that this treatment is effective at reducing VSC concentrations (Lopes *et al* 2014). However there is little data in the reported literature on the ability of light in combination with a photosensitizer to inactivate bacteria, in particular Gram-positive bacteria, that cause halitosis. In the study reported here we have investigated the capacity of the photosensitizer methylene blue in combination with red light to inactivate important Gram-positive and Gram-negative halitosis causing bacteria.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The bacteria used in this study were *P. gingivalis* (W50), *F. nucleatum* (NCTC 10562), *Pr.intermedia* (DSMZ 20706), *Pt. anaerobius* (NCTC 11460) and *S. moorei* (DSMZ 22971). Bacteria were maintained on fastidious anaerobe agar (FAA; LabM, Bury, UK) supplemented with 5% v/v defibrinated horse blood (E&O Laboratories, Bonnybridge, Scotland) under anaerobic conditions (80% N_2; 10% H_2; 10% CO_2) at 37 °C. For the photodynamic inactivation studies bacteria were grown in liquid culture until they reached stationary phase. The Gram-negative bacteria, *P. gingivalis*, *F. nucleatum*, *Pr. intermedia* were grown anaerobically for 18 h in Brain Heart Infusion broth (BHI broth; Sigma, Dorset, UK) supplemented with 5 μg ml^{-1} hemin (Sigma) and 0.5 μg ml^{-1} menadione (Sigma). *Pt. anaerobius* was grown anaerobically for 22 h in Brain Heart Infusion broth supplemented with 5 μg ml^{-1} hemin and 0.5 μg ml^{-1} menadione. *S. moorei* was grown anaerobically for 24 h in tryptic soy broth (TSB; Sigma) supplemented with 5% v/v horse serum (Sigma).

2.2. Photosensitiser and laser

The phenothiazine dye methylene blue (Sigma, Dorset, UK) was dissolved in sterile water and filtered through a 0.22 μm syringe filter (Merck Millipore). All solutions were stored in the dark at room temperature prior to use.

A low power, non-thermal, Periowave diode laser (Ondine Biomedical Inc.) was utilised in this study (wavelength 665 nm, P_{max} 160 mW). The laser was clamped onto a stand in such a way that the light emitting end was positioned directly above a well of a 96 well microtiter plate, at a height that allowed the laser beam to cover the entire well upon irradiation. The power delivered by the laser to the irradiated well was measured using a laser power meter (Gentec, TPM-300).

2.3. Photosensitisation procedure

Bacterial cells were pelleted from liquid culture by centrifugation (15 093 g, 3 min) and resuspended in pre-reduced PBS. Each bacterial suspension was adjusted to an optical density at 600 nm which corresponded to approximately 1×10^8 cells ml^{-1}. The four test groups were as follows: light plus photosensitiser (L^+S^+), light alone (L^+S^-), photosensitiser alone (L^-S^+) and the control which was neither treated with photosensitiser nor light (L^-S^-). For the L^+S^+ group, 100 μl of methylene blue was added to an equal volume of the bacterial culture in triplicate wells of a 96 well flat bottomed plate (VWR, Leicestershire, UK) and irradiated with 665 nm laser light with an energy density of 14.53 J cm^{-2}, corresponding to a 30 s exposure to light. Three additional wells containing 100 μl of methylene blue and 100 μl of the bacterial suspension were kept in the dark (L^-S^+) to assess the toxicity of the photosensitiser alone (L^-S^+). Controls, L^+S^- and L^-S^- in triplicate, consisted of 100 μl of pre-reduced PBS added to an equal volume of the bacterial cultures. The L^+S^- wells were irradiated with laser light, while the L^-S^- wells, were kept in the dark. Following irradiation/dark incubation, each sample was serially diluted 10-fold in pre-reduced PBS. A 25 μl portion of each dilution was spotted and spread plated onto 5% horse blood Fastidious anaerobe agar plates in triplicate, and the plates were incubated anaerobically at 37 °C for 48–72 h. The surviving CFU/well were determined by enumerating the viable bacteria on plates. Experiments were performed on three different occasions in triplicate.

