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A Possible Mechanism for Visible Light-Induced Wound Healing

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Background and Objectives: Chronic wounds resistant to conventional therapy have been treated successfully with low energy lasers and light emitting diodes (LEDs) in the visible and near IR region. It has been proposed that production of low level reactive oxygen species (ROS) following illumination is the first step of photobiomodulation. It was also shown that white light (400–800 nm) has similar stimulatory effects as lasers and LEDs. ROS at higher levels are toxic to cells and bacteria.

Study Design/Materials and Methods: In the present study, we examined the phototoxicity of broadband (400–800 nm, 120 J/cm^2) visible light on the survival of several pathogenic bacteria: *Staphylococcus aureus 195*, *Pseudomonas aeruginosa 1316*, *Escherichia coli 1313*, and *Serratia marcescens*. These bacteria were chosen due to their high prevalence in infected wounds. The survival of bacterial cells following illumination was monitored by counting the number of colony forming units before and after exposure to light.

Results: Illumination with white light, 120 J/cm^2, caused a reduction of 62%, 83%, and 56% in the colony count of *E. coli 1313*, *S. aureus 195* and *S. marcescens,* respectively, though no reduction in the viability of *P. aeruginosa 1316* was demonstrated. The phototoxic effect was found to involve induction of ROS production by the bacteria. It was also found that illumination of *S. aureus 195* and *E. coli 1313* in the presence of pyocyanin, known to be secreted by *P. aeruginosa,* had a stronger bactericidal effect compared to illumination alone.

Conclusion: Visible light at high intensity can kill bacteria in infected wounds. Thus, illumination of infected wounds with intense visible light, prior to low intensity illumination for stimulating wound closure, may reduce infection and promote healing. Lasers Surg. Med. 40:509–514, 2008. © 2008 Wiley-Liss, Inc.

Key words: wound healing; bacterial infections; illumination; ROS

INTRODUCTION

Phototherapy, the use of light to induce healing, has recently received increased interest. This modality, as demonstrated in the use of laser light and light emitting diode (LED) technology, has been shown to be beneficial for a wide and diverse array of indications, including the healing of chronic and acute wounds [1].

The stimulatory effects of low energy laser (LEL) irradiation on cell activation have been largely demonstrated in vitro in a variety of cell lines. For example, studies with fibroblasts and keratinocytes indicate that specific relatively low energy doses of He–Ne laser or 780 nm diode laser, induce accelerated mitosis [2]. Recently, green light (570 nm) was found to enhance fibroblast growth impaired by high glucose levels [3].

Human studies with laser light have demonstrated greater amounts of epithelialization resulting in wound closure, and stimulation of skin graft healing [1,4].

It was reported by Whelan et al. [5] that a 50% faster healing of wounds was shown when treated with light from an LED array with 3 wavelengths combined in a single unit (670, 720, and 880 nm).

Visible and near IR light can be absorbed by cellular photosensitizers such as cytochromes, flavins/riboflavins and NADP [6]. Light absorption by photosensitizers causes their excitation and subsequent relaxation by transferring electrons to O_2, thereby generating reactive oxygen species (ROS) [7]. ROS are probably best known in biology for their ability to cause oxidative stress. This phenomenon is exploited in photodynamic therapy (PDT), where cancer cells are loaded with exogenous photosensitizers and irradiated by lasers in the visible range. However, low ROS fluxes play an important role in the activation and control of many cellular processes, such as the release of transcription factors, gene expression, muscle contraction and cell growth [8,9].

In a recent study [10], we tried to identify the endogenous photosensitizers responsible for ROS production by visible light. We used electron paramagnetic resonance (EPR) coupled with the probe trapping technique, to monitor oxyradical produced in various cell cultures as a function of illumination wavelength. We found that oxyradicals were created mainly by the flavins at 400–500 nm range of visible light [10]. Wavelengths above 500 nm probably stimulate the cell by accelerating the mitochondrial

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respiratory electron transfer, as first suggested by Karu [11].

