T.3: Light induced therapy for inactivation of bacteria and enhancement of wound healing

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Abstract

Light-induced therapy provides much better selectivity compared to the more established treatment modalities because the treatment is localized to light-irradiated regions. It involves the administration/application of a light-activable drug, which is preferentially retained by the tumor cells or diseased tissue. The drug, when excited with light of appropriate wavelength, leads to generation of cytotoxic level of reactive oxygen species (ROS), killing the target cells. Another clinically relevant application of this approach; Antibacterial photodynamic therapy (APDT), has drawn increasing attention from the scientific society in the last one decade for its potential to effectively kill multidrug-resistant pathogenic bacteria and low tendency to induce drug resistance that bacteria can rapidly develop against traditional antibiotic therapy. But, the effect of APDT on wound healing is not clearly established. There are even concerns of oxidative damage to inflammatory and other cells of wound site by APDT induced ROS. At LBAS, RRCAT we have focused our attention on the possibility of use of light induced therapeutic effects for inactivation of bacteria and promotion of healing. In the present article, I shall provide an overview of the salient outcomes of various investigations undertaken to prove whether light induced therapeutic approaches are able to elicit beneficial effects on wound healing.

1. Introduction

In today's society, chronic wounds represent a major health care burden [1]. Normally wounds heal in about 2-3 weeks and the healing has four overlapping phases: coagulation, inΔammation, proliferation, and remodeling [2]. Abnormality in any of these phases, caused by either systemic factors like vascular insufficiency, diabetes, neuropathy, or local factors such as pressure and infection of wounds can cause delay in healing. These factors, coupled with the growing emergence of antibiotic resistance in bacteria commonly infecting wounds, is a big concern for diabetic, immunocompromised individuals and also in ulcers of patients undergoing radiation therapy [3-5]. In India, because of the rise in diabetes population, the incidences of chronic ulcers are expected to increase further.

Chronic wound management is complicated and pose huge economic burden. In spite of the multidisciplinary approaches undertaken such as glycemic control, local care, antibiotic therapy and surgical revascularization, chronic ulcers require long time to heal. A variety of pharmacological interventions in form of antimicrobials and antiseptics are also available. However, because of the possible adverse side effects, much reduced efficacy for virulent factors and presence of biofilms in wounds, there is urgent need to develop new modalities that can attenuate bacteria, their virulent factors and promote healing. Some of the approaches currently being explored are (i) antimicrobial peptides, (ii) efflux pump inhibitors, and (iii) antimicrobial photodynamic therapy (APDT). Of these, APDT is particularly attractive, because, while bacteria are expected to develop resistance mechanisms against the other approaches, the possibility of developing resistance against APDT is considered remote [6, 7] .

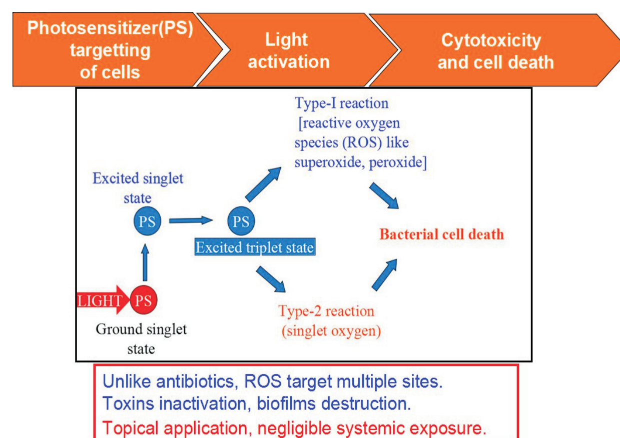

Fig. T.3.1: Principle of Antimicrobial Photodynamic Therapy (APDT). PS: Photosensitizer

APDT involves excitation of an exogenously applied or endogenously synthesized photosensitizer (PS) by visible light [8]. This can lead to generation of lethal level of singlet oxygen via energy transfer to molecular oxygen present in the wound tissue, referred to as type II process. The other process contributing to the inactivation of microbes, the type I process, involves generation of radicals through electron transfer to nearby molecules (*Fig. T.3.1*). In contrast to the conventional antibiotics that are designed to target a specific bacterial site, in APDT, the generated reactive oxygen species

(ROS) destroy all cellular components. In addition, PDT can inactivate wide spectrum of microbe strains, virulent factors [9], biofilms [10] and attenuate proinflammatory cytokines [11].

APDT efficacy depends upon the nature of PS and the composition, charge of the bacterial envelope. Gram positive bacteria cell wall constitutes a peptidoglycan wall constituting molecules like LTA, negatively charged teichuronic acids, and displays a relatively high degree of porosity. Macromolecules with a molecular weight of 30–60 kilodalton (kDa) can diuse into the inner plasma membrane through the peptidoglycan wall, which does not act as a permeability barrier for the most commonly used PS such as phenothiazines and porphyrins, with molecular weight generally in the range of 1.5–1.8 kDa. In contrast, Gram negative bacteria contains an additional outer membrane outside the peptidoglycan layer, which shows an asymmetric lipid structure composed of anionic LPS, saccharine, protein and proteins with transport function (porins). This layer inhibits the penetration of compounds with MW >700 Da across the outer envelope of these bacteria .