To assess the effect of light dose on lethal photosensitization, bacteria were grown as previously detailed until they had reached the stationary phase of growth. Methylene blue was diluted in PBS and added to an equal volume of bacteria in 96 well plates to give a final concentration of 10 μM. The L^+S^+ wells were irradiated with 665 nm laser light energy doses of 2.42 J cm^{-2}, 4.84 J cm^{-2} or 9.69 J cm^{-2}, corresponding to 5, 10 or 20 s irradiation respectively. Three additional wells containing 100 μl of methylene blue and 100 μl of the bacterial suspensions were kept in the dark (L^-S^+) and 100 μl of PBS was also added to 100 μl of the bacterial suspensions in a further six wells, three of which were irradiated with laser light (L^+S^-), while the remaining three were kept in the dark (L^-S^-).

Following irradiation/dark incubation, each sample was serially diluted 10-fold in pre-reduced PBS. A 25 μl aliquot of each dilution was spotted and spread plated onto 5% horse blood Fastidious anaerobe agar plates in triplicate, and the plates were incubated anaerobically at 37 °C for 48–72 h. The surviving CFU/well were enumerated by viable counting. Experiments were performed on three different occasions in triplicate.

2.4. Statistics

The columns and error bars shown in the graphs represent the mean and standard deviations of three individual experiments performed in triplicate. Statistical comparisons between groups were carried out using two-way ANOVA with post-hoc Bonferroni analysis, and comparisons within groups were carried

out using one-way ANOVA with post-hoc Bonferroni analysis. A P value of less than 0.0001 was considered statistically significant for two-way ANOVA, and a P value of less than 0.05 was considered statistically significant for one-way ANOVA.

3. Results

3.1. The effect of photosensitizer dose on lethal photosensitization of oral malodour causing bacteria

The effects of increasing photosensitiser dose on the viability of oral malodour causing bacteria are shown in figure 2. When the bacteria were treated with different concentrations of methylene blue and 665 nm laser light, there was a significant ($P < 0.0001$) reduction in the viable cell counts. When *P. gingivalis* was treated with methylene blue and exposed to 14.53 J cm^{-2} of laser light, a significant ($P < 0.0001$) 3 $\log_{10}$ reduction in the number of viable cells was seen with the lowest concentration of methylene blue used. When treated with the highest concentration of methylene blue, there was a 5 $\log_{10}$ reduction in viable cells (figure 2(A)). Treatment of *Pr. intermedia* with methylene blue and exposure to 14.53 J cm^{-2} laser light caused a 1.6 $\log_{10}$ ($P < 0.0001$) reduction in the number of viable bacteria at 2 μM methylene blue. At the highest concentration of methylene blue, there was a 4.5 $\log_{10}$ reduction in viable cells (figure 2(B)). In the case of *F. nucleatum*, in the groups treated with either laser light alone (L^+S^-) or with methylene blue alone (L^-S^+) the viable counts were no different from the untreated control (L^-S^-). When exposed to 2 μM methylene blue and irradiated with 665 nm laser light, there was a 3.1 $\log_{10}$ ($P < 0.0001$) reduction in the number of viable bacteria. The number of viable bacteria fell below the limit of detection with the highest concentration of methylene blue used upon exposure to 665 nm light. The bactericidal effect was dependent on photosensitizer concentration, with significantly ($P < 0.05$) more kills at 5, 10 and 20 μM methylene blue as compared to treatment with 2 μM methylene blue (figure 2(C)). Interestingly with *P. gingivalis* and *Pr. Intermedia*, a 1 and a 0.5 $\log_{10}$ reduction in viable cells occurred upon irradiation with laser light alone, respectively.

The Gram-positive bacteria associated with oral malodour were also sensitive to lethal photosensitization. At the lowest concentration of methylene blue, a 2.2 $\log_{10}$ ($P < 0.0001$) reduction in the number of viable *Pt. anaerobius* was achieved upon exposure to 665 nm laser light, as seen in figure 2. Treatment with 20 μM methylene blue and exposure to 14.53 J cm^{-2} laser light resulted in a 5.0 $\log_{10}$ ($P < 0.0001$) reduction in viable cells (figure 2(D)).

Irradiation of *S. moorei* in the presence of 2 μM methylene blue resulted in 0.9 $\log_{10}$ ($P < 0.0001$) reduction in viable bacterial cells. Treatment with the highest concentration of methylene blue and exposure to laser light resulted in a 4.6 $\log_{10}$ reduction in viable cells. As was the case with *F. nucleatum*, photodynamic inactivation of *S. moorei* was dependent on photosensitiser concentration, with a significant ($P < 0.05$) reduction in viable colony counts at 5, 10 and 20 μM methylene blue as compared to 2 μM (figure 2(E)).

