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The Susceptibility of Anaerobic Bacteria Isolated from Periodontal Diseases to Photodynamic Inactivation with Fotolon (Chlorin e6)

ZUZANNA DRULIS-KAWA[1,2*], ARTUR BEDNARKIEWICZ[3], GABRIELA BUGLA-PŁOSKONSKA[1], WIESŁAW STRĘK[3] and WŁODZIMIERZ DOROSZKIEWICZ[1]

[1] Institute of Genetics and Microbiology, University of Wrocław, Przybyszewskiego 63/77, 51-148 Wrocław, Poland
[2] Lower Silesian Centre of Paediatrics in Wrocław, Kasprowicza 64/66, 51-147 Wrocław, Poland
[3] Institute of Low Temperatures and Structure Research, Polish Academy of Sciences, Okólna 2, 50-422 Wrocław, Poland

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Abstract

Photodynamic inactivation (PDI) may be a very promising alternative method for the antimicrobial treatment of periodontitis. Several studies have demonstrated the sensitivity of subgingival flora to PDI using toluidine blue, methylene blue, and chlorin e6 derivatives. In the present study we report the activity of the Fotolon sensitizer, composed of chlorin e6 and polyvinylpyrrolidone (PVP), against anaerobic bacteria isolated from periodontal diseases. Over 99.9% reduction in colony forming units in 20 Gram-positive and 30 Gram-negative clinical anaerobic strains was obtained.

Key words: photodynamic inactivation, Fotolon (chlorin e6), anaerobic bacteria

Introduction

Periodontal diseases result from the accumulation of bacterial plaque on the tooth surface. The occurrence of anaerobic bacteria such as *Porphyromonas gingivalis*, *Prevotella* sp., *Treponema* sp., *Veillonella* sp., *Bacteroides* sp., *Capnocytophaga* sp., and *Actinomyces* sp. in periodontal pockets results from the accumulation of metabolic products. Later, inflammation begins together with posterior destruction which leads to periodontal disease. The common treatments of periodontitits involve mechanical removal of the biofilm (plaque) and antimicrobial chemotherapy (Sbordone *et al.*, 1990; Slot, 1979; Wirkström *et al.*, 1993). However, the overuse of antibiotics favours the natural selection of drug-resistant oral pathogens (Kleinfelder *et al.*, 1999). The alternative method of periodontal disease treatment could be photodynamic inactivation (PDI). To be efficient, PDI requires a proper dose of light delivered to the appriopriate amount of photo sensitizer (PS) accumulated inside the bacteria or on their cell walls. The (laser) light illumination leads to the formation of reactive singlet oxygen or/and redox reactions (Wainwright, 1998). The effects of PDI on microorganisms depend on the structure of the PS and the incubation time of the drug with bacteria cells. PDI can cause damage to the cell wall and increase cytoplasmic membrane permeability and nucleic acid strand breakage (Wainwright, 1998; Jacob and Hamann, 1975; Menezes *et al.*, 1990). The photodynamic eradication of oral and wound pathogens (localised infection) seems to be a very promising technique (Bertoloni *et al.*, 1990; Embleton *et al.*, 2002; Hamblin *et al.*, 2002; Hamblin *et al.*, 2003; Merchat *et al.*, 1996; Soncin *et al.*, 2002; Soukos *et al.*, 1996). Several studies have demonstrated the sensitivity of subgingival flora to PDI using cationic charged photosensitizers: toluidine blue (Dortbudak *et al.*, 2001; Kömerik *et al.*, 2003; Sarkar and Wilson, 1993; Wilson, 1994), methylene blue (Chan and Chern-Hsiung, 2003), and poly-L-lysine- chlorin e6 conjugates (Rovaldi *et al.*, 2000; Soukos *et al.*, 1998).

* Corresponding author. Mailing address: Dr. Zuzanna Drulis-Kawa, Institute of Genetics and Microbiology, University of Wrocław, Przybyszewskiego 63/77, 51-148 Wrocław, Poland, Tel./fax: +48-71-325-21-51, E-mail: kawa@microb.uni.wroc.pl

In the present study we demonstrate that Fotolon (chlorin e6), having been successfully used in diagnosing tumours and in treating surface tumours as well as mucous and open organs (Parkhots *et al*., 2003), could also be applied in the photodynamic inactivation of local oral infections.

