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Photodynamic efficacy of toluidine blue O against mono species and dual species bacterial biofilm

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ABSTRACT

Aim: The purpose of this study was to investigate how *Enterococcus faecalis* and *Streptococcus mutans* behave in mono and dual species biofilm after photodynamic treatment.
Background: Antimicrobial photodynamic therapy (aPDT) leads to the generation of reactive oxygen species (ROS) that destroys bacterial cells in presence of a photosensitizer, visible light, and oxygen.
Material and methods: We have taken *Enterococcus faecalis* and *Streptococcus mutans* as monospecies culture and their dualspecies culture biofilm. Antibacterial effect was evaluated by colony forming unit while antibiofilm action by crystal violet and congored binding assays.
Results: We found that dual species biofilm are more resistant than monospecies biofilm and *S. mutans* shows dominance over *E. faecalis* in dual species biofilm, it inhibited the growth of *E. faecalis* in dual species biofilm. Antibiofilm efficacy of TBO also validated that dualspecies show less inhibition than monospecies biofilm this may be due to different EPS constitution in dualspecies biofilm, hence less inhibition was observed in EPS production of dualspecies biofilm than monospecies biofilm. Reactive oxygen species and singlet oxygen yield was found to be light dose dependent and antimicrobial photodynamic efficiency is directly related to the ROS production.
Conclusion: We conclude that dual species biofilm shows resistance over monospecies biofilm and *S. mutans* in dual species inhibits the growth of *E. faecalis*.

1. Introduction

Dental caries is a chronic disease that causes irreversible tooth decay. It is a most prevalent disease among children [1]. *Streptococcus mutans* is considered as the most cariogenic bacteria which plays an important role in the caries formation. This bacterium is known to metabolize the ingested carbohydrates to form acid that results in the damage of dentine hard tissue and formation of caries [2,3]. This bacterium is also known to form a biofilm on the tooth surface called plaque. Biofilm is the surface dependent, three-dimensional and heteromorphic communities of microorganism embedded in a self-producing extracellular polymeric substance (EPS) [4,5]. EPS provides structural stability to the biofilm and it also protects biofilm against harsh environmental contditions and antibacterial agents [6]. Human oral microflora contains a different type of genetically distinct organisms. Naturally occurring oral biofilm are polymicrobial or multispecies and they are more resistant to the antibacterial agent than mono species [5]. Endodontic biofilm is multispecies and the reason for endodontic treatment failure is incomplete irradiation of bacterial biofilm from the root canal [7]. *Enterococcus faecalis* has been frequently found in persistent infection, these species are known to grow in a lethal environment after treatment [8]. Current treatment includes either mechanical disruption of oral biofilm or use of antibacterial agents, both of them has limitations. The emergence of biofilm-related and antibiotic resistance strains led to the urgent need to use an alternative technique that does not show resistance.

Antimicrobial photodynamic therapy is a treatment for microbial biofilm infection and it also offers a mean for selectively damage the target cells while sparing neighboring cells. aPDT is depending upon nontoxic light sensitive dye known as a photosensitizer (PS) which absorbs appropriate wavelength of light in presence of oxygen to produce reactive oxygen species [9–11]. In antimicrobial photodynamic therapy, there are two type of mechanism: type I and type II. Type I reaction involves transfer of electron or hydrogen atom from excited

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triplet state PS to a substrate, leads to the production of radicals derived from water. These radicals then combine with molecular oxygen and produce free radicals like hydroxyl radicals (HO•), superoxide ($^{\cdot}O_2^-$) and hydrogen peroxide (H_2O_2). [12]. Type II depends upon the transfer of energy from excited PS to molecular oxygen that leads to the emergence of singlet oxygen ($^1O_2$), one PS molecule before destroying can generate thousands molecule of singlet oxygen ($^1O_2$) [13]. Both type I and type II mechanisms operate in the cell at the same time but which ROS shows dominance is totally depends upon the type of PS and on the microenvironment [14]. In human oral cavity, many bacteria are considered as main pathogens responsible for the development of infection and the causes of infection is due to poor oral hygiene, diabetes mellitus, use of antibiotics for a long time and heavy smoking habits and immunosuppressive drugs that can infect the individuals to this opportunistic infection [15]. The oral microflora involves a huge number of different species and they are living in coordination; in view of the above background we have initiated our study with a mixed culture of *S. mutans* and *E. faecalis.* We are trying to create more or less natural condition by mixing two bacteria. Multispecies culture is difficult because there are many conditions that are different from bacteria to bacteria such as growth in the same media, PH, dietary product and so on. It was reported earlier that bacterial-fungal mixed biofilm was effectively reduced by aPDT as compared to classical endodontic irrigants [16]. aPDT is also effective against single and mixed fungal biofilm of *Candida albicans* and non *albicans candida* species. It was also reported that dual species biofilm is hard to eliminate compared to single species biofilm [17]. The aim of this study is to compare the effect of aPDT on *S. mutans* and *E. faecalis* monoculture and as well as on dual culture [18].