3.2. The effect of light dose on lethal photosensitization of oral malodour causing bacteria

To study the effect of different light doses in combination with methylene blue on the viability of oral malodour causing bacteria, cells were exposed to light doses of either 2.42 J cm^{-2}, 4.84 J cm^{-2} or 9.68 J cm^{-2} in the presence of 10 μM methylene blue.

Upon irradiation with the lowest light dose in combination with 10 μM methylene blue, there was a significant ($P < 0.0001$) reduction in the number of viable *P. gingivalis* cells (figure 3(A)). At the lowest light dose, there was a 2.7 $\log_{10}$ reduction in the number of viable cells, which increased to a 3.5 $\log_{10}$ reduction in the number of viable cells at the highest light dose.

Irradiation of *Pr. intermedia* with varying light doses in the presence of 10 μM methylene blue resulted in a significant ($P < 0.0001$) reduction in viable cell counts (figure 3(B)). Exposure to a light dose of 2.42 J cm^{-2} with 10 μM methylene blue caused a 1.4 $\log_{10}$ ($P < 0.0001$) reduction in cell numbers. At the longest exposure time there was a 3.1 $\log_{10}$ reduction in the number of viable cells. Photodynamic inactivation of *Pr. intermedia* was dependent on light dose, with a significant ($P < 0.05$) reduction in viable cells after 10 and 20 s exposure as compared to 5 s exposure. Treatment of *F. nucleatum* cells with 10 μM methylene blue in combination with 665 nm laser light for 5 s were reduced in number by 3.6 $\log_{10}$ ($P < 0.0001$). The number of viable cells fell below the limit of detection (LoD = 10 cells) after 20 s exposure (figure 3(C)). For *P. gingivalis* and *Pr. intermedia*, there was also a significant ($P < 0.0001$) reduction in viable cell numbers when treated with light alone (L^+S^-).

Upon irradiation of *Pt. anaerobius* with 2.42 J cm^{-2} laser light in the presence of methylene blue, viable cell numbers fell by 2 $\log_{10}$ ($P < 0.0001$). At the highest light dose, a 2.9 $\log_{10}$ reduction in the number of viable cells was observed (figure 3(D)). In the case of *S. moorei*, a significant 1 $\log_{10}$ ($P < 0.0001$) reduction in the number of viable cells was observed with 2.42 J cm^{-2} of laser light and 10 μM methylene blue. With a 20 s exposure time, there was a 3.9 $\log_{10}$ ($P < 0.0001$) reduction in the number of viable *S. moorei* cells.

4. Discussion

Oral malodour can arise due to a number of reasons, including transient, lifestyle, oral and non-oral causes. Whatever the cause may be, oral malodour is an extremely important condition due to the adverse impact it can have on the professional and personal

