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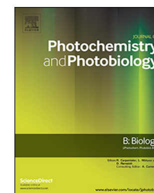

Efficacy of photodynamic therapy against *Streptococcus mutans* biofilm: Role of singlet oxygen

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ABSTRACT

In photodynamic therapy (PDT), killing is entirely based on the ROS generation and among different types of ROS generated during PDT, singlet oxygen is considered as the most potential as illustrated in many studies and therefore it is predominantly responsible for photodamage and cytotoxic reactions. The aim of this study was to check whether singlet oxygen (Type II photochemistry) is more potential than free radicals (Type I photochemistry) against *Streptococcus mutans* biofilm. We have taken two phenothiazinium dyes i.e. toluidine blue O (TBO) and new methylene blue (NMB). TBO was found to have better antibacterial as well as antibiofilm effect than NMB. Antibacterial effect was evaluated by colony forming unit while antibiofilm action by crystal violet and congo red binding assays. We have also evaluated the disruption of preformed biofilm by biofilm reduction assay, confocal laser electron and scanning electron microscopy. More singlet oxygen production was detected in case of TBO than NMB while more Free radical (HO·) was produced by NMB than TBO. TBO showed better antibacterial as well as antibiofilm effect than NMB so; we conclude that potency of a photosensitizer is correlated with the capability to produce singlet oxygen.

1. Introduction

Biofilm is an assemblage of sessile microbial communities that remain associated with substratum and are embedded in the pool of self-produced extracellular polymeric substances (EPS) matrix consisting of protein, polysaccharides and nucleic acid [1]. EPS serves as a physical barrier that augments resistance against antimicrobial agents [2]. *Streptococcus mutans* is considered as one of the dominant residents of oral biofilm and therefore have the largest contribution in the development of dental caries, which is characterized by irreversible tooth decay [3]. Moreover, *S. mutans* is also known to cause infective endocarditis that leads to morbidity and mortality even up to 50% [4]. Due to the emergence of multi drug resistance strains, there is an urgent need to explore novel approaches rather than conventional antibiotic therapy whose effect is diminishing day by day.

Photodynamic therapy has emerged as an effective process to combat multi drug resistance strain. It is as effective against resistant strain of bacteria as their naive counterparts. PDT involves delivering visible light of the appropriate wavelength to excite non-toxic photosensitizers (PS) molecules in an oxygen rich environment. The non-toxic PS absorbs photons of light of the correct wavelength to get excited. Once excited, PS gets converted from excited singlet state to a long lived triplet excited state through intersystem crossing which subsequently reacts with ground (triplet) state O_2 in two different ways [5]. In Type I reaction it either transfer electron from triplet excited state of PS to substrate or in Type II reaction transfer energy from triplet excited state of PS to molecular oxygen. Type I reaction involves generation of free radicals like hydroxyl radicals (OH·), hydrogen peroxide (H_2O_2) and superoxide (O^{2-}) while Type II reaction involves generation of singlet state oxygen. The $^1O_2$ Σg form of singlet oxygen is too short lived; it is the $^1O_2$ Δg form that is involved in photodynamic response [6]. These reactive oxygen species are highly toxic and consequently damage the microbial cell by targeting membrane lipids, proteins, nucleic acids and other cellular components [7]. Singlet oxygen is known to modify guanine residue selectively and few stand break and site of base loss (AP site), the major product of base modification caused by singlet oxygen is 8-hydroxyguanine (7,8- dihydro-8-oxoguanine) [8]. The susceptibility of proteins is due to double bond or sulfur moieties, sulphides are generally oxidized to sulfoxides, while disulphides react to form thiolsulfinates [9,10]. Singlet oxygen reacts with unsaturated lipids to form lipid hydroperoxides, and with the aid of ferrous iron can play an important role in initiating free radical chain reactions [11]. Both the mechanisms operate in the cells simultaneously but their relative proportions may depend upon the type of PS being employed and essentially on the microenvironment of the PS molecule [12].

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Although free radicals contribute to photodamage but singlet oxygen ($^1O_2$ Δg) is considered as the major ROS responsible of cell death in PDT [13]. Singlet oxygen is considered as highly reactive species and it is most desirable in PDT due its oxidative ability. Direct excitation from molecular oxygen ($^3O_2$) to singlet oxygen ($^1O_2$) is forbidden, for that a photosensitizer is required. In the light of present background, we have initiated our study by comparing the singlet oxygen production of the two PS of same class and also evaluated the antibiofilm efficacy of PDT on *S. mutans*.

2. Materials and Methods

2.1. Bacterial Strain

Streptococcus mutans MTCC 497 (Institute of Microbial Technology, Chandigarh, India) was used in this study. The microorganism was subcultured in Brain Heart Infusion (BHI) Broth (Himedia Labs, Mumbai, India) and was incubated at 37 °C.

2.2. Photosensitization and Light Source

Toluidine Blue O (TBO), and New methylene blue (NMB) were used as photosensitizers, all were obtained from (Sigma-Aldrich, St. Louis, MO). A stock solution of 1 mg/mL was prepared in HPLC grade water and stored at 4 °C in the dark. A 100 mW laser of 630 nm wavelength was used and the effective radiant exposure of the light source was calculated as described by Rolim et al. [14]. The applied PD was 0.130 W cm^{-2} and energy fluency were set to 5 J/cm^2, 50 J/cm^2 and 100 J/cm^2 when irradiated for 39 s, 6 min 25 s and 12 min 50 s respectively.