The important parameters that an ideal PS for APDT should have include (i) high ROS quantum yield, (ii) cationic charge as well as amphiphilic nature to ensure higher accumulation at the polyanionic microbial cell surfaces [12] and (iii) a strong absorbance in the red region to minimize the energy deposition in tissue. A cationic PS is preferentially taken up by bacterial cells over human cells , because bacterial outer cell envelope has predominantly anionic phospholipids and molecules. Mammalian cell membranes, in contrast, have mostly zwitterionic phospholipids, cholesterol [13]. Amphiphilic PS is preferred over hydrophobic or hydrophilic PS because this helps in partitioning of the cationic amphiphilic PS into microbial membrane. This has been proven in studies comparing the efficiency of synthetic meso substituted cationic porphyrin with tetra-, tri-, di- or monocationic charges on bacterial inactivation.

Cationic phenothiaziniums have been the most widely investigated photosensitive drugs currently being investigated for APDT [14, 15]. However, these have relatively lower triplet yield [14], hydrophilic nature, and efflux pump dependent uptake into bacterial cells [15]. Therefore, there is interest in exploring other drugs. Chlorophyll derivatives like chlorine p6 (cp6) are attractive because of good ROS yield, amphiphilicity and strong absorbance in the red (660 nm) region [16]. Since cp6 is anionic, its conjugation with cationic peptides like poly-l-lysin (pl-cp6) is expected to enhance the targeting of the cp6 to bacterial cells.

2. Use of APDT for bacteria inactivation and enhancement of wound healing

It is known that both hyperglycemia and oxidative stress can directly activate cell adhesion, pro and anti-inflammatory molecules, enzymes, growth factors in cells [17]. Thus, APDT induced ROS generation can generate many beneficial response in wounds. Also, bacterial cell degradation products released upon APDT induced bacterial cell destruction can generate chemotactic response for inflammatory cells [18] which can modulate innate immunity. However, the light fluence and PS concentration that would maximize bacterial inactivation and inflammatory cell accumulation simultaneously in wounds need to be carefully optimized. Therefore, we carried out detailed studies the photobactericidal efficacy of APDT for both Gram positive and negative bacteria and investigated the use of APDT for the healing of bacteria infected and uninfected wounds in normal and diabetic mice.

2.1 Photobactericidal efficacy of APDT induced by photoactivable drug and light

A disadvantage of many of the PS used currently for APDT is that these act on a single class of bacteria and do not have the desired broad spectrum activity. While Gram positive bacteria are photosensitive to both anionic and cationic photosensitizers, Gram negative bacteria are less susceptible to photoinactivation by anionic drugs due to the structural complexity of cell envelope [19]. We investigated the phototoxicity of cp6, and its poly-l-lysine conjugate (pl-cp6 on methicillin resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa* and HaCaT, a keratinocyte cell line, used as a model system for host cells.

Results show that singlet oxygen and other ROS yield of cp6 is not diminished significantly due to its conjugation to poly-l-lysin. As expected, the uptake of pl-cp6 observed to be higher than cp6 for all the bacteria strains studied. This leads to much higher photobactericidal efficacy of pl-cp6 than that of cp6 *(Fig. T.3.2A)*. Also, for these bacteria, photokilling efficacy of pl-cp6 is independent of growth phase, which is a

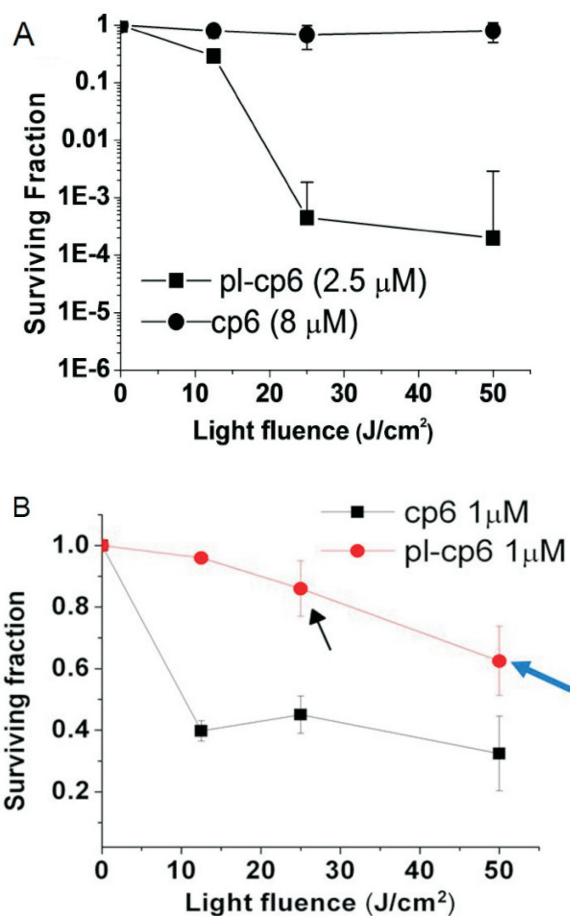

Fig. T.3.2: Photobactericidal efficacy and selectivity of APDT induced by pl-cp6 and red light (660 nm ± 25 nm). (A): Surviving fraction of P.aeruginosa treated with cp6 or pl-cp6. (B): Phototoxicity in HaCaT (keratinocyte cells). Cells were incubated with drugs for 15 min followed by exposure to red light.

desirable feature, since in wound milieu bacteria may be in different growth phases. Furthermore, our results show, for PS concentration and light fluence resulting in 2-3 log survival loss in bacteria, sub lethal (~ 20 %) level of survival loss was observed in HaCaT cells (keratinocytes) , suggesting selectivity of this approach *(Fig. T.3.2B)*.