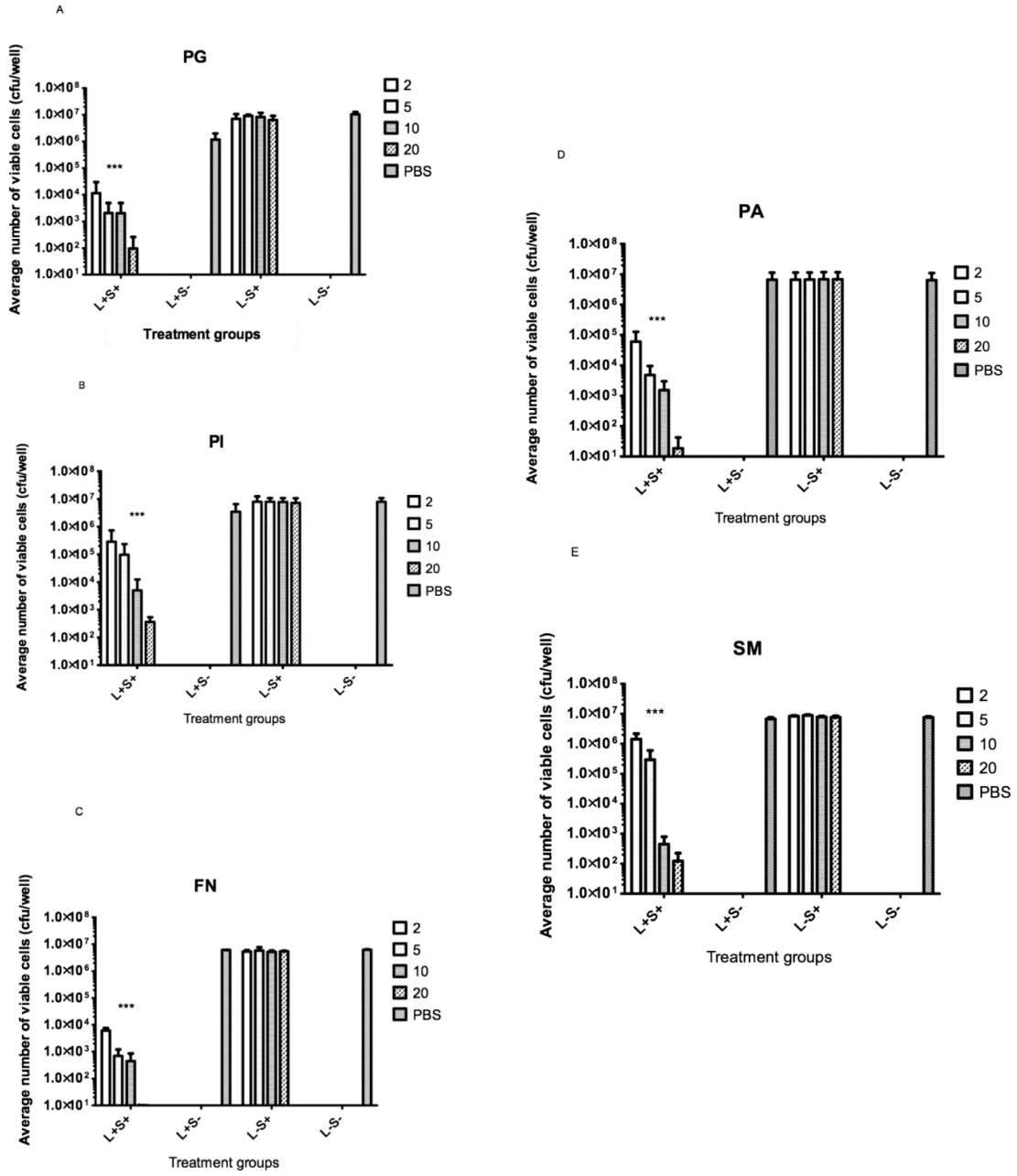

Figure 2. The capacity of methylene blue at doses of 2, 5, 10 or 20 μM in combination with a 665 nm laser light dose of 14.53 J cm^{-2} to cause photodynamic inactivation of oral malodour casing bacteria. An equal volume of either PBS (S^-) or methylene blue (S^+) (concentrations ranging from 2–20 μM) was added to 100 μl of the bacterial suspensions and either kept in the dark (L^-) or exposed to 665 nm laser light with an energy density of 14.53 J cm^{-2} (L^+). After irradiation/dark incubation, samples were serially diluted and the surviving CFU/well were enumerated. Error bars represent the standard deviation from the mean. *** $P < 0.0001$ (two-way ANOVA). Experiments were performed three times in triplicate and the combined data are shown. (A) Lethal photosensitisation of *P. gingivalis*. (B) Lethal photosensitisation of *Pr. intermedia*. (C) Lethal photosensitisation of *F. nucleatum*. (D) Lethal photosensitisation of *Pt. anaerobius*. (E) Lethal photosensitisation of *S. moorei*.

lives of sufferers. Suffering from oral malodour can lead to psychological changes, which can in turn, lead to social and personal isolation (Bollen and Beikler 2012). As such oral malodour sufferers can have a poorer quality of life compared to those that do not suffer. Current treatments include the advocation of proper oral hygiene, mechanical removal of the tongue coating that is responsible for the malodour and the use of antimicrobial toothpastes and mouthwashes to reduce the bacterial load in the oral cavity (De Geest *et al* 2016). The ability of light activated antimicrobial agents (LAAAs) to cause photodynamic inactivation of a number of bacteria has been well documented (Demidova and Hamblin 2004, Jori *et al* 2006). In particular, there have been numerous *in vitro* and *in vivo* studies on the use of photodynamic inactivation for the treatment of oral pathogens (Komerik *et al* 2003, Wilson 2004, Fimple *et al* 2008, Schiffner *et al* 2014).