2. Material and methods

2.1. Bacterial strain

Enterococcus faecalis, ATCC 2729 and *Streptococcus mutans,* MTCC 497 (Institute of Microbial Technology, Chandigarh, India) were used in this study. Both bacteria were cultured in Brain Heart Infusion (BHI) broth supplemented with 5% sucrose to the media followed by incubation at 37° C.

2.2. Photosensitizers

Toluidine blue O (TBO, Sigma-Aldrich, St. Louis, MO) was used as a photosensitizer. 1 mg/mL of solution was diluted in HPLC water, filtered sterilized the solution and stored at 4∘C in the dark. 100 mW of laser light (Model No-MRL-III, CNI LASER, China) of wavelength 630 nm used to expose the Photosensitizer. The Beam diameter is 3 mm and the ray height is 24.8 mm. The applied power density was 0.130 W/cm^2 and energy fluency was set to 5 J/cm^2 and 10 J/cm^2 when irradiated for 39 and 77 s [19].

2.3. Effect of aPDT on the planktonic cell

aPDT inactivation of bacterial cells by phenothiazinium dyes was assessed on two Gram-positive bacterial species. 1:100 dilution of overnight culture of *E. faecalis* and *S. mutans* were taken in a fresh media separately and mixed into fresh media to form mixed culture. Then the mixed suspension was again incubated overnight at 37 °C after that secondary culture was prepared. 10^8 CFU/ml of separate culture as well as a mixed culture were incubated with 10μM of TBO for 10 min and then treated with light for 5 J/$cm^2$and 10 J/cm^2. Control were left untreated, after treatment 10-fold serially dilution was prepared in PBS and then spread the treated and untreated cells on BHI agar plates. We used BHI agar with and without 5% NaCl only for mixed culture these two type of BHI plates were used just to differentiate *E. faecalis* from *S. mutans*. Plates were kept at 37 °C for 24 h for *E. faecalis* and 48 h for *S. mutans* and mixed culture. Numbers of colonies were then counted. Only TBO treated group without light (dark toxicity of PS) was also evaluated.

2.4. ROS detection

ROS detection was measured by fluorescence spectroscopy using 2′, 7′dichlorofluoresceindi acetate (DCFH-DA) [20]. The first step was to pellet down the overnight grown bacterial culture the pellet was then washed with phosphate buffered saline (PBS) and then resuspended the pellet in PBS. 10^8 CFU/mL of both the bacterial suspensions was taken in eppendorf separately as well as their mixed culture was incubated for 10 min with 2.5 μM (DCFH-DA) followed by the addition of 10 μM of TBO in each eppendorf and then exposed to appropriate light doses. The fluorescence intensity was measured just after treatment at 525 nm with slit width 1.5 nm using spectrofluorometer (Tokyo, Japan Hitachi F-4500X). Fluorescence intensity of control was recorded without treatment.

2.5. Singlet oxygen detection ($^1O_2$)

Measure the relative singlet oxygen quantum yield of the phenothiazinium dye by 9, 10-anthracenediylbis (methylene) dimalonic acid (AMDA). A solution containing 10^8 CFU/ ml of *E. faecalis, S. mutans* and mixed culture separately was taken in 1 mL eppendorf then 10 μM AMDA was added followed by the addition of 10 μM concentration of TBO. After incubation for 10 min, the 630 nm light was exposed for 5 J/cm^2 and 10 J/cm^2. Singlet oxygen production was determined by the decreased intensity of 399 nm absorption peak of AMDA, lower the intensity of 399 nm peak is the direct measure of singlet oxygen production. The 399 nm O.D deterioration is an amount of the quantity of singlet oxygen produced [21].