Experimental

Materials and Methods

Microorganisms. In our study, 50 anaerobic clinical strains isolated from periodontal infections were used. These were: *Prevotella oralis* (n = 8), *Prevotella denticola* (n = 7), *Leptotrichia buccalis* (n = 4), *Veillonella* sp. (n = 11), *Actinomyces* sp. (n = 4), *Clostridium* sp. (n = 4), *Eubacterium* sp. (n = 7), *Peptostreptococcus* sp. (n = 4), and *Propionibacterium* sp. (n = 1). The microbiological investigations were performed at the Bacteriology Department of the Lower Silesian Center of Paediatrics in Wrocław. The samples were taken from the periodontal pockets of patients. The growth of anaerobic bacteria was carried out on Schaedler Agar +5% Sheep Blood plates in an anaerostat Genbox System (bioMerieux, France) at 35°C for 7 days. The bacteria were identified on the basis of microscopic morphology in Gram-stained preparations and their metabolic properties using ID32 A tests (bioMérieux).

Photosensitizer. Fotolon (Haemato-Polska, Wrocław, Poland) is a complex of the trisodium salt chlorin e6 and polyvinylpyrrolidone (PVP). It is a water-soluble, lyophilic, dry substance of green-black colour. Chlorin in suspension tends to be unstable and it aggregates quickly when applied without PVP, leading to a decrease in bioavailability. PVP in an aqueous medium forms hydrophobic cavities into which various molecules can be built (Parkhots *et al.*, 2003). The admixture of PVP to chlorin e6 increases quantum yield and fluorescence lifetime compared with chlorin e6 in 0.9% sodium chloride alone, indicating some changes in the local environment and reduced aggregation of chlorin e6. Nevertheless, absorption spectra measured with freshly prepared Fotolon solutions and those several days/weeks old showed the Soret band shifting towards red (data not shown), decreasing in intensity and increasing in its full width at half-maximum in old solutions. This is a clear indication of polimerisation. To keep Fotolon's bioavailability high, a PS solution in 0.9% sodium chloride was prepared just before the experiments. The manufacturer of Fotolon recommends intravenous application of 2.5–3 mg of PS per kg of body weight. The calculated PS dose is dissolved *ex tempore* in 100 ml of 0.9% sodium chloride and administered by i.v. infusion over 30 minutes. The initial solution used for cancer treatment is around 1.7 mM of chlorin e6 and is still safe (Parkhots *et al.*, 2003).

Photobleaching monitoring. Photobleaching of the Fotolon solution was experimentally monitored by means of quantitative absorption and fluorescence spectra measurements while illuminating the solution with a laser diode. Ten miligrams of Fotolon was dissolved in 1 ml of 0.9% sodium chloride, then dilutions were made to obtain concentrations of $c_{e6} = 31$ mg ml^{-1} and $c_{4xe6} = 125$ mg ml^{-1}. Four ml of the Fotolon solution was then placed in an absorption-emission 10.00 mm quartz cuvette in a cuvette holder dedicated to absorption and emission spectra. Absorption and fluorescence were measured with an OceanOptics SD2000 two-channel miniature spectrophotometer. The absorption spectra in the 450–800 nm range were measured with an LS-1 tungsten-halogen miniature lamp after subtraction of dark current and proper baseline correction. The fluorescence was monitored by the second channel of the spectrophotometer under 405 nm excitation with an LED (Light Emitting Diode from Roithner-Laser-Technik, Austria). The excitation light (651 nm), perfectly matching the absorption spectrum of Fotolon in 0.9% sodium chloride, was delivered through a fibre to the second transparent surface of the cuvette. A Coherent FieldMaster with an LS-10 head shadowed with a 0.5 cm^2 hole was used to measure the light flux (power density), which was fixed at 500 mW cm^{-2} at the cuvette surface for the photostability experiments.

Light source. A pigtailed laser diode (LaserSecura, Wroclaw, Poland) was used as an excitation source emitting at 651 nm, exactly matching the Q absorption band maximum of chlorin e6 (Fotolon) in 0.9% sodium chloride. The microtitration plate was fixed about 4 cm above the tip of the laser fibre. A light power meter (Coherent, Field Master) equipped with a measuring head (Coherent, LM-10) shielded with a 0.5 cm^2 diaphragm was used to obtain a light flux of 250 mW cm^{-2} by diode current changes. Sixty seconds of illumination was used, giving a total light dose of 15 J cm^{-2}. Photostability experiments have shown that this light dose does not completely photobleach the chlorin e6 solution and, in fact, a higher (*e.g.* ≥30 J cm^{-2}) light dose could be used for further enhancement of PDI. The manufacturer of Fotolon recommends delivering a minimum of 300 J cm^{-2} at a power density of 200–300 mW cm^{-2} for efficient photodynamic therapy of cancer. The power density used (250 ± 10 mW cm^{-2}) is high enough to increase the local temperature; however, no cfu decreases were noted compared with untreated control groups.

