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A comparison of antibacterial and antibiofilm efficacy of phenothiazinium dyes between Gram positive and Gram negative bacterial biofilm

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ABSTRACT

Background: Antimicrobial photodynamic therapy (APDT) is a process that generates reactive oxygen species (ROS) in presence of photosensitizer, visible light and oxygen which destroys the bacterial cells. We investigated the photoinactivation efficiency of phenothiazinium dyes and the effect of ROS generation on Gram positive and Gram negative bacterial cell as well as on biofilm.
Material and methods: Enterococcus faecalis and *Klebsiella pneumonia* were incubated with all the three phenothiazinium dyes and exposed to 630 nm of light. After PDT, colony forming unit (CFU) were performed to estimate the cell survival fraction. Intracellular reactive oxygen species (ROS) was detected by DCFH-DA. Crystal violet (CV) assay and extracellular polysaccharides (EPS) reduction assay were performed to analyze antibiofilm effect. Confocal laser electron microscope (CLSM) scanning electron microscope (SEM) was performed to assess the disruption of biofilm.
Results: $8\log_{10}$ reduction in bacterial count was observed in *Enterococcus faecalis* while $3\log_{10}$ in *Klebsiella pneumoniae*. CV and EPS reduction assay revealed that photodynamic inhibition was more pronounced in *Enterococcus faecalis*. In addition to this CLSM and SEM study showed an increase in cell permeability of propidium iodide and leakage of cellular constituents in treated preformed biofilm which reflects the antibiofilm action of photodynamic therapy.
Conclusion: We conclude that Gram-positive bacteria (*Enterococcus faecalis*) are more susceptible to APDT due to increased level of ROS generation inside the cell, higher photosensitizer binding efficiency and DNA degradation. Phenothiazinium dyes are proved to be highly efficient against both planktonic and biofilm state of cells.

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1. Introduction

Microbial infections remain to be one of the main causes of mortality and the major factor of this infectious diseases caused by multiple drug resistant bacteria and their aggregation as biofilm [1]. A biofilm can be defined as a sessile community of microbes that adhere irreversibly to an inert or to living surface and are embedded in a self-producing matrix of extracellular polymeric substances (EPS) [2]. EPS is an important component of biofilm it provides structural stability as well as protection to the biofilm against antimicrobial agents leads to antimicrobial resistance [3]. Owing to the severity of these infections, there is a critical need to explore novel approaches like Antimicrobial photodynamic therapy (APDT) which appears to be the most promising alternative methodology against biofilm-related infections. APDT disrupts the biofilm and inhibits its regrowth.

In APDT, photosensitizer (PS) is excited by absorbing a light corresponding to the absorption peak of PS in presence of oxygen rich environment to produce a phototoxic response, normally via oxidative damage [4]. Two pathways of oxidative mechanisms of photoinactivation (PI) are responsible for the inactivation of the target cells. Type I pathway involves the generation of free radicals like hydroxyl radicals (HO•). Type II pathway involves the generation of excited singlet-state oxygen [5]. Type I and Type II pathways occur simultaneously however, the ratio between these two pathways depends on types of PS employed and also on the photosensitizer's microenvironment [6].

Over the last few decades, the effectiveness of photoinactivation of Gram-positive and Gram-negative bacteria using different PS has been extensively studied but little information is available

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on its potential uses against bacteria organized in biofilm [7]. It has been reported that neutral, anionic and cationic photosensitizers effectively destroyed Gram-positive bacteria but only cationic photosensitizers were found to photoinactivate Gram-negative bacteria [8]. It was also found that in the case of positively charged PS, positive charge allowed it to bind to negatively charged bacterial cell membrane and in some cases penetrated to the microbial cells due to the porous nature of cell wall [9].

To the best of our knowledge, its effect on gram positive and gram negative bacterial biofilm has been less studied so far. Bridging this gap, here we examined the mechanistic insight behind photodynamic effects against both the classes of bacteria. Hence, we compared the efficacy of three phenothiazinium dyes i.e. Toluidine Blue O (TBO), Azure A (AA) and New Methylene Blue (NMB) on *Enterococcus faecalis* and *Klebsiella pneumoniae* as representative of Gram positive and Gram negative bacteria respectively. The reason behind selecting these dyes is that they show absorption in the red region of light that can penetrate tissue efficiently, limited photobleaching and less toxicity.

2. Materials and methods

2.1. Bacterial strain

The bacterial strains used in this study were *Enterococcus faecalis* MTCC 2729 (Gram positive) and *Klebsiella pneumoniae* ATCC 700603 (Gram negative) as an experimental model. The microorganisms were subcultured in Brain Heart Infusion (BHI) broth supplemented with 1% sucrose (Himedia Labs, Mumbai, India) and was incubated at 37 °C.