Because the absorption bands of cytochrome oxidase and flavoproteins are quite wide, we hypothesed [6] that white light can replace expensive lasers and LEDs for photobiostimulation. In our previous studies [12], we demonstrated that a non-laser multiwave light source in the visible range (white light) is able to induce ROS and enhance fibroblast proliferation similarly to LEL. This broadband visible light device was also used successfully to treat patients with chronic ulcers after failure of conventional treatment [13]. It is assumed that visible light, when irradiated at high intensities, induces high concentrations of ROS. This assumption led us to test the efficacy of visible light irradiation on bacterial eradication. In a previous study [14], we found that broadband visible light, 300 mW/cm^2, can reduce viability of *Staphylococcus aureus*, in the absence of exogenous photosensitizors. This led to our hypothesis that the augmentation of wound healing induced by visible light may also be a result of wound sterilization.

In the present work, we studied the lethal effect of high intensity white light on *Serratia marcescens*, *Pseudomonas aeruginosa 1316*, *S. aureus 195*, and *Escherichia coli 1313*, which predominate in wound infections. In addition to the usual endogenous bacterial photosensitizers like flavins and porphyrins, which are responsible for light induced ROS formation, some bacterial species secrete other metabolites such as prodigiosin and pyocyanin, produced by *S. marcescens* and *P. aeruginosa*, respectively, which absorb light in the visible range (absorption maxima at 537 nm for prodigiosin and 690 nm for pyocyanin [15,16]), and might act as photosensitizers. We therefore further examined the effect of visible light illumination on *S. aureus 195*, *P. aeruginosa 1316* or *E. coli 1313* in the presence of pyocyanin, and the ability of pyocyanin to produce ROS upon irradiation.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Clinical isolates of *S. aureus 195*, *E. coli* strain 1313 and *S. marcescens* (obtained from the Clinical laboratories of the Meir Medical Center, Kfar-Saba, Israel) were examined in this study, as well as ATCC strain 1316 of *P. aeruginosa*.

The bacterial strains were grown overnight on plates containing Nutrient Agar (NA) (Difco, Detroit, MI, USA) at 37°C for *E. coli 1313* and *S. aureus 195*, or 30°C for *S. marcescens* and *P. aeruginosa 1316*.

For *S. aureus* 195 or *E. coli 1313*, 1–2 colonies from each strain were transferred from NA into 20 ml Nutrient Broth (NB) (Difco). The initial optical density of each culture at 660 nm was between 0.1 and 0.13. The cultures were grown aerobically at 37°C with aeration until they reached the middle logarithmic phase at an OD of 0.5 at 660 nm.

S. marcescens and *P. aeruginosa 1316* were grown in a similar manner, but with several changes: *S. marcescens* was grown in 50 ml of NB for at lest 6 h (end of logarithmic phase), *P. aeruginosa 1316* was grown in 100 ml of "Pseudomonas P" medium Difco), for 24 hours. Both bacteria were grown at 30°C in the dark to enable pigment production.

Pyocyanin Extraction

Cultures of *P. aeruginosa 1316* were allowed to grow for 24 hours in Pseudomonas P medium. Pigment was extracted according to Wagner et al. [17]. Briefly, pyocyanin was extracted with chloroform from supernatant (5 ml) of an overnight culture grown in liquid media; the chloroform layer (1 ml) was removed, and an additional 1.5 ml of chloroform was added, and extracted with 0.5 ml of 0.025 nmol/L HCl-H_2O. The aqueous layer was removed and 5 μl of 10 nmol/L NaOH added. Aliquots were transferred to a cuvette, and absorbance at 690 nm was measured in a spectrophotometer.

Light Sources

A homemade setup consisting of a halogen lamp equipped with appropriate filters for irradiation in the white (400–800 nm) region was used in these experiments. The halogen lamp was coupled to an optical fiber. The distance between the light source and the exposed sample surface was adjusted to obtain controlled power densities. The light intensity was measured with a power meter (Ophir, Jerusalem, Israel).