In this study we found that using the phenothiazine dye methylene blue and 665 nm red laser light, lethal photosensitization of the oral malodour causing bacteria *P. gingivalis*, *Pr. intermedia*, *F. nucleatum*, *Pt. anaerobius* and *S. moorei* could be achieved. The five bacteria

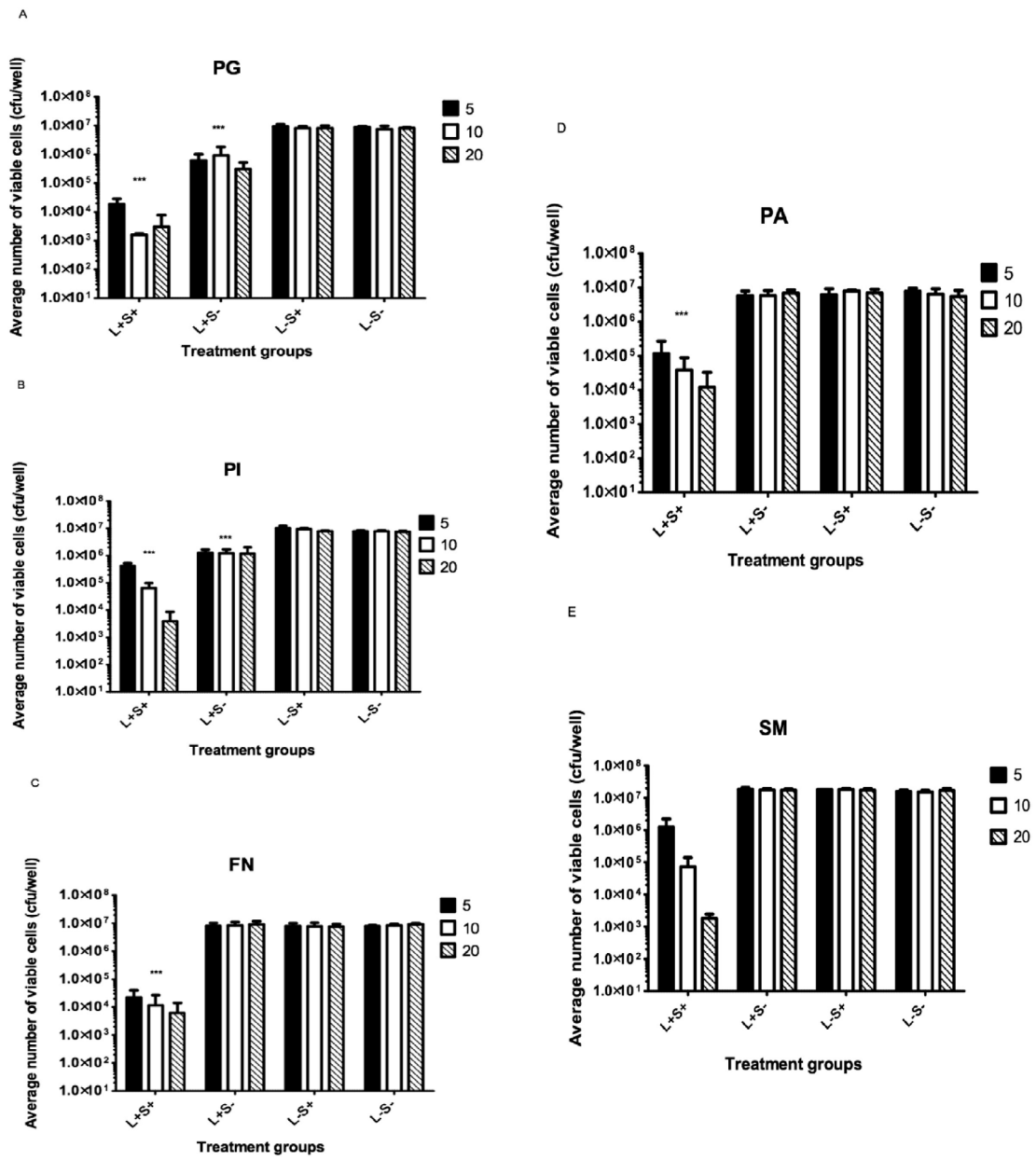

Figure 3. The capacity of 665 nm laser light at doses of 2.42 J cm^{-2}, 4.84 J cm^{-2} and 9.69 J cm^{-2} (corresponding to 5, 10 and 20 s exposure) in combination with 10 μM methylene blue to cause photodynamic inactivation of oral malodour causing bacteria. An equal volume of either PBS (S^-) or methylene blue (S^+) (10 μM) was added to 100 μl of the bacterial suspensions and either kept in the dark (L^-) or exposed to 665 nm laser light (L^+) (energy doses ranging from 2.42 J cm^{-2}–14.53 J cm^{-2}). After irradiation/dark incubation, samples were serially diluted and the surviving CFU/well were enumerated. Error bars represent the standard deviation from the mean. *** $P < 0.0001$ (two-way ANOVA). Experiments were performed three times in triplicate and the combined data are shown. (A) Lethal photosensitisation of *P. gingivalis*. (B) Lethal photosensitization of *Pr. intermedia*. (C) Lethal photosensitisation of *F. nucleatum*. (D) Lethal photosensitisation of *Pt. anaerobius*. (E) Lethal photosensitisation of *S. moorei*.

tested in this study were all found to be susceptible to lethal photosensitization when treated with either different concentrations of methylene blue or different light doses, with significant reduction in the number of viable cells seen for each of the bacteria.

Photodynamic inactivation was found to be dependent on photosensitiser dose in the case of *F.nucleatum* and *S. moorei*, while it was dependent on laser light dose in the case of *Pr. intermedia*. Significant kills were also demonstrated with the lowest concentration of methylene blue (2 μM) and the lowest laser light dose (2.42 J cm^{-2}) for each of the five bacteria tested. Low photosensitizer and light doses would be beneficial in a clinical setting because the laser light dose corresponds to a short irradiation time of 5 s, and at such a low concentration of methylene blue, temporary staining of the tongue blue would be minimal. While significant kills were achieved with low energy doses, complete eradication of any of the bacteria was not achieved with low methylene blue concentrations and low light doses.