2.2. Mechanism of bacterial cell membrane damage induced by APDT

The interaction of photosensitizer with bacterial cell envelop plays a critical role in the initiation of photoinactivation process [20]. Hence, observation of morphological changes on the cell surface induced by the photodynamic treatment provides useful information for understanding the mechanism of action of different PS in different types of bacteria. Changes in cellular morphology resulting from APDT induced by cp6, pl-cp6 and a standard phenothiazinium, toluidine blue (TBO) were monitored using atomic force microscopy (AFM). Fluorescence microscopy of cell impermeable dye (Propidium iodide) uptake and

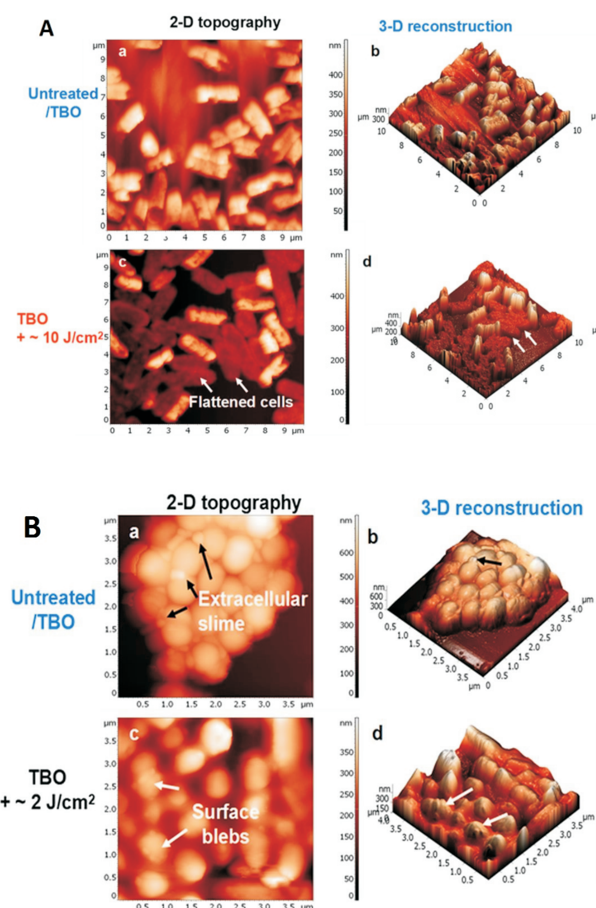

Fig. T.3.3: Photosensitization induced morphological alteration in bacteria. (A): AFM images of gram negative bacteria E. coli cells treated with 25 μM TBO alone (a & b) and irradiated with light fluence of 10 (c & d). Scan area: 10 m x 10 m.(B): AFM images of S. aureus cells treated with 25 μM TBO alone (a & b) and irradiated with light (c & d). Scan area: 4 m x 4 m.

absorbance spectroscopy were used to monitor damage to cell membrane and leakage of intracellular contents, respectively.

APDT mediated by both TBO and pl-cp6 resulted in a significant reduction in the mean cell height, flattening of Gram negative bacteria; *P.aeruginosa, and E.coli* (Fig T3.3.A). These observations suggest damage to the bacterial membrane and reduction of cell volume due to the loss of cytoplasmic materials [21]. For *S.aureus*, the change in morphology observed subsequent to APDT mediated by TBO and pl-cp6 was significantly different. There were blebs in outerwall of *S.aureus* subjected to APDT mediated by TBO indicating breakage in the contact between the cell wall and the membrane (Fig T.3.3 B). In contrast, pl-cp6 mediated APDT resulted in damage primarily to outer peptidoglycan layer.

2.3. Effect of topical APDT on healing of *P. aeruginosa* infected wounds of mice.

P. aeruginosa is a common opportunistic Gram negative bacterium. This bacterium produces several extracellular virulent factors [22] which can cause hyperinflammatory response, delayed collagen remodeling, bloodstream invasion, and dissemination. In addition, *P.aeruginosa* can easily form biofilms on ulcers. Considering the intrinsic resistance of this bacterium to many antibiotics, there is a need to develop alternate antimicrobial approaches for treatment of wound infections caused by this pathogen.