Light Exposure

For *S. marcescens*, *E. coli 1313, S. aureus 195*, or *P. aeruginosa 1316*, 1 ml of bacterial suspension was exposed to illumination with the white light source (power density of 400 mW/cm^2) for 5 minutes (equivalent to light flux of 120 J/cm^2). For *S. marcescens*, the culture used was of end log-phase, and was diluted to obtain an OD of 0.5 at 660 nm. Cells were illuminated for 1 minute, under a light flux of 24 J/cm^2. Bacterial cultures grown or maintained under the same conditions but without light exposure served as controls.

In the experiments where pyocyanin extracts were used, samples of *S. aureus 195* or *E. coli 1313* were mixed with the extracted pyocyanin at 1:1 volume ratio and incubated in the dark for 1 hour. Next, 1 ml of the suspension was exposed to the maximum output of the light source (power density of 400 mW/cm^2) for 5 minutes under aerobic conditions. Light flux was equivalent to 120 J/cm^2.

Determination of Bacterial Survival

The survival of bacterial cells following illumination was monitored by counting the number of viable colony forming cells after exposure of the suspended bacteria to light. This was performed by counting the number of colony forming units (CFUs) after appropriate dilution on agar plates, incubation overnight, and calculating the number of colonies per ml of irradiated suspension. Bacterial cultures grown under the same conditions but without light exposure served as controls. The survival rate was calculated according to the equation:

$$\frac{-(N_0 - N)}{N_0} \times 100 = \text{CFU percentage difference}$$

where N_0 is the number of CFU per ml at time zero and N is the number of CFU per ml in the samples following illumination. All experiments were repeated at least three times.

Spin Trapping Measurements Coupled With Electron Paramagnetic Resonance (EPR) Spectroscopy

To detect ROS, the EPR-spin trapping technique coupled with the spin trap, DMPO (Sigma, Milwaukee, WI, USA) was used. Samples containing 8×10^6 bacterial cells per ml with 0.02 M DMPO were drawn by a syringe into a gas-permeable Teflon capillary (Zeus Industries, Raritan, NJ) and inserted into a narrow quartz tube that was open at both ends. Then the tube was placed into the EPR cavity, and spectra were recorded before and after illumination (5 minutes, 400 mW/cm^2 white light) through the EPR cavity on a Bruker EPR 100d X-band spectrometer. The EPR measurement conditions were as follows: Frequency, 9.75 GHz; power, 20 mW; scan width, 65 G; resolution, 1024; receiver gain, 2×10^5; conversion time, 81.92 milliseconds; time constant, 655.36 milliseconds; number of scans, 2.

Temperature Measurement During Illumination

Temperature measurements for each light source and medium were performed using a thermocouple (MRC, Holon, Israel).

Statistical Analysis

All the experiments were repeated at least three times, and their means were used for data analysis. Statistical significance was calculated by ANOVA one-way test.

RESULTS

Effect of Illumination With Broadband Visible Light on the Viability of *E. coli 1313*, *S. aureus 195*, *P. aerugionosa 1316*, and *S. marcescens*

We first wished to determine whether exposure to white light had bactericidal effects. Illumination with white light (400–800 nm) at 24 and 120 J/cm^2 produced a dose-dependent effect on *S. marcescens* viability. A 56%, reduction in viability was measured after 5 min illumination with white light at 120 J/cm^2 (Fig. 1). Illumination for 1 minute (24 J/cm^2) resulted in a reduction of only 24.5% (results not shown). White light (400–800 nm) illumination resulted in a reduction of viability of 83% and 62% for *S. aureus 195* and *E. coli 1313*, respectively. However, no effect was observed *for P. aeruginosa 1316* (mid log phase bacteria) which was tested under the same illumination conditions (Fig. 1).

