Lethal photosensitisation of *Porphyromonas gingivalis* by their endogenous porphyrins under anaerobic conditions: An *in vitro* study

Abstract

Background: Lethal photosensitisation has been previously demonstrated in *Porphyromonas gingivalis*, but oxygen is considered to be essential to this process. However, since *Porphyromonas gingivalis* is a periodontal pathogen which grows in the low oxygen conditions found in the subgingival crevice, it was considered prudent to study its photosensitivity in anaerobic conditions.

Methods: A series of experiments were undertaken to attempt to induce lethal photosensitisation in *Porphyromonas gingivalis* (ATCC 33277) under strict anaerobic conditions using two different 405 nm light sources. Samples of *Porphyromonas gingivalis* were grown on a blood–containing, solid growth medium before being suspended in saline and then exposed to 405 nm light delivered by either a hand-held light source (Toothcare™) (11.4 mW/cm^2) or a laser pointer (328.5 mW/cm^2). With the exception of the adjustment of the *Porphyromonas gingivalis* suspensions to a fixed optical density, the experiments were carried out in their entirety within an anaerobic chamber.

Results: The lowest Toothcare light dose tested (0.34 J/cm^2; 30 seconds) yielded a statically significant kill of 63.4% which increased to 94.1% kill at higher light doses (3.42 J/cm^2; 300 seconds). The laser pointer similarly achieved kills of 90.2% at the lower light dose tested 9.86 J/cm^2; 30 seconds) and 94.5% kill at the highest light dose (98.55 J/cm^2; 300 seconds).

Conclusions: Lethal photosensitisation can be instigated in planktonic suspensions of *Porphyromonas gingivalis* at 405 nm delivered by hand-held devices under anaerobic conditions. This suggests the possibility that lethal photosensitisation occurred by the oxygen-independent type I pathway as oppose to the oxygen-dependent type II pathway.

Keywords: *Porphyromonas gingivalis*, lethal photosensitisation, photodynamic therapy, protoporphyrin IX, light

Introduction

Upon adsorption of a photon of light of a specific wavelength, a photosensitiser can undergo a transition from a low energy 'ground' state to an excited 'triplet' state which can then interact by one of two specific reactions with the *exterior milieu* [1]. Type I reactions (typically) involve the transfer of an electron from the substrate to the photosensitiser which results in a cationic substrate and an anionic photosensitiser, both of which can be highly reactive. In an oxygenated environment, these unstable ions can react further with biological substrates to produce reactive oxygen species including superoxide anions (O_2^-), hydroxyl radicals (·OH) and peroxide (O_2^{-2}). Type I reactions are favoured by high substrate and low oxygen concentrations, due to oxygen competing with the substrate for interaction with the photosensitiser [2]. The more common type II reactions involve the generation of singlet oxygen ($^1O_2$) radicals, whereas type I reactions produce ·OH. All of these reactive oxygen species can destructively interact with neighbouring biological molecules such as proteins, lipids [3], DNA [4] or the photosensitiser itself [5].

Lethal photosensitisation, mediated by an exogenous photosensitiser in the form of porphyrins, chlorophylls or dyes, can be used for treating pre-malignant / neoplastic disorders [6] and non-oncological conditions such as lichen planus [7] and actinic keratosis [8]. Lethal photosensitization has also been used to treat urinary tract infections [9], localised infections of the skin [10] and has shown to be effective against oral bacteria growing as a biofilm in an in vitro model [11] as well as against *Porphyromonas gingivalis* in an animal model [12]. In the case of treating bacterial infections, the effectiveness of lethal photosensitisation is largely dictated by the ability of the photosensitiser to associate with the bacterial cell, either by binding to its surface or by internalisation. However, it is not always easy to achieve such an association and the difficulties of undertaking effective lethal photosensitisation with gram-negative bacteria has been widely reported.