As shown previously by us, APDT induced by pl-cp6 and red light (660 nm ± 25 nm) leads to ~4 log of *P.aeruginosa in vitro*. Therefore, it may be expected that that pl-cp6 mediated APDT can be a good alternative for treatment of infections of wounds caused by this bacterium. Previously, studies of our group [23] and others have shown that APDT can inactivate bacterial virulent factors [24] which are known stimulators of proinflammatory cytokines. In contrast, PDT is known to induce release of proinflammatory mediators and matrix metalloproteinases in tumors [18]. Therefore, in bacteria infected wounds, which already has a hyperinflammatory condition, APDT is expected have profound influence. So, apart from monitoring the decrease in bacterial load, the levels of different cytokines like interleukin-6 (IL-6), Tumor necrosis factor-α (TNF-α) were measured to quantitate the effect of APDT on the inflammatory response.

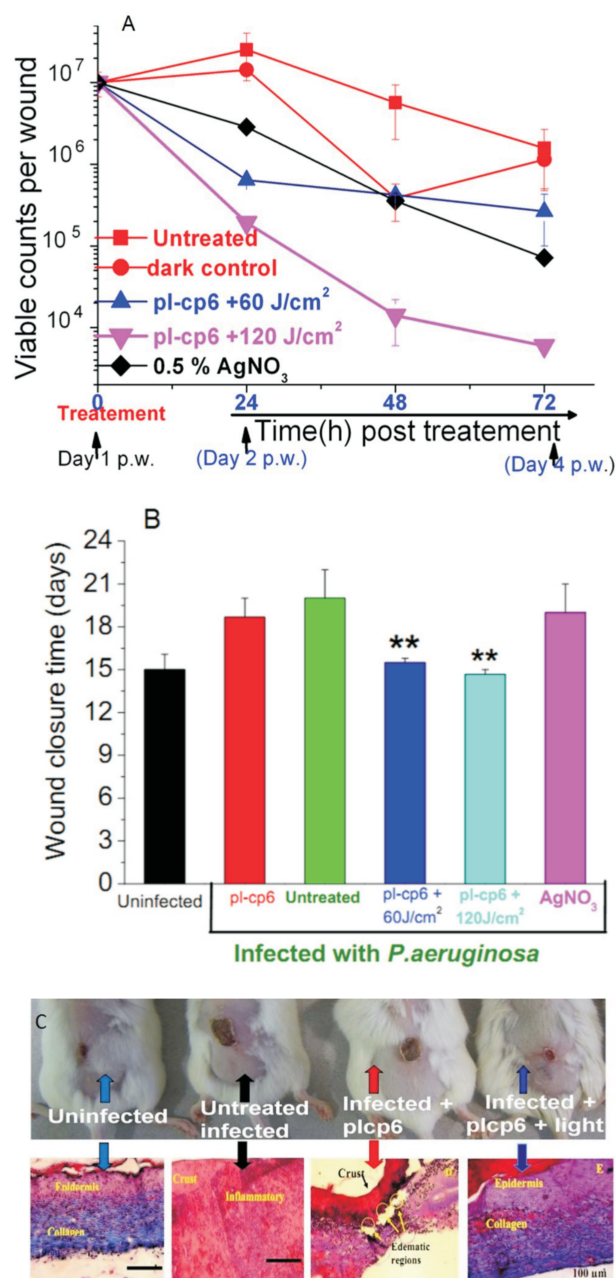

*Fig. T.3.4: Bactericidal (A) and wound healing efficacy (B) of APDT versus silver nitrate ($AgNO_3$) in wounds infected with P.aeruginosa. **: $p < 0.05$ comparison of means between the untreated and photodynamically treated wounds. (C): Enhanced inflammation resolution in wounds subjected to APDT.*

Our results also showed that 96 hr post APDT, bacteria load (*Fig. T.3.4*), IL-6 and, TNF-α level in the infected wounds were reduced by ~1.5 log , ~6 times and ~ 4 times, respectively [25]. A further mechanistic study revealed that this attenuation of hyperinflammatory response is observed because of the down regulation of inflammatory stress response master controller, Nuclear Factor Kappa B and Toll like receptor-4 in wounds post APDT [26]. There was also increase in levels of markers of cell proliferation in wounds, which may also have contributed to the observed faster healing (*Fig. T.3.4 B &C*). It may be further inferred from the results that efficacy of APDT is better compared to $AgNO_3$, a standard antibacterial agent *(Fig T.3.4 B)*. This may be because of enhanced inflammation resolution in wounds subjected to APDT as observed by histology *(Fig T.3.4 C)*.

2.4. Effect of topical APDT on collagen remodeling response of MRSA or *P.aeruginosa* infected wounds of mice.

S. aureus is a major cause of community acquired and nosocomial infections. It employs a wide variety of defensive mechanisms to develop antibiotic resistance and produces many virulent factors for evasion of the immune systems. These include proteases and toxins which can kill leukocytes, inhibit neutrophil chemotaxis, resist phagocytosis, inactivate complement and neutralise host antimicrobial peptides. It has been shown that wound infections caused by this bacteria often results in hyperinflammation and impairment of collagen remodeling. Invasion of host tissue component by bacterial pathogen can initiate destruction of structural proteins including collagen. This is caused by either bacterial proteases [27] or bacteria proeases induced activation of latent host collagenases [28]. Inactivation of bacteria and the bacterial proteases is expected to reduce inflammation and restore collagen remodeling in wounds. At the same time, it is known that reactive oxygen species (ROS) may lead to the activation of Matrix Metalloproteases (MMPs) [29]. Therefore, the effect of pl-cp6 and red light mediated APDT on collagen restoration in murine excisional wounds infected with *MRSA* and *P. aeruginosa* was studied. Histology and Masson's trichrome staining is used to study collagen deposition in wounds during healing. However, histology provides semi quantitative information on collagen remodeling and because of its invasiveness leads to random sampling. Another approach is hydroxyproline level quantitation, which is also an invasive method.