Experimental procedure. After 48 h of incubation (stationary phase) on Schaedler Agar + 5% Sheep Blood plates in anaerobic conditions, the bacteria were diluted in 0.9% sodium chloride to the optical density of McFarland No 0.5. One hundred µl of bacterial culture at an approximate concentration of 10^7 cells ml^{-1} was placed in the wells of a flat-bottom 96-well microtitration plate in triplicate. Five µl of chlorin e6 solution was added to each well to a final chlorin e6 concentration of 50 µg ml^{-1} (84 µM). Before the 60-sec illumination, the bacteria were incubated for 5 min in the dark at room temperature. To determine the number of colony-forming units (cfu), aliquots of 10 µl were taken from each well, serially diluted in 0.9% sodium chloride, and placed on Schaedler Agar + 5% Sheep Blood. The anaerobic bacteria were cultured in an anaerostat Genbox System at 35°C for 3 days in the dark. The respective controls were bacteria strains (i) treated with neither PS nor light, (ii) exposed to light in the absence of PS, and (iii) treated with PS but not illuminated.

To determine the optimal experimental conditions, preliminary tests were carried out with various concentrations of e6: (i) 50 µg ml^{-1} (84µěM) with 30 and 60 s of illumination; (ii) 25 µg ml^{-1} (42 µM) with 30 and 60 s of illumination; (iii) 2.5 µg ml^{-1} (4.2 µM) with 30 and 60 s of illumination; and (iv) 50 µg ml^{-1} , 25 µg ml^{-1}, and 2.5 µg ml^{-1} with 60 s of illumination and 5 min of incubation. Two clinical isolates were taken for preliminary tests: the *Prevotella oralis* strain 9 as a Gram-negative and *Actinomyces naeslundii* strain 52 as a Gram-positive representative were used.

Statistical analysis. Statistical analysis was performed using the t-test for independent samples (Statistica 5.0 StatSoft, Polska).

Results

Since PDI requires short incubation and illumination times with limited light flux in order not to sensitise or kill eukaryotic cells, it is potentially useful to increase the PS concentration to obtain PDI-induced cfu reduction. However, applying high doses of photosensitizer in PDI experiments may reduce PDI efficiency due to self-shielding. Increasing the light flux is another way to increase the efficiency of PDI. In cancer treatment with Fotolon the suggested light flux is no less than 200–300 mW cm^{-2} (Parkhots, 2003). To assess the self-shielding effect experimentally we carried out Fotolon photobleaching studies. Figure 1 presents the photobleaching of the Fotolon 0.9% NaCl solution. Absorption was monitored as intensity of Q-band at 653nm and normalised to the absorption coefficient for the not irradiated sample. The emission was monitored as luminescence intensity of Q-band at 660 nm when excited with 405 nm by Light Emitting Diode and normalized to luminescence intensity for the not irradiated sample. Saturation of the absorption curve starts at 30 J cm^{-2}, indicating that delivering more than 30 J cm^{-2} will not have any therapeutic effect due to the lack of active PS molecules (Fig. 1). It can also be noticed that increasing the PS concentration reduces the photobleaching rate, and about 60 J cm^{-2} was needed to complete photobleaching of the PS.