2.2. Photosensitizers

Toluidine blue O (TBO), Azure A (AA), and New methylene blue (NMB) were obtained from (Sigma-Aldrich, St. Louis, MO). A stock solution of 1 mg/mL was prepared in HPLC grade water. This solution was filtered-sterilized and then stored at 4 °C in the dark. A 100 mW laser of 630 nm wavelength was used to irradiate all three PS. The effective radiant exposure of the light source was calculated as described by Rolim et al. [10]. Beam diameter is 3 mm and beam height from the base is 24.8 mm. The applied PD was 0.130W cm^{-2} and energy fluency were set to 100 J/cm^2 when irradiated for 13 min.

2.3. Photodynamic inactivation of planktonic cells

Efficacy of Antimicrobial photodynamic therapy (APDT) of all the three phenothiazinium dyes on the viability of Gram positive and Gram negative bacteria was evaluated. 10^8 CFU/mL suspensions of bacteria (*E. faecalis* and *K. pneumoniae)* in phosphate buffer saline (PBS) were incubated with each dye at a concentration of 10 μM in the dark for 10 min and were then treated with different light doses. After irradiation, 10-fold serial dilution in PBS was performed followed by the spread of cells on BHI agar (supplemented with 1% sucrose) plate. No treatment was given to the control and dark toxicity was tested without light, following incubation at 37° C for 24 h, numbers of grown colonies were counted [11].

2.4. Bacterial binding studies

Cationic dye has the ability to bind to the bacterial cell wall, therefore it is important to determine the number of dye binds to the bacterial cells. Cells were incubated with 10 μM concentration of each dye for 15 min in the dark. After incubation, cells were centrifuged at 10,000*g* for 10 min and the obtained pellets were washed twice with PBS. The cell pellet was then dissolved in the same volume of 0.1% SDS [12]. Absorption spectra were recorded between 500–700 nm by using a UV-visible spectrophotometer (Perkin Elmer Life and Analytical Sciences, Shelton, USA).

2.5. Biofilm formation assay

Biofilm formation was assessed by using the protocol of Loo et al. with few modifications [13]. Briefly overnight culture of *E. faecalis* and *K. pneumoniae* was diluted to 10^7 CFU/mL into fresh BHI supplemented with 1% sucrose with 10 μM concentration of TBO, AA and NMB with respective control and blank after irradiation with appropriate wavelength (630 nm) of light for different light doses, plates were incubated at 37 °C for 24 h. After incubation media having unattached cells were decanted the remaining planktonic cells were removed by gentle rinsing with PBS. The adhered biofilms in the wells were fixed with formalin (37%, diluted 1:10) and 2% sodium acetate. Each well was stained with 200 μL of 0.1% crystal violet at room temperature for 15 min. Bound dye was released with 100 μL of 95% ethanol, after twice washing with PBS. Plates were then set on a shaker for 5 min to allow full release of dye. Biofilm formation was then quantified by measuring the optical density of the suspension at 630 nm by a microplate reader (BIO-RAD iMark ™ Microplate reader, India). The absorbance for the blanks was subtracted from the test values to minimize background interference.

2.6. Influence of PDT on EPS production

The Congo red (CR) binding assay was used to evaluate exopolysaccharide (EPS) production, as reported earlier [14]. 50 μL of overnight growth culture of bacteria was diluted to 10^8 CFU/mL into fresh BHI with 1% sucrose which was then treated with 10 μM of each dye and irradiated for 60 s in the case of Gram-positive and 300 s for Gram-negative bacteria. However, controls were left untreated in both the cases. After incubation for 24 h at 37 °C, the medium was removed and biofilms were washed with PBS and then fresh medium (100 μL) was added to each well including the controls. 50 μL of CR (0.5 mM) was then added to each well. Medium (100 μL) along with 50 μL CR was added to another well for blank measurements (Blank CR). Plates were incubated for 2 h. The medium in each well was transferred to 200 μL microcentrifuge tubes and centrifuged at 10,000*g* for 5 min. The supernatant was transferred to empty wells in microtitre plates. Absorbance was taken at 490 nm. The absorbance value of the supernatant was subtracted from the absorbance value of the ‘blank CR’. The resultant value represents the amount of bound CR or EPS produced. This experiment was conducted in triplicate.

2.7. Photodynamic inactivation of biofilms

The efficacy of APDT using TBO, AA and NMB on the viability of Gram positive and Gram negative bacterial biofilm was investigated using following protocol [15]. Overnight cultures of *E. faecalis* and *K. pneumoniae* were re-suspended to a final concentration of 1.0×10^8 CFU/mL in fresh BHI supplemented with 1% sucrose. Aliquots (100 μL) of the diluted bacterial suspension were inoculated in each well of 96 well microtitre plate and were incubated for 48 h at 37° C, exhausted growth medium and planktonic cells were replaced after 24 h with fresh media. Prior to the treatment, the remaining non-adherent bacteria were removed by twice washing with sterile PBS. Preformed biofilm was incubated with 50 μM of each dye in the dark for 30 min and then irradiated with a final light dose of 100 J/cm^2. Control was left untreated. Biofilms were then disrupted by vortexing followed by serial dilution by 10 fold.

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