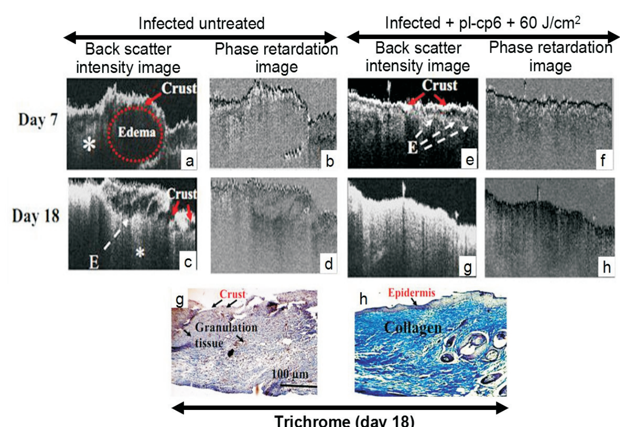

*Fig. T.3.5: Effect of APDT on collagen remodeling of MRSA infected wounds. Image size (OCT images): 1.5 mm x 3 mm. E: New epithelium layer. *: Granulation tissue. Lowermost panel: infected wound untreated (g) and APDT group (h), scale bar: 100 µm.*

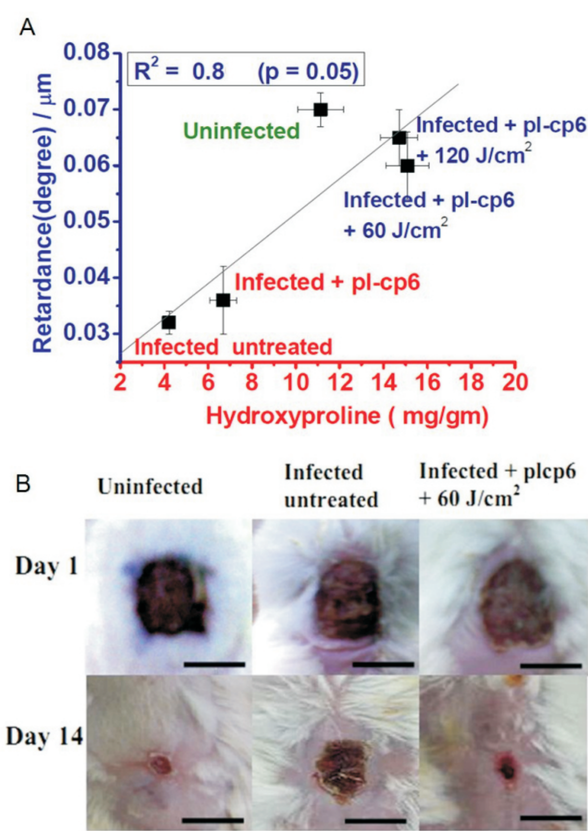

Fig. T.3.6: APDT induced increase in hydroxyproline content of MRSA infected wounds (A) and wound closure (B). Scale bar: 1 cm.

Collagen ordering contributes to tissue birefringence. It is expected that by measuring tissue retardance by Polarization Sensitive Optical Coherence Tomography (PSOCT), information on collagen remodeling can be obtained. Using PSOCT, we quantified the decrease in collagen remodeling in bacteria infected wounds [30] and monitored effect of APDT. The PSOCT data was correlated with hydroxyproline content and immunoblotting of matrix metalloproteases (MMP-8 and MMP-9) level in wounds.

As expected, compared to the uninfected wounds, slower collagen restoration *(Fig T.3.5)* and higher MMP-8,9 expressions were observed in infected wounds on day 5 post wounding[31]. Further, compared to the infected wounds treated with pl-cp6 alone, in infected wounds treated with both pl-cp6 and light the hydroxyproline content and retardance *(Fig T 3.6 A)* were higher by a factor of ~3 and ~2, respectively but MMP 8, 9 levels were lower [31]. These results suggest that APDT leads to promotion of collagen remodeling of wounds which may have contributed to faster wound closure *(Fig T.3.6 B)*.

2.5. Effect of topical APDT healing response of MRSA infected wounds of diabetic mice.

Impaired wound healing is a serious complication for diabetic patients. Infections of by antibiotic resistant has been reported as one of the leading causes of lower limb amputation and mortality in diabetic patients [32].Therefore, alternative therapeutic methods, which can eliminate antibiotic resistant bacteria and improve healing, are essential for management of wounds in diabetic conditions. An important concern pertaining to the use of APDT for infected wounds in diabetes is possible oxidative damage to inflammatory cells by ROS. Therefore, we monitored effect of APDT bacteria regrowth, oxidants and antioxidant levels in wound tissues of diabetic mice. Further, to quantify the effect of APDT on inflammatory cells, measurements were also made on the level of myeloperoxidase (MPO), a marker for number of inflammatory cells and neutrophil elastase (NE), a marker of neutrophil function.