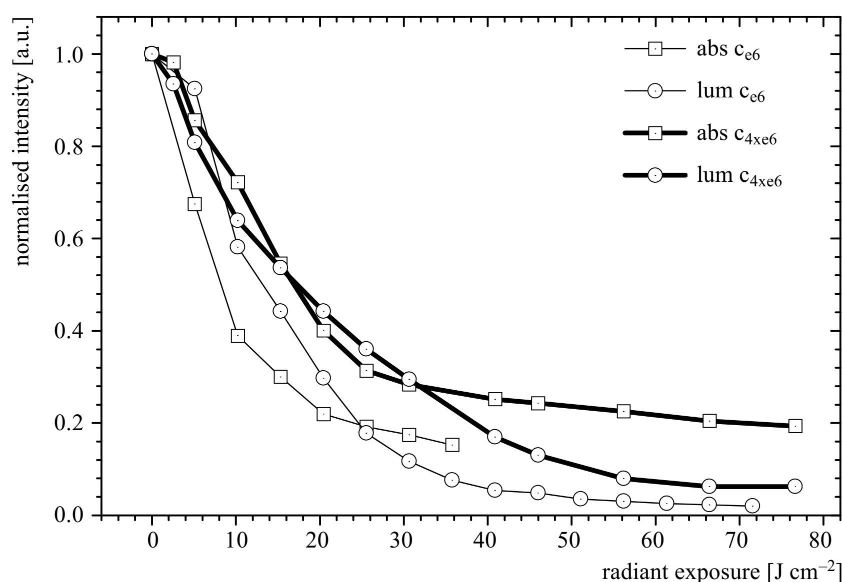

Fig. 1. The photobleaching of the Fotolon 0.9% NaCl solution

Normalized absorbance (rectangles) and emission (circles) intensity versus the radiant exposure delivered to the Fotolon solution (31 μg ml^{-1} – thin lines and 125 μg ml^{-1} – bold lines)

In the preliminary anti-bacterial tests (Fig. 2) in the three control groups (i) treated neither with PS nor light, (ii) exposed to light in the absence of PS, and (iii) bacteria treated with PS but not illuminated, no changes of the viable count were observed. The smallest survival fraction (a decrease by 5 orders of magnitude) was obtained for the chlorin e6 in concentration of 50 μg ml^{-1}, 60 s of illumination, and 5 min of incubation. The *Actinomyces naeslundii* strain 52 was similarly sensitive to both the 25 μg ml^{-1} and the 50 μg ml^{-1} PS concentrations. In the case of *Prevotella oralis* isolate 9, the best antimicrobial effect was exhibited by the PDI with 5 min of incubation. Therefore these conditions (60s of activation preceded by 5 min of incubation with PS concentration 50 μg ml^{-1}) were used for all further experiments on the clinical strains.

The survival fractions of the tested clinical strains are shown in Fig. 3. There were no differences observed among the three control groups in spite of the relatively high light flux applied. The presented data are the number of colony forming units (cfu) normalised to the respective initial number of cfu to compare the different strains easily. The behaviour of the clinical strains could not be predicted from the Gram-positive or Gram-negative character of the bacteria. Among the Gram-negative isolates, the *Prevotella oralis* strains were the most sensitive (4–5 log of cfu decrease). Statistically significant susceptibility variations ($p<.05$) were observed for the *P. oralis* strains 9 and 32. The *Prevotella denticola* and *Leptotrichia buccalis* strains showed various susceptibilities to PDI with Fotolon, maintaining decreases in cfu of 2–4 orders of