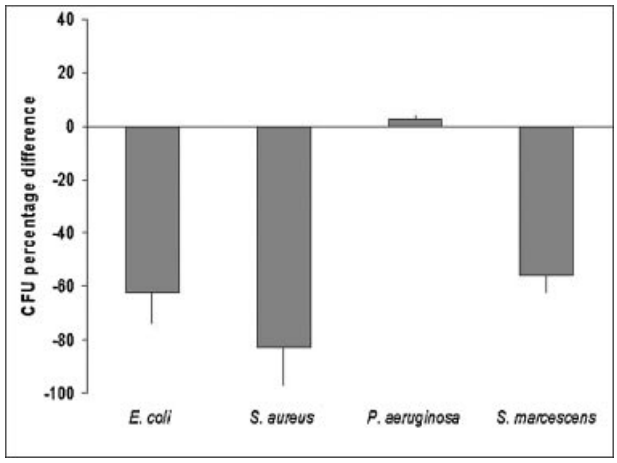

Fig. 1. The effect of broadband visible light illumination on *S. aureus 195*, *E. coli 1313*, *S. marcescens*, and *P. aeruginosa 1316*. Viability was determined by colony forming units. Each value represents the mean of at least three experiments.

Effect of Light Exposure on *S. aureus 195*, *E. coli 1313*, or *P. aeruginosa 1316* in the Presence of Pyocyanin

Mid log phase suspensions of *E. coli 1313*, *P. aeruginosa 1316* or *S. aureus 195* were incubated with pyocyanin extracted from a 24-hour-old culture of *P. aeruginosa 1316*, and bacterial viability following exposure to light (120 J/cm^2) in the presence or absence of pyocyanin was measured.

As can be seen in Figure 2, illumination with white light (400–800 nm) in the presence of pyocyanin resulted in viability reduction of 97 and 92% for *S. aureus 195* and *E. coli 1313* respectively, whereas in cultures illuminated without incubation with pyocyanin, the reduction was 83% and 62% for *S. aureus 195* and *E. coli 1313*, respectively. No effect of illumination was observed for *P. aeruginosa 1316*. Bacterial viability was not changed following incubation with pyocyanin in the dark (Fig. 2). The differences between light exposure and light exposure plus pyocyanin were found to be significant only for *E. coli 1313* and *P. aeruginosa* with $P<0.001$, but not for *S. aureus*.

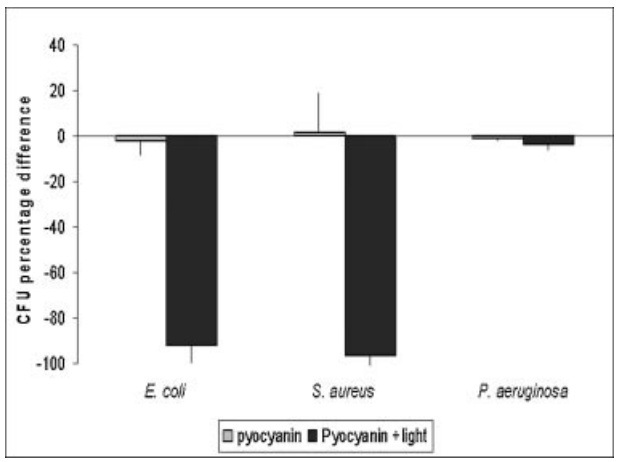

Fig. 2. Effect of light exposure on *S. aureus 195*, *E. coli 1313*, or *P. aeruginosa 1316* in the presence of pyocyanin. Viability was determined by colony forming units. Each value represents the mean of at least three experiments.

Light Induced ROS by Bacteria

The EPR spectrum of illuminated *E. coli 1313* (Fig. 3), revealed four peaks characteristic of DMPO-OH adduct, which can be assigned to formation of hydroxyl and superoxide radicals [18,19]. Illumination of *S. aureus 195* resulted in a similar spectrum. No detectable EPR signals were found in illuminated *P. aeruginosa 1316*.

Figure 4b presents the typical EPR spectrum of the pyocyanin radical in preilluminated pyocyanin extracts. In Figure 4a EPR signals of DMPO-OH can be seen, indicating formation of hydroxyl or superoxide radical by pyocyanin following illumination.