The results presented in *Fig T.3.7 A* show that, compared to untreated wounds, in MRSA infected wounds of diabetic mice subjected to APDT at a fluence of ~120 J/cm^2 there was ~ 1.5 log decrease in bacteria load after APDT. However, at 24h

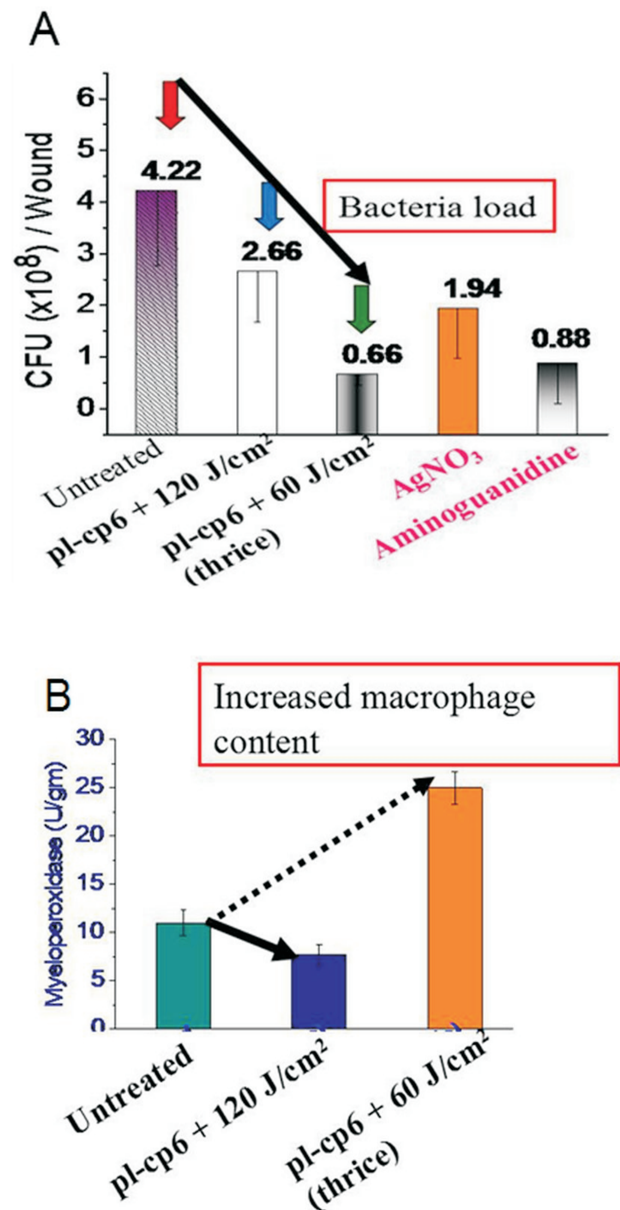

Fig. T.3.7: (A) Effect of low fluence multiple APDT, Amioguanidine (AG) and silver nitrate ($AgNO_3$) on bacterial load. (B): Effect of single and multiple APDT on level of myeloperoxidase, in wounds.