The problem of photosensitiser delivery is obviated in the case of a number of gram-negative anaerobic oral bacteria such as *Porphyromonas gingivalis*,

Prevotella intermedia and *Prevotella nigrescens*. These putative periodontal pathogens accumulate (relatively) large amounts of endogenous porphyrins in the form of dimeric iron protoporphyrin IX (μ-oxo bishaem) on the cell surface of *Porphyromonas gingivalis* [13] and monomeric iron protoporphyrin IX (haematin) in *Prevotella* species. [14]. These endogenous porphyrins, can act as photosensitisers and generate reactive oxygen species [15]. The presence of protoporphyrin IX on the cell surface results in black-pigmented colonies after 3 – 6 days anaerobic incubation on blood / haemin containing agars. These pigments are purported to protect these strictly anaerobic bacteria from oxygen [14] but notwithstanding this adaptation, exposure to an aerobic environment for as little as 4 minutes has been shown to be fatal to both *Porphyromonas gingivalis* and *Prevotella* species. [16]. There is a well-established link between *Porphyromonas gingivalis* and periodontal disease and it has been recently shown that even very low colonization levels of this bacterium can lead to a dynamic shift in the oral microbiota which ultimately leads to inflammatory bone loss [17]. This suggests that steps to limit the abundance of *Porphyromonas gingivalis* within the commensal oral microflora may improve periodontal health.

In the laboratory setting, digital quantitative light-induced fluorescence has been used to induce and quantify red fluorescence in *in vitro* grown biofilms of microcosm and single-species cultures of black-pigmented anaerobes [18]. These experiments demonstrated that continued exposure to 405 nm light caused significant photobleaching of the fluorophores. Since photobleaching and lethal photosensitisation have the same underlying mechanism, in that reactive oxygen species reacts destructively with biological molecules [19], it was postulated that 405 nm illuminations could potentially be used to kill these black-pigmented anaerobes *in vitro*. 405 nm light is not cytotoxic to mammalian tissue, including lens epithelial cells [20].

The aim of this study was to determine if the 405 nm light delivered by a Toothcare hand-piece and / or a laser pointer was capable of instigating lethal photosensitisation in *Porphyromonas gingivalis* under anaerobic conditions. A decision was made to obviate the confounding cytotoxic effects of oxygen upon

this strict anaerobe despite the fact that oxygen is considered requisite for lethal photosensitisation.

Materials and Methods

Microbiological Culture

Swab cultures of *Porphyromonas gingivalis* (ATCC 33277) were grown on anaerobic basal agar (Oxoid) supplemented with 5% defibrinated horse blood (TCS Biosciences, Botolph Claydon, UK) in an anaerobic chamber (80% N_2, 10% CO_2, 10% H_2) (Don Whitley, Shipley, UK) at 37ºC. After 4 – 7 days incubation, colonies were harvested from the agar using a sterile loop and suspended in 2 mL of phosphate-buffered saline (Oxoid) which had been pre-reduced by storing in an anaerobic atmosphere immediately after autoclaving. The optical density of this suspension was adjusted to 0.1 units at 650 nm (Spectrophotometer, Model 2021, Cecil Instruments, Cambridge, UK) using additional phosphate-buffered saline. Eight, 200 µL aliquots of the bacterial suspension were transferred to a 96-well plate containing micro magnetic stirrer bars. The wells were spaced out as much as possible in order to reduce the effects of stray light between the different legs of the experiment. A parallel control experiment was conducted using *Enterococcus faecalis* (NCTC 775) instead of *Porphyromonas gingivalis*.

Light Exposure

The 96-well plate containing the samples was located on top of a magnetic stirrer (Variomag mono, Fisher Scientific UK, Loughborough, UK) to ensure that the samples were well mixed during the light exposure. The plastic wave-guide was first removed from the Toothcare device so as to reveal the naked, internal light-emitting diodes. The power output of the Toothcare was measured (PM200 power meter with S121C detector head; Thorlabs Ltd, Ely, UK) at 3.2 mW. The light source was mounted in a retort stand perpendicular to the target well and touching the lid of the 96-well plate. During light exposure, the lid was removed from the plate to leave a discrete distance between light source and the surface of the liquid samples, which was nominally 5 mm. The exposure times used were 30, 60 and 300 seconds which correspond to light energy intensities (total light dose) of 0.34, 0.68 and 3.42 J/cm^2 respectively. During a single experiment, each light dose variable was conducted in duplicate and included controls that were not exposed to light. Following light exposure, the samples were serially diluted

in phosphate-buffered saline and plated onto anaerobic basal agar to enumerate the total number of viable bacteria remaining in terms of colony forming units. The experimental protocol is summarised in Figure 1. Although the cultures of *Enterococcus faecalis* used in the control experiments were similarly grown on anaerobic basal agar before exposure to Toothcare illumination, the post-illumination samples were plated onto nutrient agar (Oxoid) as this bacterium does not actually require blood supplemented media in order to enumerate the colony forming units.