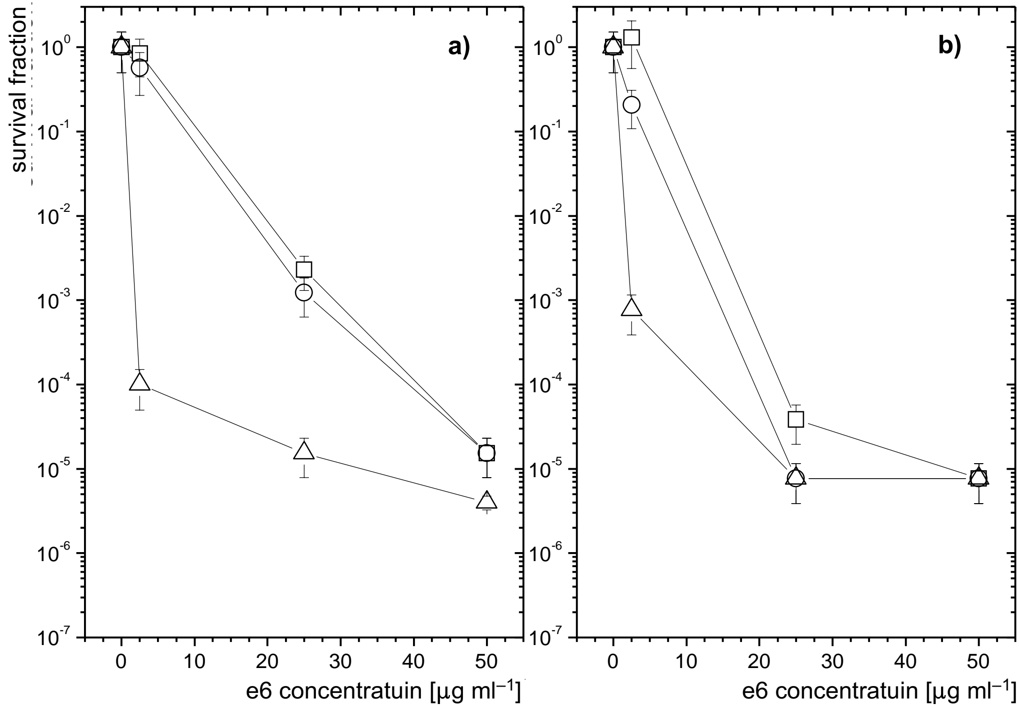

Fig. 2. Effect of the incubation time and photosensitizer concentration on phototoxicity against (a) *Prevotella oralis* and (b) *Actinomyces naeslundii*

Rectangles describe 30s of PS activation, circles describe 60s of PS activation and triangles 60s of activation preceded by 5min of incubation.

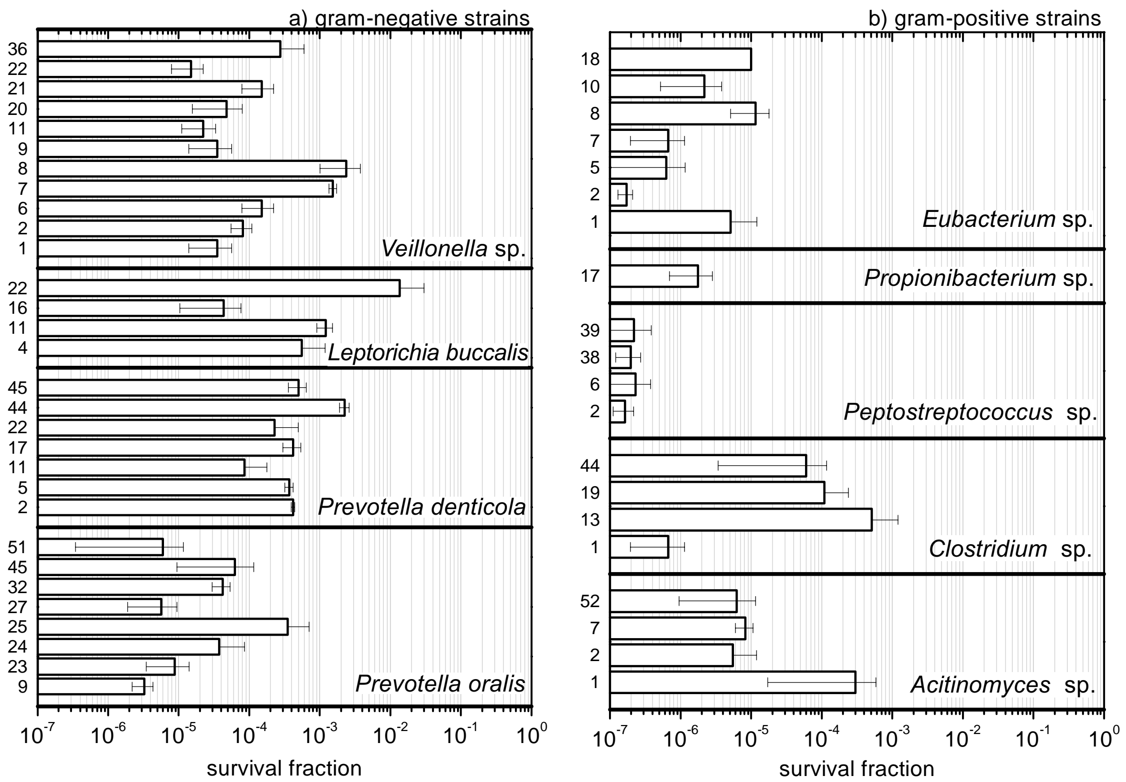

Fig. 3. Effect of the Fotolon PDI on (a) Gram-negative, (b) Gram-positive clinical anaerobic isolates

The data are normalized to respective control groups' cfu and then averaged over two experiments. Data error bars are standard deviation. Digits beside bars denote the isolated strains.