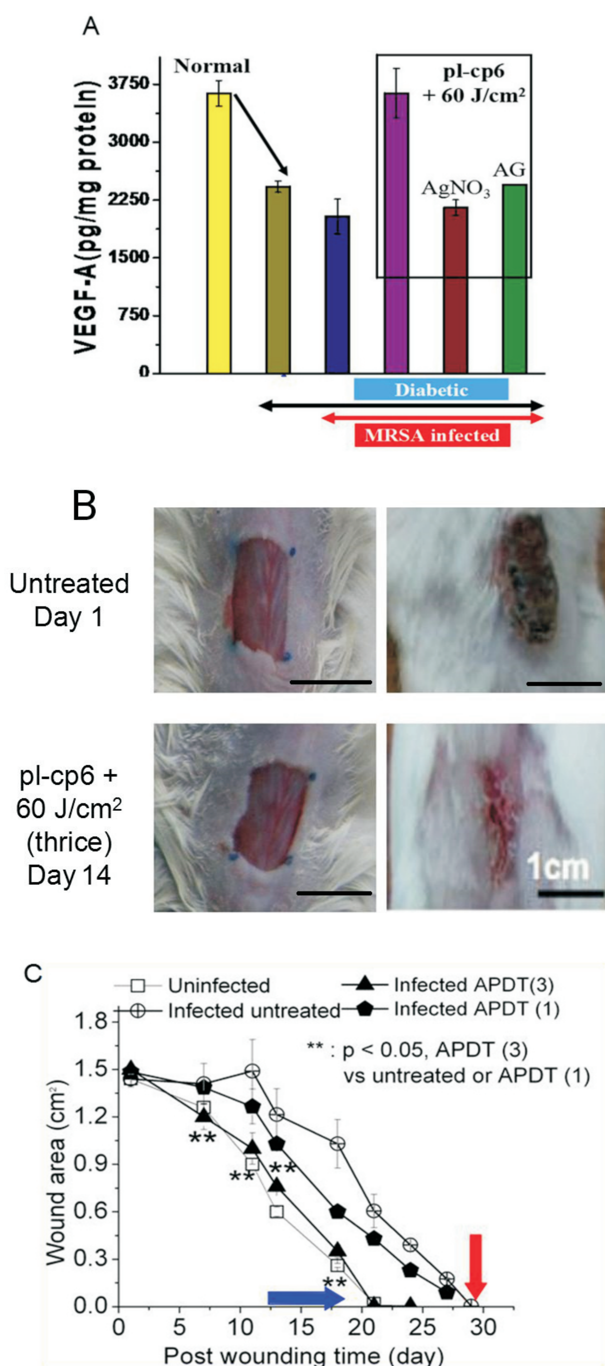

*Fig. T.3.8: (A) Effect of APDT on angiogenic growth factor (VEGF-A) level of MRSA infected wounds on day 3 p.w. *: $p < 0.05$ compared to wounds of non diabetic mice, **; compared to untreated control. (B & C): Effect of multiple APDT on healing of wounds of diabetic mice. Treatment frequency is denoted in parentheses. Scale bar : 1 cm.*

post APDT (day 3 post wounding) there was ~ 5-fold bacteria regrowth. The use of APDT repeated at 24 h intervals for 3 days was investigated to address this issue. APDT at a higher fluence (~120 J/cm^2) led to a significant increase in oxidative stress, inflammatory cell damage and depletion of antioxidants contributing to delayed wound healing. Multiple APDT at a lower fluence (~60 J/cm^2) led to better responses like reduction in bacterial regrowth by a factor of ~ 2.5, increase in antioxidants but spared the inflammatory cells (*Fig. T.3.7 A*). The latter was suggested by the increased MPO and NE level of wounds subjcted to multiple APDT at lower fluence of ~60 J/cm^2 *(Fig T.3.7 B)*.

Further, we also investigated the effect of multiple APDT at this lower fluence on angiogenesis of wounds. The efficacy of APDT was compared with two wound healing promoting agents; silver nitrate ($AgNO_3$), a standard antibacterial agent and Aminoguanidine (AG), an inhibitor of advanced glycated end product (AGE). Measurements were made on the levels of nitric oxide (NO) and vascular endothelial growth factor-A (VEGF-A) of wound tissue, which play important role in angiogenesis and healing of wounds. The results of this investigation show that multiple APDT leads to an increase in the levels of NO and VEGF-A (*Fig. T.3.8A*) by factor of ~3.5 and ~60 %, respectively, on day 3 [33]. Compared to $AgNO_3$ and AG, efficacy of APDT were observed to be more pronounced (*Fig. T.3.8A*). While $AgNO_3$ kills bacteria, it does not affect the bacterial virulent factors. Similarly, while AG inhibits AGE formation, it has no direct effect on either bacteria or virulent factors. On the other hand, APDT not only kills bacteria but also inactivates virulent factors. In addition, other beneficial effects such as attenuation of hyperinflammation, enhanced angiogenesis caused by APDT may also lead to improvement in wound healing (*Fig. T.3.8 B & C*).

3. Low level Light Therapy as an adjuvant for enhancement of healing of diabetic foot ulcers

Non-healing DFUs are resistant to conventional treatment [32]. Several adjuvant therapies which have been tried to stimulate healing process are ultra-sound, laser therapy, electrical stimulation, hyperbaric oxygen, and vacuum-assisted closure [34]. In this respect, while high level of ROS generated by PDT has cytotoxic effects, excitation of endogenous chromophores such as cytochromes by visible light leads to increase in metabolic rate, proliferation capability, growth factor secretion in cells *(Fig T.3.9)*. This therapeutic approach, also known as photobiostimulation or

low level light therapy (LLLT), has been shown to improve wound repair out come in numerous studies on animal models and a limited number of clinical trials [35,36]. Lack of reproducible results have hampered the wide spread use of laser therapy [37].

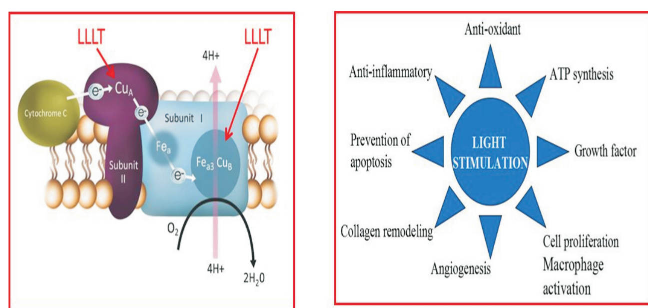

Fig. T.3.9: Principle of LLLT . Cytochromes in electron transport series of inner mitochondrial mebrane are excited by visible light , leading to enhnaced electron transport and energy synthesis by cells.

In our recent study [38] we observed that LLLT, used as an adjuvant to conventional antibiotics treatment options can improve rate of healing in diabetic foot ulcers. Patients in study group received LLLT (660 ± 20 nm, ~3 J/cm^2) or 15 days along with conventional therapy and those in control group were treated with conventional therapy alone. From the images presented *(Fig. T.3.10)*, it can be observed that the ulcers of LLLT group has more granulation tissue (red) compared to the control group which show some amount of pus (yellow). Percentage ulcer area reduction was 37 ± 9% in the LLLT group and 15 ± 5.4 % in the control group ($p < 0.001$, t-test) on day 15 *(Fig. T.3.11)*.