A laser pointer (Shenzhen Ifoxmart Import and Export Co., Ltd, Shenzhen, China) producing a collimated, coherent 405 nm beam was also used as an alternative light source. Despite being labeled as a 10 mW model, the actual power output of the laser pointer was measured as 42.7 mW. The exposure times used were 0.5, 1 and 5 minutes which corresponded to light energy intensities of 9.86, 19.71 and 98.55 J/cm^2 respectively. The laser pointer device was held by hand during its use with the button manually depressed. New alkaline batteries were fitted to the laser pointer daily prior to its use. It should be noted that the continual operation of the laser pointer, especially under the conditions found within an anaerobic cabinet, is contrary to the manufacturer's instructions and ultimately led to the failure of the unit.

With the exception of the optical density measurements, all of the other procedures (i.e. cell harvesting, sample preparation, light exposure, serial dilution and plating) involving *Porphyromonas gingivalis* were carried out within the confines of the anaerobic chamber to protect the cells from the cytotoxic effects of oxygen. The details of the radiant properties of the Toothcare and laser pointer together with the light dosing regimens used are shown in Table 1. The experiments which employed the Toothcare and laser pointer against *Porphyromonas gingivalis* were conducted on four occasions (n = 8 due to internal duplicates) whilst the porphyrin-free control experiments with *Enterococcus faecalis* were conducted twice (n = 4).

Results

The antimicrobial effects of different 405 nm light doses upon planktonic suspensions of *Porphyromonas gingivalis* are shown in Figure 2. The lowest Toothcare light dose employed (0.34 J/cm^2; 11.4 mW/cm^2 for 30 seconds) yielded a significant antimicrobial effect with a 63.41% kill ($n = 8$, $p<0.05$; student's t-test). The maximum light dose used (3.42 J/cm^2; 11.4 mW/cm^2 for 300 seconds) corresponded with a kill of 94.11% ($n = 8$, $p<0.05$). The observed antimicrobial effect was dependent upon the light dose.

The lowest laser pointer light dose employed (9.86 J/cm^2; 328.5 mW/cm^2 for 30 seconds) yielded a significant antimicrobial effect in a 90.21% kill ($n = 4$, $p>0.05$). The maximum light dose used (98.55 J/cm^2; 328.5 mW/cm^2 for 300 seconds) corresponded with a kill of 94.50% ($n = 8$, $p<0.05$). Similarly to the Toothcare experiments, the antimicrobial effect was dependent upon the light dose. There was no statistically significant difference between the antimicrobial effects of the Toothcare and laser pen at any of the light doses employed.

The maximum light dose delivered by the Toothcare did not have an antimicrobial effect in the 'porphyrin-free' control experiments using similar cultures of *Enterococcus faecalis* ($n = 4$; $p = 0.23$).

Discussion

Enterococcus faecalis was chosen for the control experiments as a counterpoint to *Porphyromonas gingivalis* since it is a fast-growing, gram-positive, oxygen-tolerant, facultative anaerobe which does not possess the enzymes required for porphyrin biosynthesis [21] and has been used as a control in similar studies of lethal photosensitisation [22, 23]. The samples of *Enterococcus faecalis* that were exposed to the light in the experiments described herein were grown on anaerobic basal agar despite not requiring such a complex media or blood supplements. This decision was made in order to eliminate the possibility that the carry-over of material containing haemoglobin from the anaerobic basal agar growth media being responsible for any photodynamic effect. The observed

ineffectiveness of 405 nm light against *Enterococcus faecalis* corroborates a previous study [23] which observed no measurable kill at an energy density of 72 J/cm^2 (10 mW/cm^2 for 2 hours). Direct measurement of the temperature within the samples was not considered necessary due to the plethora of data which indicate that sample heating is not an issue at 405 nm, even at comparatively high light doses [22, 24-26]. Notwithstanding this, *Porphyromonas gingivalis* can grow relatively uninhibited at 43 ºC [27].