2.3. Inactivation of Planktonic Cells

S. mutans (10^8) was incubated with photosensitizers at a concentration of 10 μM in the dark for 10 min and was then irradiated with 5 J/cm^2 of light dose. After irradiation, 10-fold serial dilution in phosphate buffer saline (PBS) was performed followed by the spread of cells on BHI agar (supplemented with 5% sucrose) plate. Control was left untreated and dark toxicity was checked for photosensitizers without light and light toxicity was checked only with light, followed by incubation at 37 °C for 48 h. Numbers of grown colonies were then counted [15].

2.4. Biofilm Formation Assay

Biofilm formation after PDT was assessed on *S. mutans* using the protocol of Loo et al. with few modifications [16]. Briefly overnight culture of *S. Mutans* was diluted to 10^7 CFU/ml into fresh BHI supplemented with 5% sucrose. The bacterial cells were incubated with 10 μM concentration of photosensitizers for 15 min and then irradiated with appropriate wavelength (630 nm) of 5 J/cm^2. Plates were left for 24 h at 37 °C for biofilm formation, after incubation media having unattached cells were decanted and remaining biofilms were washed twice with PBS. The adhered biofilm were fixed with formalin (37%, diluted 1:10) and 2% sodium acetate. Each well containing biofilms were stained with 200 μl of 0.1% crystal violet dye at room temperature for 15 min. After washing with PBS, bound dye was released with 100 μl of 95% ethanol on shaker for 5 min. Biofilm was then quantified by measuring the optical density at 630 nm by a microplate reader (BIORAD iMark™Microplate reader, India).

2.5. Quantification of Extracellular Polysaccharides Substance Reduction After PDT

The Congo red (CR) binding assay was used to quantify the extracellular polysaccharides substances (EPS) reduction, as reported earlier [17]. 10^7 CFU/ml *S. Mutans* cells were maintained in into fresh BHI with 1% sucrose; cells were incubated with 10 μM concentration of photosensitizers for 15 min and then irradiated with appropriate wavelength (630 nm) of 5 J/cm^2 as described above. Controls were left untreated, after incubation for 24 h at 37 °C, the medium was removed and biofilms were washed with PBS. Fresh medium (100 μl) and 50 μl of CR (0.5 mM) was then added to each treated and control wells and same were added to another well for blank, plates were then incubated for 2 h. After then whole content of the well was transferred to 200 μl microcentrifuge tubes and centrifuges at 10,000 ×*g* for 5 min. The supernatants were transferred to empty wells in microtitre plate and a colorimetric change in the solution was measured using a microtitre plate reader (BIORAD iMark TM Microplate reader) at 490 nm. This experiment was conducted in triplicate.

2.6. Endogeneous ROS Detection

ROS generated inside the cells after APDT were quantified by fluorescence spectroscopy using 2′, 7′-dichlorfluorescein-diacetate (DCFH-DA). 10^7 CFU/ml of *S. mutans* cells were incubation with 5 μM DCFH-DA for 10 min in PBS and were then treated with 10 μM of each photosensitizer and irradiated with 5 J/cm^2 of light dose. Controls were left untreated; the fluorescence intensity was measured by exciting at 485 nm using slit width 1.5 nm [18].

2.7. HO• Estimation

In order to detect type I photochemistry, total concentration of HO• was estimated. We have used fluorescence probe 3′-*p*-hydroxyphenyl-fluorescein (HPF) for the same. *S. mutans* (10^7 CFU/ml) cells were incubation with 5 μM HPF for 10 min in PBS and were then treated with 10 μM of each photosensitizer and irradiated with 5 J/cm^2 of light dose. Controls were left untreated; the fluorescence intensity was measured by exciting at 492 nm using slit width 1.5 nm [19].

2.8. Quantification of Singlet Oxygen Detection

9,10-anthracenediylbis (methylene) dimalonic acid (AMDA) was used to measure the relative singlet oxygen quantum yields of the phenothiazinium dyes in PBS. 10^7 CFU/ml of *S. mutans* cells were incubation with 5 μM AMDA for 10 min and were then treated with 10 μM of each photosensitizer and irradiated with 5 J/cm^2 of light dose. The decrease in the 399-nm absorption band of AMDA after treatment corresponds to the amount of singlet oxygen generated [20].

2.9. Antimicrobial Photodynamic Inactivation of S. mutans Biofilms

48 h preformed biofilm of *S. mutans* whose medium and planktonic cells were replaced after 24 h with fresh media were incubated with10 μM and 50 μM of TBO and NMB in the dark for 30 min and then irradiated with 5 J/$cm^2$and 50 J/cm^2 of light doses. Control was left untreated. Biofilms were then disrupted by vortexing followed by serial dilution by 10 fold and were then plated onto BHI agar plate, further incubated for 24 h at 37 °C. Numbers of grown colonies were then counted [20].

2.10. Confocal Laser Scanning Microscopy (CLSM)

Biofilm was grown in covered glass bottom confocal dishes (Genetix Biotech Asia Pvt. Ltd., New Delhi, India) with a dish size of 35 mm, 22 mm cover glass for 48 h. Exhausted media was replaced with fresh media after 24 h. Preformed biofilm were then incubated with 50 μM TBO and NMB in the dark and then irradiated with 50 J/cm^2 of light dose. After irradiation, confocal dishes were washed with sterile PBS and biofilm was stained with PI and syto9 followed by incubation at 37 °C for 1 h. Fluorescence emission was observed using Fluo View

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