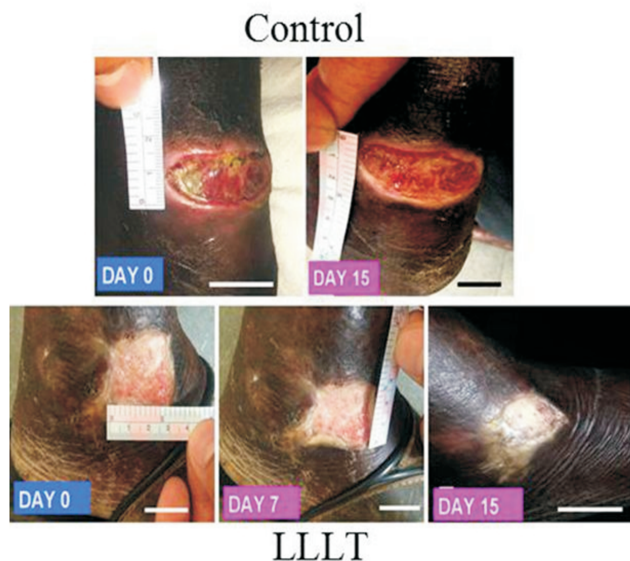

Fig. T.3.10: Wound contraction with respect to initial wound area, in control and LLLT group on day 15. Scale bar : 2 mm.

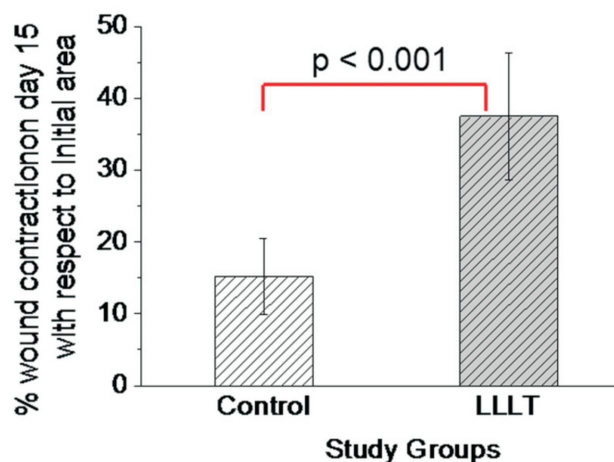

Fig. T.3.11: Effect of LLLT on mean wound area contraction of control and LLLT group on day 15. The data here represent means ±SD (n =15) for each group.

Apart from the reduction in wound area, it was observed that the majority of the wounds of LLLT groups were devoid of pus and exhibited granulation. In contrast, the wound that received conventional treatment, showed more pus, lesser granulation and required more debridement and dressing changes. The patients of the LLLT groups did not feel any discomfort with the procedure during this duration. The LLLT procedure is therefore a good adjuvant for the treatment of DFUs.

3. Summary

To summarize, in this article we have presented the important outcomes of our investigations at LBAS, RRCAT, on healing of wounds mediated by topical APDT as well as LLLT. While APDT causes significant reduction of bacteria load in wounds, in tumors PDT has been shown to induce acute hyperinflammatory reaction. Since this may prove counterproductive for chronic wound management, our current studies were intended to address this discrepancy. By measuring the level of biochemical markers of inflammation,

cell proliferation and by performing optical imaging on tissue retardation in bacteria infected wounds subjected to APDT, we have conclusively shown that that low fluence topical APDT not only kills bacteria but also leads to reduction of the hyperinflammatory response, while improving cell proliferation, collagen remodeling. Our results clearly suggest that there exists an APDT parametric window by using which possible adverse effect of APDT on host cells can be minimized while improving the wound healing outcome and we hope this would motivate further studies on clinical application of APDT.

4. Future directions

Our results show the potential of APDT for applications in wound healing. Further research may be aimed at developing suitable formulation for photosensitizer such as use of gel matrix like collagen for uniform and sustained drug release at the wound site.

As recent clinical studies suggest, LLLT can promote healing in foot ulcers of diabetic patients after the bacteria load is brought down to a manageable level by antibiotic treatment. However, for scenario wherein high load of antibiotic resistant bacteria such as MRSA are observed, it would be desirable to carry out studies on combined approaches involving more effective antibacterial treatment such as APDT and LLLT. In fact, a recent clinical trial from one Indian group shows that in patients with chronic periodontitis, a combination of a single application of PDT (980 nm laser and methylene blue) and LLLT provide additional benefit to scaling and root treatment planning in terms of clinical parameters, at 6 months following the interventions [39].Therefore, use of a combined approach involving APDT to reduce antibiotic resistant bacteria load followed by LLLT should be explored for management of wound healing in diabetes. It is conceivable that APDT and LLLT, in combination might have synergistic effects on control of wound infections and the resultant inflammatory response as well as on the promotion of wound healing.

5. Acknowledgements

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