Phototoxicity due to the presence of endogenous porphyrins has been demonstrated previously in cultures of *Porphyromonas gingivalis* in a number of studies including those which employed broad-spectrum light [25, 26, 28] and monochromatic / laser light [12, 29]. Although it is difficult to directly compare the different methodologies, power densities, energy densities and incident light spectra employed in this wide variety of studies; survival fractions of the order of 1% were typically reported. Violet light (defined as 380 – 450 nm), specifically 405 nm, has been demonstrated to have the greatest photodynamic effect against *Porphyromonas gingivalis* [30]. The antimicrobial properties of 405 nm light has also been described in a range of bacteria other than black-pigmented anaerobes including; *Fusobacterium nucleatum* [26], *Pseudomonas aeruginosa*, *Staphylococcus aureus* [31], methicillin-resistant *Staphylococcus aureus* [32], *Campylobacter jejuni* [33], *Propionibacterium acnes* [34], *Helicobacter pylori* [35], *Clostridium perfringens*, *Klebsiella pneumoniae* and *Proteus vulgaris* [23]. Despite the Soret band of protoporphyrin IX being around 405 nm, this wavelength of light is avoided in anti-cancer photodynamic therapy due to its poor tissue penetration as a result of the Soret band of hemoglobin [36]. It is possible to circumvent the barrier presented by hemoglobin in the subgingival crevice by utilizing a fiber optic light source to directly illuminate the bacteria.

In the present study, it was considered important to protect the strict anaerobe, *Porphyromonas gingivalis* from the confounding effects of cytotoxic oxygen. The anaerobic chamber used in these experiments was routinely checked for the presence of oxygen using anaerobic indicator strips (Oxoid). Additionally, an *ad hoc* measurement taken using an oxygen meter (Portable O_2 meter, Analox,

Stokesley, UK) confirmed the absence of oxygen. Whilst the dissolved oxygen concentration in the 200 µl samples was not determined, mixing a volume of liquid within an oxygen-free gaseous atmosphere is analogous to well-established methods for deoxygenating water [37].

Previous lethal photosensitisation studies embarked upon by other workers using endogenous bacterial porphyrins focused on experiments executed under conditions that were not anaerobic. Although some of these studies did include a cohort of experiments that were carried out in an anaerobic environment, none of them reported a measurable kill in the absence of oxygen [22, 26, 29, 38]. One piece of work in particular went as far as to state that oxygen was 'essential' to the process [39], but their incorporation of oxygen scavengers such as ascorbic acid could have quenched the reactive molecular species produced by photodynamic reactions rather than eliminating oxygen from the system *per se*. These statements regarding the importance of oxygen contradict our findings which demonstrated light-induced cell death under anaerobic conditions. The reasons for this contradiction are unclear, but may be in part due to our use of a reducing, anaerobic environment and the selection of a gram-negative anaerobe which has a relatively large amount of an intrinsic photosensitiser (protoporpyrin IX) incorporated onto the cell surface. A recent study has demonstrated that gram-negative bacteria are more susceptible to the ·OH radicals, whose production is favoured in low-oxygen conditions, whereas gram-positive bacteria are more susceptible to $^1O_2$. Their study demonstrated lethal photosensitisation in the presence of NaN_3, which is known quench singlet oxygen meaning that the type I reaction was responsible. Although type I and type II reactions can occur simultaneously, their ratio depends upon the photosensitiser in question as well as the local microenvironment [40]. The type I mechanism has previously been proposed as the most likely source of ·OH radicals, as opposed to $^1O_2$ by the type II mechanism, in the lethal photosensitisation of *Porphyromonas gingivalis* [26].

The power densities delivered by the laser pointer (42.7 mW/cm^2) and particularly Toothcare (3.2 mW/cm^2) and were lower than the power densities