Chan and Lai demonstrated that *F. nucleatum* was highly susceptible to photoinactivation with methylene blue, achieving reductions in the number of bacteria of 1.3 logs (Chan and Lai 2003). A previous study conducted by Williams *et al* found that treatment with

20 μg ml^{-1} of tolonium chloride, also known as toluidine blue, a phenothiazine dye closely related to methylene blue, and exposure to 4.8 J cm^{-2} of 633 nm laser light resulted in a maximum mean log reduction of 2.81 in the number of *F. nucleatum* as compared to 1.14 mean logs for *Pr. intermedia* and 2.48 mean logs for *Peptostreptococcus (now Parvimonas) micros* also tested in their study (Williams *et al* 2006). Theirs is similar in the case of the results presented within this study with *F. nucleatum* being the most affected by photodynamic inactivation, as it was the only bacterium tested that had viable cell counts fall below the limit of detection. This demonstrates that with an increased energy dosage, higher levels of kill for *F. nucleatum* can be achieved. The results of this study are favourable however, as the concentration of methylene blue utilised in this study was much lower compared to the concentration of tolonium chloride, and the 665 nm light source used in this study would have been able to penetrate more deeply as compared to the 633 nm laser device used by Williams *et al* therefore suggesting that our regime using methylene blue and 665 nm light produces a more promising outcome.

Energy doses in excess of 20 J cm^{-2} from a 633 nm diode laser in combination with derivatives of chlorin e6, have been demonstrated to completely eradicate cultures of *P. gingivalis* and *F. nucleatum* (Pfitzner *et al* 2004). In our study, the maximum energy dose tested was 14.53 J cm^{-2} which resulted in significant bacterial reduction, and in the case of *F. nucleatum*, reduced cells counts below our limit of detection. It may be possible that our regime completely eradicated *F. nucleatum* cells, and as such the findings of this study are an improvement on those already reported due to the low photosensitiser and light dose combination used in comparison to previous studies.