Temperature Changes During Illumination

The temperature elevation was measured by placing each light source at a distance of 0.5 cm from the suspension. Plates were always illuminated when placed on a white surface. The maximum recorded temperature elevation was 4°C under the maximal energy dose (240 J/cm^2), of the white light illumination. These results suggest that in the present experiments, the decrease in bacterial viability during illumination is not due to over-heating of the medium, but rather to the phototoxic effect.

DISCUSSION

The microflora of leg and foot ulcers is usually polymicrobial, the predominant bacteria being *S. aureus* (present in 34% of wounds), *Pseudomonas aeruginosa* (21%), *Streptococcaceae* (30.2%), *Enterobacteriaaceae* (48.4%), and *E. coli* (8%) [20–25].

Hansson et al. [26] observed that 86% of ulcers with no clinical signs of infection nevertheless contained more than one bacterial species.

Intravenously delivered broad-spectrum antibiotics and surgical debridement are the methods most often used to treat the infected wounds [27]. However, the effectiveness of antibiotic treatment is decreasing, due to the development of resistant strains [28]. On the other hand, surgical treatment has the disadvantage of being highly invasive, and is impractical in many cases.

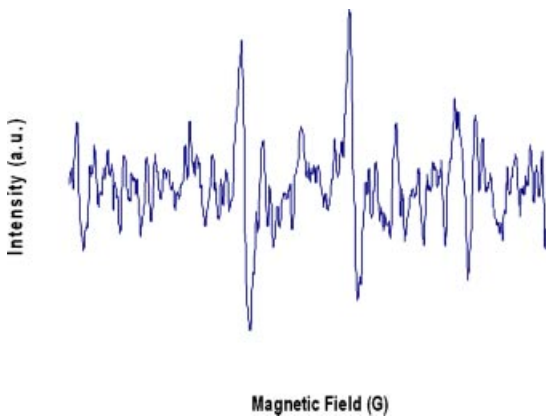

Fig. 3. ROS formation in irradiated *E. coli 1313*.

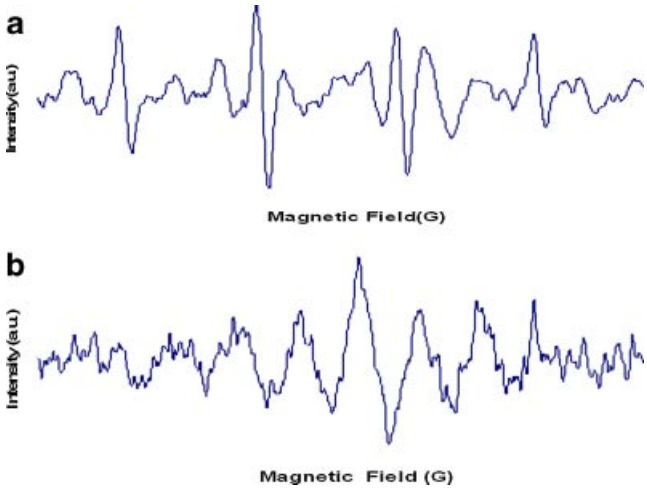

Fig. 4. Visible light induced ROS production by pyocyanin: (**a**) following illumination; (**b**) before illumination.

An alternative approach for killing bacteria present in topical wounds is by lethal photosensitization, that is, the use of photodynamic therapy (PDT), which was suggested some years ago [29–31]. In PDT, ROS generated by an illuminated photosensitizer introduced exogenously into the bacteria induce a toxic effect [32–34]. This photochemical treatment has been used successfully in vitro [7]. However, its main disadvantage is the difficulty of introducing photosensitizers into certain bacteria [7]. It should also be noted that introducing photosensitizers into an infected wound is problematic, as the photosensitizer could also be taken up by the surrounding mammalian cells, resulting in their death.