used in comparable studies which also employed 405 nm light against *Porphyromonas gingivalis* (10 to 800 mW/cm^2) [18, 23, 24, 30]. Despite their relatively low power outputs, the Toothcare was capable of delivering a 1.72 log reduction in the viability of *Porphyromonas gingivalis* suspension at an energy density of 3.42 J/cm^2, whereas the laser pen achieved 2.36 log kill at 98.55 J/cm^2. The observed antimicrobial effects of the Toothcare were commensurate with the most directly comparable study which achieved a 1.34 log reduction at an energy density of 2 J/cm^2 [24]. Another important finding of the Kotoku study was that when comparing identical energy densities, power density was far more important than time in terms of maximising the antibacterial effect – in other words; at equivalent light doses, more power for less time is more effective than less power for longer. Nevertheless, despite the relatively low power density delivered by the 405 nm light-emitting diodes incorporated into the Toothcare device, a significant antimicrobial effect was observed against *Porphyromonas gingivalis* in an *in vitro* model system under anaerobic conditions. Further work should be undertaken to elucidate the importance of oxygen to this process together with a study of the effects of digital quantitative light-induced fluorescence lighting on biofilms of *Porphyromonas gingivalis* at higher power densities. It should be noted that a number of similar 405 nm laser pens that were labeled as 10 mW (two of), 50 mW and 100 mW all had essentially the same power output of the order of 40 - 45 mW. The timeframe of the light exposure is within practicable and clinically acceptable parameters.

The blue light sources which are readily available in dental clinics, such as curing lamps, have already been proposed as a means of inducing lethal photosensitisation in susceptible oral bacteria [41]. The results of our experiments indicate that the 405 nm lighting incorporated into Toothcare can instigate lethal photosensitisation in *Porphyromonas gingivalis* and that this can be achieved under anaerobic conditions, which suggests that this is mediated by type I photochemical reactions. Further *in vitro* work is required in order to demonstrate the effectiveness of this and similar illumination regimes against oral bacteria within a multi-species biofilm under anaerobic conditions as well as

the effects of microaerophilic levels of oxygen as found in the subgingival crevice [42].

Acknowledgements

JAH and ZK contributed equally to this work as an undergraduate elective research project. The project was internally funded by the Department of Health Services Research, University of Liverpool. Steve Scribbins (University of Liverpool, School of Electronics and Electrical Engineering) and Stuart Edwardson (University of Liverpool, School of Engineering) independently measured the power output of the illumination sources.

References

[1] Ochsner M. Photophysical and photobiological processes in the photodynamic therapy of tumours. Journal of photochemistry and photobiology B, Biology. 1997;39:1-18.
[2] Yamakoshi Y, Umezawa N, Ryu A, Arakane K, Miyata N, Goda Y, et al. Active oxygen species generated from photoexcited fullerene (C-60) as potential medicines: $O_2^{-\bullet}$ versus $^1O_2$. J Am Chem Soc. 2003;125:12803-9.
[3] Grossweiner LI, Patel AS, Grossweiner JB. Type I and type II mechanisms in the photosensitized lysis of phosphatidylcholine liposomes by hematoporphyrin. Photochem Photobiol. 1982;36:159-67.
[4] Bertoloni G, Lauro FM, Cortella G, Merchat M. Photosensitizing activity of hematoporphyrin on Staphylococcus aureus cells. Biochimica et biophysica acta. 2000;1475:169-74.
[5] Chen TS, Zeng SQ, Zhou W, Luo QM. A quantitative theory model of a photobleaching mechanism. Chinese Physics Letters. 2003;20:1940-3.
[6] Copper MP, M T, Tan IB, Ruevekamp MC, Stewart FA. Photodynamic therapy in the treatment of multiple primary tumours in the head and neck, located to the oral cavity and oropharynx. Clinical Otolaryngology. 2007;32:185-9.
[7] Aghahosseini F, Arbabi-Kalati F, Fashtami LA, Fateh M, Djavid GE. Treatment of oral lichen planus with photodynamic therapy mediated methylene blue: a case report. Med Oral Patol Oral Cir Bucal. 2006;11:E126-9.
[8] Tschen EH, Wong DS, Pariser DM, Dunlap FE, Houlihan A, Ferdon MB. Photodynamic therapy using aminolaevulinic acid for patients with nonhyperkeratotic actinic keratoses of the face and scalp: phase IV multicentre clinical trial with 12-month follow up. Br J Dermatol. 2006;155:1262-9.
[9] Wainwright M, Stanforth A, Jones R, Loughran C, Meegan K. Photoantimicrobials as a potential local approach to geriatric UTIs. Letters in Applied Microbiology. 2010;50:486-92.
[10] Hamblin MR, O'Donnell DA, Murthy N, Contag CH, Hasan T. Rapid control of wound infections by targeted photodynamic therapy monitored by in vivo bioluminescence imaging. Photochem Photobiol. 2002;75:51-7.
[11] Wilson M, Burns T, Pratten J. Killing of Streptococcus sanguis in biofilms using a light-activated antimicrobial agent. JAntimicrobChemother. 1996;37:377-81.
[12] Komerik N, Nakanishi H, MacRobert AJ, Henderson B, Speight P, Wilson M. In vivo killing of Porphyromonas gingivalis by toluidine blue-mediated photosensitization in an animal model. Antimicrobial Agents and Chemotherapy. 2003;47:932-40.
[13] Smalley JW, Silver J, Marsh PJ, Birss AJ. The periodontopathogen Porphyromonas gingivalis binds iron protoporphyrin IX in the mu-oxo dimeric form: an oxidative buffer and possible pathogenic mechanism. Biochemical Journal. 1998;331:681-5.
[14] Smalley JW, Silver J, Birss AJ, Withnall R, Titler PJ. The haem pigment of the oral anaerobes Prevotella nigrescens and Prevotella intermedia is composed of iron(III) protoporphyrin IX in the monomeric form. Microbiology. 2003;149:1711-8.
[15] Zenkevich E, Sagun E, Knyukshto V, Shulga A, Mironov A, Efremova O, et al. Photophysical and photochemical properties of potential porphyrin and chlorin