2.6. Biofilm formation

We have followed the protocol reported earlier by Loo et al. with few modifications [22]. Concisely overnight culture of *S. mutans* and *E. faecalis* were diluted to 10^8 CFU/mL into the fresh broth of BHI supplemented with 5% sucrose. Aliquots (100 μL) of the diluted bacterial suspension were inoculated in each well of 96 well microtitre plate the bacterial cell was incubated with 10μM of the concentration of photosensitizers (TBO) for 10 min and then irradiated with appropriate wavelength (630 nm) of 5 J/cm^2 and 10 J/cm^2 light. After treatment the bacterial cells were incubated for 24 h of *E. faecalis*, 48 h for *S.mutans* and Mixed biofilm at 37∘C for biofilm formation. After incubation, the planktonic cells were decanted and gentle washed the residual biofilm with PBS. Each biofilm wells were stained with 100 μL of 0.1% crystal violet for 5 min on a shaker at room temperature. The dye was removed and 100 μL of 95% ethanol was added in each well for the purpose of removing bound CV from a bacterial biofilm. Then optical density of the suspension was recorded by a microplate reader (BIO-RAD I Mark TM Microplate, India) at 630 nm.

2.7. Mixed biofilm

S. mutans and *E. faecalis* incubated overnight separately in BHI supplemented with 5% sucrose and then 10^8 CFU/ml cells from each bacterial suspension were mixed together in fresh BHI and again incubated for 24 h at 37 °C to form mixed culture.The bacterial culture broth was centrifuged at 10,000 rpm to pellet down the cells for 10 min and discard the supernatant. The cell pellet was then resuspended in sterile PBS. Again 10^8 CFU/ml of mixed culture was taken and treated the way as defined above.

2.8. Reduction of extracellular polysaccharides after aPDT

Extracellular polymeric substance (EPS) production was estimated by using Congo red (CR) binding assay, as reported earlier [23]. The overnight bacterial culture was diluted to10^8 CFU/mL into BHI broth with 5% sucrose then treated with 10μM of TBO dye and irradiated for two different times 39 s and 77 s with 630 nm light. Controls were left untreated. After treatment bacterial suspension were incubated for 24 h at 37 °C. After incubation exhausted media were removed and biofilms were washed with PBS followed by the addition of 50 μl of congo red (0.5 mM) in each well. For blank, 100 μl of fresh media along with 50 μl CR was added to well. Then Plates was incubated for 2 h at 37 °C. The medium in each well was transferred to 200 μl microcentrifuge tubes and centrifuged at 10,000 *g* for 5 min. Then the supernatant was transferred into the empty wells of new 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 value of result represents the amount of bound CR or EPS produced. This experiment was done in triplicate.

2.9. Statistical analysis

All experiments were performed in triplicate. The results were presented as the mean ± standard deviation (SD) of individual experiments in triplicate and compared with those of the control groups were analyzed using Student's t-test. Free online software One-way analysis of variance (ANOVA) was used for the comparison of multiple means. Data with P-value ≤ 0.05 were considered statistically significant. http://www.physics.csbsju.edu/stats/anova.html.

3. Results

3.1. Photoinactivation of planktonic bacteia

Photoinactivation of *S. mutans, E. faecalis,* and Mixed culture planktonic cells by TBO with 10μM concentration was evaluated by the plate count method. We have taken four groups for *S.mutans*, *E.faecalis* and Mix culture i.e. Control (C), Light (L), Toluidine blue with irradiation light of 5 J/cm^2 (TBO + 5 JL) and Toluidine blue with irradiation of 10 J/cm^2 light (TBO + 10 J L). After treatment with respective light doses, treated cells of *S. mutans* and *E. faecalis* were plated over BHI agar plates. However, after treatment mix culture was plated in BHI with NaCl and without NaCl as shown in Fig. 1. *S.mutams* showed 3.3 log and 5.4 log reduction after photoinactivation by 5 J/cm^2 and 10 J/cm^2. *E.faecalis* showed 3.8 log and 5.5 log reduction with light irradiation of 5 J/cm^2 and 10 J/cm^2. The group which are treated with only 630 nm light showed no reduction. However, mixed culture with NaCl showed 5.2 log reduction after being irradiated with 5 J/cm^2 of light dose and with 10 J/cm^2 of light not a single colony were observed i.e 100% reduction. Without NaCl the reduction is 4.2 log and 5.4 log with 5 J/cm^2 and 10 J/cm^2 respectively. Only TBO treated group (Dark toxicity) does not affected the viability of the bacterial cells.