magnitude. Among *Prevotella denticola*, isolate 44 was significantly ($p<.05$) more resistant to PDI than were strains 2, 5, 11, 17, and 22. The same dependency was observed between isolates 2 and 11. Statistically significant susceptibility variations ($p<.05$) were noticed for the 11 and 16 *Leptotrichia buccalis* strains. Most of the *Veillonella* sp. isolates demonstrated similar susceptibility of 3–4 log cfu reduction, but only one isolate, 7, was significantly ($p<.05$) more resistant than the remaining isolates. We found that the clinical strains of *Clostridium* sp. and *Actinomyces* sp. behaved similarly to Gram-negative bacteria, with level decreases of 3–4 and 3–5 orders of magnitude, respectively. No significant susceptibility differences were observed between these two bacterial groups. The other Gram-positive isolates (*Eubacterium* sp., *Propionibacterium* sp., and *Peptostreptococcus* sp.) were more susceptible and showed 4–6 log of cfu reduction. Statistically significant resistance ($p<.05$) was noticed for 18 compared with the 2, 5, 7 and 10 *Eubacterium* sp. strains.

Discussion

The spectroscopic investigation of the photostability of Fotolon exhibited a lack of reciprocity between light flux and treatment time. According to Wainwright (Wainwright, 1998), high power density over a short time period may give different anti-microbial effects from those of low power density over a longer time even though the light dose is the same in both cases. The smaller photobleaching rates for solutions with higher chlorin e6 concentrations can be explained by the self-shielding effect. When the dye concentration becomes higher, the distance travelled by the excitation light is reduced due to its loss in intensity down to e^{-1} of the initial value (defined by the absorption coefficient a). In such a case, superficial layers of the dye absorb the light very efficiently and thus block its penetration into deeper layers. As the photobleaching of superficial dye layers proceeds, these layers become transparent while deeper layers still strongly absorb the light. This is why radiant exposures for a highly concentrated photosensitizer solution may be underestimated, leading to reduced PDI efficiency. During the antibacterial experiments, 60-sec illumination was used, giving a 15 J cm^{-2} total light dose. This light dose does not completely photobleach chlorin e6 solution and, in fact, a higher light dose (*e.g.* ≥30 J cm^{-2}) could be used for further enhancement of PDI.

Several studies have demonstrated the efficacy of toluidine blue, methylene blue, and chlorin e6 derivatives in the photodynamic inactivation (PDI) of subgingival flora. Soucos (Soukos *et al.*, 1998), Rovaldi (Rovaldi *et al.*, 2000), and Pfitzner (Pfitzner *et al.*, 2004) determined the antimicrobial activity of poly-L-lysine chlorin e6 conjugates and new photosensitizers BLC 1010, BLC 1014 on anaerobic bacteria compared with pure chlorin e6. We have compared our results gained during preliminary tests with a concentration of 2.5 μg ml^{-1} (4.2 μM) chlorin e6 (see Fig. 2) with those of pure chlorin e6 obtained by the three other groups. As one could expect, the Gram-positive bacterial strains exhibited higher susceptibility to PDI with chlorin e6 than did the Gram-negative bacteria. This indicates that the crucial attack points of reactive species generated during photodynamic action are located on the cell cover , regardless of whether Gram-positive or Gram-negative bacteria were treated.

We have also performed experiments in which much higher concentrations of the PS were used than in experiments performed by Soukos (Soukos *et al.*, 1998), Rovaldi (Rovaldi *et al.*, 2000), and Pfitzner (Pfitzner *et al.*, 2004). Since (i) Fotolon is not phototoxic up to 10 mg kg^{-1} of body weight, (ii) it does not accumulate in normal tissue up to 1 hour after administration, and (iii) the only “affinity” to bacteria comes from the shorter time needed for the PS compound to destroy crucial bacterial cell structures (mostly the cell membrane) compared with eukaryotic cells, there is no argument against using Fotolon concentrations as high as 80–100 μM of chlorin e6. The highest concentration used by us was in fact the concentration of Fotolon infusion solution for standard cancer treatment. Both Gram-positive and Gram-negative bacteria were sensitive to PDI with Fotolon in higher concentration however, clinical isolates displayed various PDI susceptibilities. In our opinion, besides standard strains, larger clinical strain collections should be tested to assess the therapeutic effects of photosensitizers. The amount of cfu reduction after PDI was 3 to 6 logs (viable count <0.01%) and 2 to 5 logs (viable count < 0.1%) for 20 Gram-positive and 30 Gram-negative wild strains, respectively. A reduction in cfu of over 99.9% is regarded as bactericidal and sufficient for bacteria eradication.

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