photosensitizers for PDT. Journal of Photochemistry and Photobiology B-Biology. 1996;33:171-80.
[16] Bradshaw DJ, Marsh PD, Watson GK, Allison C. Oral anaerobes cannot survive oxygen stress without interacting with facultative/aerobic species as a microbial community. Letters in Applied Microbiology. 1997;25:385-7.
[17] Hajishengallis G, Liang S, Payne MA, Hashim A, Jotwani R, Eskan MA, et al. Low-Abundance Biofilm Species Orchestrates Inflammatory Periodontal Disease through the Commensal Microbiota and Complement. Cell Host & Microbe. 2011;10:497-506.
[18] Hope CK, de Josselin de Jong E, Field MR, Valappil SP, Higham SM. Photobleaching of red fluorescence in oral biofilms. J Periodontal Res. 2011;46:228-34.
[19] Tong-Sheng CS-Q, Z.; Wei, Z.; Qing-Ming, L. A quantitative theory model of a photobleaching mechanism. Chinese Physics Letters. 2003;20:1940-3.
[20] Andley UP, Lewis RM, Reddan JR, Kochevar IE. Action spectrum for cytotoxicity in the UVA-wavelength and UVB-wavelength region in cultured lens epithelial-cells. Investigative Ophthalmology & Visual Science. 1994;35:367-73.
[21] Frankenberg L, Brugna M, Hederstedt L. Enterococcus faecalis heme-dependent catalase. Journal of Bacteriology. 2002;184:6351-6.
[22] Henry CA, Dyer B, Wagner M, Judy M, Matthews JL. Phototoxicity of argon laser irradiation on biofilms of Porphyromonas and Prevotella species. J Photochem Photobiol B. 1996;34:123-8.
[23] Maclean M, MacGregor SJ, Anderson JG, Woolsey G. Inactivation of bacterial pathogens following exposure to light from a 405-nanometer light-emitting diode array. Applied and environmental microbiology. 2009;75:1932-7.
[24] Kotoku Y, Kato J, Akashi G, Hirai Y, Ishihara K. Bactericidal effect of a 405-nm diode laser on *Porphyromonas gingivalis*. Laser Physics Letters. 2009;6:388-92.
[25] Soukos NS, Som S, Abernethy AD, Ruggiero K, Dunham J, Lee C, et al. Phototargeting oral black-pigmented bacteria. Antimicrob Agents Chemother. 2005;49:1391-6.
[26] Feuerstein O, Ginsburg I, Dayan E, Veler D, Weiss EI. Mechanism of visible light phototoxicity on Porphyromonas gingivalis and Fusobacterium nucleatum. Photochem Photobiol. 2005;81:1186-9.
[27] Chen W, Honma K, Sharma A, Kuramitsu HK. A universal stress protein of Porphyromonas gingivalis is involved in stress responses and biofilm formation. Fems Microbiology Letters. 2006;264:15-21.
[28] Loe H, Silness J. Periodontal disease in pregnancy. I. Prevalence and severity. Acta odontologica Scandinavica. 1963;21:533-51.
[29] Henry CA, Judy M, Dyer B, Wagner M, Matthews JL. Sensitivity of Porphyromonas and Prevotella species in liquid media to argon laser. Photochem Photobiol. 1995;61:410-3.
[30] Fukui M, Yoshioka M, Satomura K, Nakanishi H, Nagayama M. Specific-wavelength visible light irradiation inhibits bacterial growth of Porphyromonas gingivalis. J Periodontal Res. 2008;43:174-8.
[31] Guffey JS, Wilborn J. In vitro bactericidal effects of 405-nm and 470-nm blue light. Photomed Laser Surg. 2006;24:684-8.