In the case of *P. gingivalis*, photodynamic inactivation was not dependent on the laser light dose. This is in contrast to results reported in another study using toluidine blue O (TBO) in combination with a helium/neon gas laser with a wavelength of 632.8 nm (Bhatti *et al* 1997), which resulted in complete bacterial kills using 2.2 J cm^{-2} of light. Conversely, an *in vivo* study investigating the killing of *P. gingivalis* also using Toluidine blue, found that photodynamic inactivation was not dependent on light dose response using a 630 nm diode laser, but was dependent on photosensitizer concentration at lower light doses. This effect was thought to be due to sufficient doses of energy being supplied to the sample in order to activate all of the photosensitiser molecules present, thereby generating enough oxygen radicals to kill the bacteria (Komerik *et al* 2003). It has also been found that as well as killing of *P. gingivalis* cells by photodynamic inactivation, virulence factors of this pathogen are also inactivated by the process, thereby providing another advantage of this therapy over conventional treatments (Komerik *et al* 2000).

Exposure to 665 nm laser light alone resulted in a significant reduction in the numbers of viable *P. gingivalis* and *Pr. intermedia* cells, albeit in a lower magnitude compared to the L^+S^+ group, as seen in figures 2(A), (B), 3(A) and (B). *P. gingivalis* and *Pr. intermedia* are black pigmented anaerobes, both of which have been found to accumulate the endogenous porphyrins, dimeric protoporphyrin IX and monomeric protoporphyrin IX. These endogenous porphyrins have a strong absorption peak at approximately 400 nm which corresponds to blue light, and numerous studies have shown that upon irradiation with blue light, both of these pathogens can be photoinactivated in the absence of an exogenous photosensitiser (Soukos *et al* 2005, Hope *et al* 2013, 2016, AbdulAzeez *et al* 2014). Whilst those studies focused on blue light, which strongly activates these endogenous porphyrins, there is a smaller absorption peak at approximately 600–700 nm, corresponding to the red light which was utilized in our study. Another study utilized red light only to kill bacteria via photodynamic action, and found that following a single exposure to red light at 632.8 nm at a 100 mW cm^{-2} light intensity and with a 360 J cm^{-2} energy density, there was a pronounced photodynamic effect on *P. gingivalis* and *Pr. intermedia* (Konig *et al* 2000). This supports our data reported herein where there is a reduction in viable bacteria in the L^+S^- group. However although our study utilises a higher power laser, the energy dose is almost 25 times lower with a similar effect, therefore the regime utilised in this study is favourable compared to those previously reported. These results also demonstrate that using a laser light source in combination with a photosensitiser, in the case of our study methylene blue, much lower energy doses can be delivered to the target area or tissue, with significant bactericidal effects and reduced chances of causing damage to the surrounding areas.

It is interesting to note that in our study the Gram-negative bacteria were more susceptible to photodynamic inactivation than the Gram-positive bacteria, *Pt. anaerobius* and *S. moorei*. It is widely thought that Gram-negative bacteria are less susceptible to photodynamic inactivation due to structural differences in the cells as compared to Gram-positive bacteria, namely the presence of an outer membrane which serves to act as a permeability barrier (Maisch *et al* 2004). This apparent paradox with our findings is probably because uptake of methylene blue may be increased in Gram-negative bacteria due to its cationic nature, low molecular weight and hydrophilicity, thereby allowing the photosensitiser to pass across the porin-protein channels in the outer membrane of Gram-negative bacteria. As well as passing across the outer membrane, methylene blue is also able to localise to the anionic lipopolysaccharide found within the outer membrane and form methylene blue dimers, which themselves add to and enhance the photodynamic effect (Maisch *et al* 2004), and this effect could account for the differences in susceptibility seen in our study.

Photodynamic inactivition studies on *Peptostreptococcus* species have demonstrated that these bacteria are able to be killed by this technique. A study using photodynamic therapy to treat endodontic infection

found that *Peptostreptococcus (now Parvimonas) micros* was completely eradicated after photodynamic therapy with 67 μM methylene blue and exposure to 30 J cm^{-2} diode laser light (Soukos *et al* 2006). Another study carried out investigating the susceptibility of anaerobic bacteria isolated from periodontal disease to PDI with 84 μM chlorin e6 and 15 J cm^{-2} laser light resulted in a 4–6 log reduction in the 4 Peptostreptococcal species treated (Drulis-Kawa *et al* 2005). These studies highlight the susceptibility of peptostreptococci to photodynamic inactivation and as such suggest that PDI is a viable option for the eradication of these bacteria.