As bacteria contain endogenous photosensitizers, that absorb light throughout the visible region [35], we recently studied the effect of white light on the survival of two *S. aureus* clinical isolates and found that illumination of *S. aureus* strains *101* and *500* with intense broadband visible light (without exogenous photosensitizors) results in ROS generation and reduces cell viability [14]. In contrast, low intensity light increased their viability, that is, induced a biostimulatory effect.

In the present study, it was determined whether light-induced ROS might help to reduce bacterial growth in irradiated infected wounds, thereby assisting wound healing. To this end, the bactericidal effect of visible light was measured on bacterial strains known for their predominance in wound infections. For the above purposes, a broadband visible light source (400–800 nm), 20–120 J/cm^2, was used for illumination of *S. aureus 195*, *E. coli 1313*, *P. aeruginosa 1316*, and *S. marcescens*.

It was shown that illumination of *S. aureus 195*, *E. coli* 1313, and *S. marcescens* with intense white light (energy dose of 120 J/cm^2) resulted in a reduction of 83%, 62%, and 56%, respectively, in their viability. It should be mentioned that the energy doses needed for bacterial killing are much higher than those needed to induce skin cell proliferation (4 J/cm^2) [36].

No effect was observed in illuminated cultures of *Pseudomonas*. This is in agreement with other studies finding *P. aeruginosa* to have high resistance to oxidative stress [37].

The EPR study, using the spin trap, DMPO, revealed the formation following irradiation, of a quartet which indicates superoxide and hydroxyl radical formation, in *E. coli 1313* (see Fig. 3), *S. marcescens* and *S. aureus 195* (not shown), which can explain their sensitivity to light. No production of ROS was found in illuminated *P. aeruginosa 1316*.

DMPO is a common spin probe which detects $^{\cdot}OH$ by forming the spin adduct, DMPO-OH. The DMPO can also trap $O_2^{\cdot -}$ to produce the spin adduct, DMPO-OOH. Nevertheless, since the latter is unstable, it decomposes to the DMPO-OH adduct [18,38].

P. aeruginosa is one of the predominant bacteria in leg and foot ulcers and it also secretes pyocyanin, a pigment which absorbs visible light [16]. The ability of pyocyanin, extracted from *P. aeruginosa 1316*, to produce ROS upon irradiation, was studied. As can be seen in Figure 4b, pyocyanin is a radical itself, with 7 typical EPR signals [39]. Upon irradiation, a quartet DMPO-OH signal indicating hydroxyl and superoxide radicals is observed (Fig. 4). It should be noted that the relative intensities of the quartet lines are distorted, possibly due to the EPR lines of the pyocyanin itself. Despite the fact that pyocyanin generates ROS upon irradiation, *P. aeruginos 1316* is resistant to light-induced toxicity (Fig. 1). Nevertheless, irradiation of *S. aureus 195, or E. coli 1313 in* the presence of pyocyanin, decreased their viability in comparison to light alone (see Figs. 1 and 2). The extended decrease in viability of *E. coli* in the presence of pyocyanin and light, was significant ($P<0.001$).

It is also known that *S. marcescens* secretes prodigiosin, a red pigment with a strong absorption band in the visible range, which was found to photosensitize cancerous cells [38]. Based on these results, it is probable that light-induced bacterial killing is not only due to endogenous bacterial photosensitizors, but also a result of the existence of various photosensitizing metabolites secreted by the bacteria.

It can be concluded that visible light, at higher intensities (around 400 mW/cm^2, 120 J/cm^2) than those needed for cellular activation, could be used for bacterial eradication. The bactericidal effect is mediated by production of high levels of hydroxyl and superoxide radicals.

When using light irradiation for wound healing, intense visible light at high flux may be useful for bacterial eradication. Then the light intensity can be lowered to about 4 J/cm^2 in order to stimulate growth factors and fibroblast proliferation for the healing process. The high intensity visible light may also stimulate human cells to secrete connecting factors like collagen, leading to faster closure of the wounds [40]. This ability of high intensity light is used widely in photo-rejuvenation.

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