[32] Enwemeka CS, Williams D, Hollosi S, Yens D, Enwemeka SK. Visible 405 nm SLD light photo-destroys methicillin-resistant Staphylococcus aureus (MRSA) in vitro. Lasers in surgery and medicine. 2008;40:734-7.
[33] Murdoch LE, Maclean M, MacGregor SJ, Anderson JG. Inactivation of Campylobacter jejuni by exposure to high-intensity 405-nm visible light. Foodborne Pathog Dis. 2010;7:1211-6.
[34] Ashkenazi H, Malik Z, Harth Y, Nitzan Y. Eradication of Propionibacterium acnes by its endogenic porphyrins after illumination with high intensity blue light. Fems Immunology and Medical Microbiology. 2003;35:17-24.
[35] Ganz RA, Viveiros J, Ahmad A, Ahmadi A, Khalil A, Tolkoff MJ, et al. Helicobacter pylori in patients can be killed by visible light. Lasers in Surgery and Medicine. 2005;36:260-5.
[36] Hanson EK, Ballantyne J. A blue spectral shift of the hemoglobin soret band correlates with the age (time since deposition) of dried bloodstains. PLoS One. 2010;5:e12830-e.
[37] Butler IB, Schoonen MAA, Rickard DT. Removal of dissolved-oxygen from water - a comparison of 4 common techniques. Talanta. 1994;41:211-5.
[38] Izzo AD, Walsh JT. Light-induced modulation of Porphyromonas gingivalis growth. Journal of photochemistry and photobiology B, Biology. 2004;77:63-9.
[39] Maclean M, Macgregor SJ, Anderson JG, Woolsey GA. The role of oxygen in the visible-light inactivation of Staphylococcus aureus. Journal of photochemistry and photobiology B, Biology. 2008;92:180-4.
[40] Huang L, Xuan Y, Koide Y, Zhiyentayev T, Tanaka M, Hamblin MR. Type I and Type II mechanisms of antimicrobial photodynamic therapy: An in vitro study on gram-negative and gram-positive bacteria. Lasers in Surgery and Medicine. 2012;44:490-9.
[41] Bouillaguet S, Wataha JC, Zapata O, Campo M, Lange N, Schrenzel J. Production of Reactive Oxygen Species from Photosensitizers Activated with Visible Light Sources Available in Dental Offices. Photomed Laser Surg. 2010;28:519-25.
[42] Mettraux GR, Gusberti FA, Graf H. Oxygen tension (pO2) in untreated human periodontal pockets. JPeriodontol. 1984;55:516-21.

Figures Legends and Tables

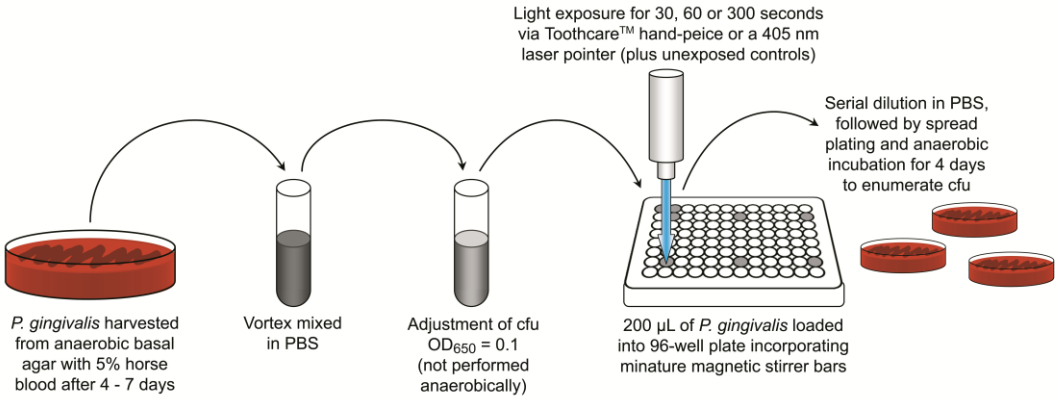

Figure 1. Experimental procedure detailing the exposure of planktonic cultures of *Porphyromonas gingivalis* to 405 nm light. With the exception of the optical density adjustment, all of the procedures were undertaken under anaerobic conditions.