PDI studies on *Pt. anaerobius* are scarce, with one study achieving a 3 log kill of the bacterium using hematoporphyrin derivative (HpD) in combination with exposure to a 100 mW projector light for 20 min (Venezio 1985). In our study a significant reduction in the numbers of this bacterium were achieved using the highest concentration of methylene blue, and there was also an observed light dose response in the viability of this bacteria, however this was not statistically significant. Our study also utilised a much lower exposure time as compared to Venezio *et al*, with our maximum exposure time of 30 s resulting in a 5 $\log_{10}$ reduction in bacteria, demonstrating that our current regime is favourable to previously reported regimes.

Our results demonstrate that a low dose of photosensitiser coupled with a low exposure time is able to cause a significant level of kill in the number of bacteria, although higher concentrations of methylene blue and longer exposure times result in higher reductions in the number of viable bacteria.

To our knowledge there have been no studies published on the use of LAAAs to kill *S. moorei*, a Gram-positive bacterium implicated in oral malodour. A study by Tanabe and Grenier has found that *S. moorei* is able to produce hydrogen sulphide from cysteine, and VSC's were also produced from serum, saliva and mucin and as such *S. moorei* has been proposed to be a major player in oral malodour (Tanabe and Grenier 2012). Previous studies of photodynamic inactivation of Gram-positive bacteria have yielded successful results and significant bacterial kills. A study into the effects of photodynamic therapy of biofilms of *Enterococcus faecalis*, a Gram-positive bacterium found in the oral cavity and which is known to be resistant to some antibiotics, found that treatment with methylene blue or TBO, with 271 J cm^{-2} and 106.4 J cm^{-2} respectively, resulted in significant levels of bacterial killing (Lopez-Jimenez *et al* 2015). Another study into the photosensitisation of *E. faecalis* biofilms determined that pre-incubation for 10 min with 60 μM methylene blue with subsequent exposure to 9.6 J cm^{-2} resulted in the disruption of the biofilms, and determined that significant levels of bacterial reduction could be achieved using this photosensitiser (Garcez *et al* 2013). *Staphylococcus aureus* and *Staphylococcus epidermidis* are also Gram-positive bacteria, most commonly associated with skin infections and implant associated infections in hospitalised patients. Biofilms of both of these organisms were treated with 40 μM TBO combined with energy doses up to 200 J cm^{-2} and it was found that a significant decrease in the bacterial biofilms could be achieved (Sharma *et al* 2008). These studies serve to show that Gram-positive have successfully been proven to be susceptible to phenothiazine photosensiters, as used in our study.

In our study, there was a significant reduction in the number of viable cells when treated with the maximum concentration of methylene blue and exposure to laser light, with the combination of the two lower in our study than previously reported by others. The levels of bacterial reduction were slightly lower for *S. moorei* as compared to *Pt. anaerobius*, however they were still significant. When compared to the other 4 bacteria in our study, *S. moorei* reported the lowest levels of bacterial kill at the lower concentrations of methylene blue used. Significant levels of bacterial reduction were achieved for *S. moorei*, proving that a combination of methylene blue and red diode laser light is effective, however higher concentrations of methylene blue may be more beneficial in eradicating this bacteria. These results are extremely positive as they suggest that treatment with red light and methylene blue, both of which have been previously used in a clinical setting, can be potentially employed as a therapeutic method to inactivate certain oral malodour associated bacteria.

Our study has demonstrated significant kills of oral malodour associated bacteria can be achieved with 665 nm laser light and methylene blue, with both low photosensitiser concentrations and low light doses.

5. Conclusion

In conclusion, the results of this study have demonstrated the photodynamic inactivation of five important oral malodour causing pathogens, namely *P. gingivalis*, *Pr. intermedia*, *F. nucleatum*, *Pt. anaerobius* and *S. moorei*. This suggests that photodynamic inactivation is a promising treatment modality for oral malodour.

The use of PDI to reduce the bacterial load of oral malodour causing bacteria would be favourable over current treatment options such as mouthwashes which serve to mask malodour or cannot be used for prolonged periods of time due to the potential effect on taste.

Additionally, the use of the Periowave diode laser as the activating light source is also favourable as it is a low power laser. This could possibly be favourable in a clinical setting as treatment times would be short, as the relatively short exposure times resulted in a significantly high level of bacterial kill and such low photosensitiser doses would stain less intensely. Although the results of this study prove to be promising, further studies are needed in order to determine whether high levels of bacterial kill can be obtained when the bacteria are in a biofilm state, as they would be when found naturally in the tongue coating.

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