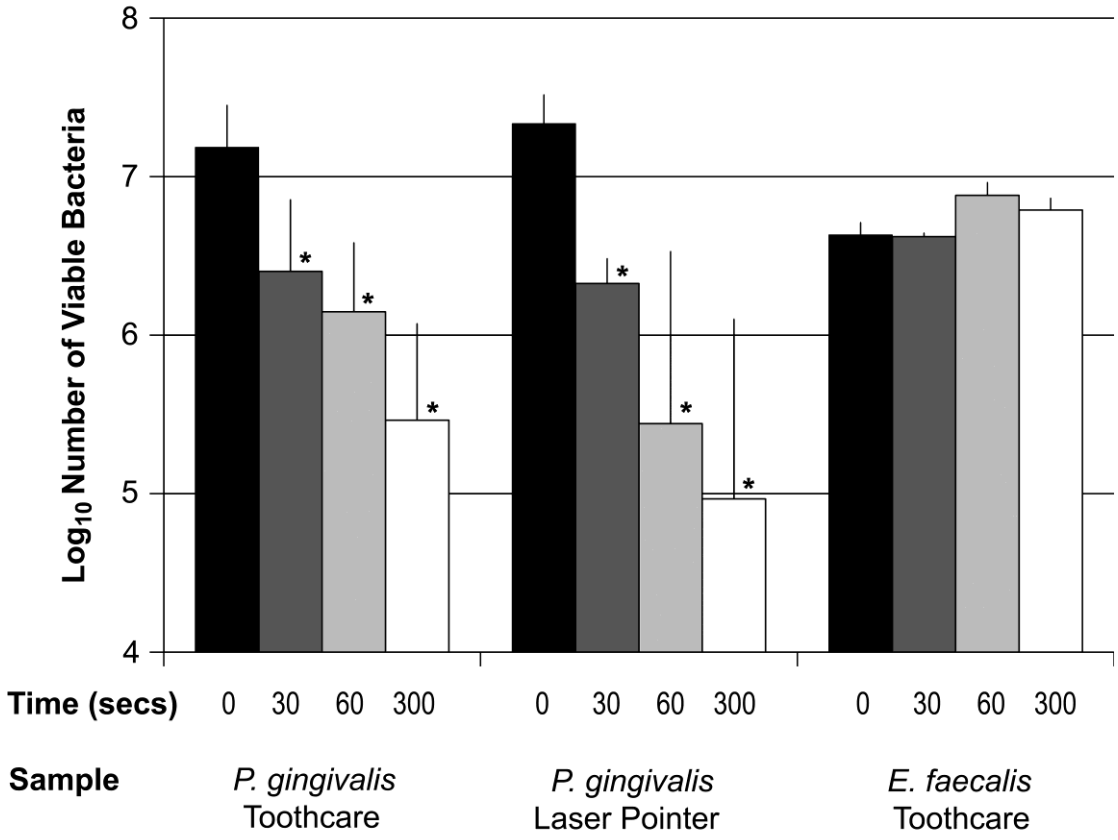

Figure 2. Viable counts of bacteria following exposure to 405 nm light. The light was delivered by Toothcare (11.4 mW/cm^2) or a laser pointer (328.5 mW/cm^2). Error bars show 95% confidence intervals (n=8; *Porphyromonas gingivalis*, n=4; *E. faecalis*), * indicates statistically significant kill ($p<0.05$) compared with unilluminated controls.

Table 1. Radiant properties of the Toothcare and laser pointer

	Toothcare	**Laser pointer**
Irradiance (mW/cm^2)	11.4	328.5
Spot size (cm^2)	0.28	0.13
Maximum radiant flux (mW)	3.2	42.7

Exposure Time (s)	**Energy Density (J/cm^2)**	
30	0.34	9.86
60	0.68	19.71
300	3.42	98.55