3.2. Detection of intracellular ROS production

E. faecalis, S. mutans and Mixed culture were exposed to laser light by using 10 μM of TBO, an increase in fluorescence of DCF was observed. Control and only light treated group did not show any increase in fluorescence. However, an increased in fluorescence intensity was observed with TBO treated group with 5 J/cm^2 and 10 J/cm^2 of 630 nm laser light by 7 fold and 12 fold, respectively, as compared with the untreated (control) group of *S. mutants* as shown in Fig. 2. *E. faecalis* showed 7.4 fold and 12.2 fold increase in fluorescence with 5 J/cm^2 and 10 J/cm^2 light, respectively, while mixed culture also showed the similar pattern of fluorescence increase, it is 7.3 fold after 5 J/cm^2 and 13.3 fold for 10 J/cm^2 light treatment.

3.3. Detection of singlet oxygen (Type II) quantum yield ($^1O_2$)

Quantum yield of singlet oxygen was calculated by measuring the degradation peak of 399 nm of AMDA. *E. faecalis, S. mutans* and mixed culture was treated the same way as described above, only light treated group abd control did not showed any singlet oxygen production. However, the phenothiazinium dye (TBO) exhibit the quantum yield of singlet oxygen in aqueous solutions after been irradiated with 5 J/cm^2 of light dose and this quantum yield is further enhanced when 10 J/cm^2 light dose was applied (Fig. 3). This shows the same pattern as observed in intracellular ROS, more the applied light doses more will be the yield of singlet oxygen.

3.4. The reduction of biofilm quantification

The biofilm inhibition was examined by crystal violet (CV) assay. We have taken three groups i.e. Control (C), Toluidine blue with 5 J/cm^2 light (D + L5 J) and Toluidine blue with 10 J/cm^2 light (D + L10 J) for each bacteria *S.mutans*, *E. faecalis* and mix biofilm. *S.mutans* inhibits biofilm formation by 34% and 50% after treated with 5 J/cm^2 and 10 J/cm^2 of laser light (630 nm). *E.faecalis* showed inhibition of 27% and 46% and mix biofilm showed inhibition of 19% and 40% after 5 J/cm^2 and 10 J/cm^2 of light treatment respectively as compared with control Fig. 4.

3.5. Reduction of EPS

Role of EPS formation is very important for the maintenance of biofilm structure, the EPS production was evaluated by Congo red binding assay for *S. mutans, E. faecalis* and their mix culture. After aPDT it was found that the production of EPS was reduced for *S. mutans* by 59% and 93% with 5 J/cm^2 and 10 J/cm^2 of light dose respectively. *E. faecalis* showed 74% and 94% reduction while mix culture showed 62% and 80% reduction after being irradiated with 5 J/cm^2 and 10 J/cm^2 of light doses respectively as shown in Fig. 5. This showed that 10 J/cm^2 light reduced EPS production to a greater extent.

4. Discussion

With the emergence of multidrug resistance and biofilm-related bacterial strain put a worldwide threat because conventional antibiotic becoming inactive. The assemblage of these strain as biofilm worsen the situation because biofilm provides its resident bacteria a safe environment and structural stability against antimicrobial agents [24]. Natural oral flora is very dynamic that undergo rapid fluctuations and the residing species are in the multispecies biofilm. aPDT evolved as a potential alternative therapeutic strategy since this modality has a broad spectrum against microorganisms. It can kill bacteria (Gram positive and Gram negative), virus, fungi and protozoa in one shot. It is fast and it can kill many bacteria within a few seconds without showing any resistance [25,26]. The prime focus of this study was to check the efficacy of aPDT against mono species and dual species biofilm because our natural flora contains multispecies biofilm. Although there are many articles available which inactivated mixed culture bacteria no report provides the exact status of bacteria after treatment. To the best of our knowledge, this is the first time we are providing the actual conclusion of the mixed culture biofilm after aPDT. We study the efficacy of phenothiazinium dye on *S. mutans*, *E. faecalis* and their dual species biofilm. We have also quantified the ROS production and singlet oxygen generation. After photoinactivation of *S. mutans* by 5 J/cm^2 and 10 J/cm^2 of laser light 630 nm showed less reduction than *E. faecalis*. It is clear from this data that *E. faecalis* are more susceptible to aPDT than *S.mutans*. After photoinactivation, we have plated mixed culture in BHI media with or without 5% NaCl. The purpose of doing the same is that *S. mutans* does not grow in NaCl containing media; from this we will separate the actual count of *E. faecalis* from *S. mutans*. Mixed culture

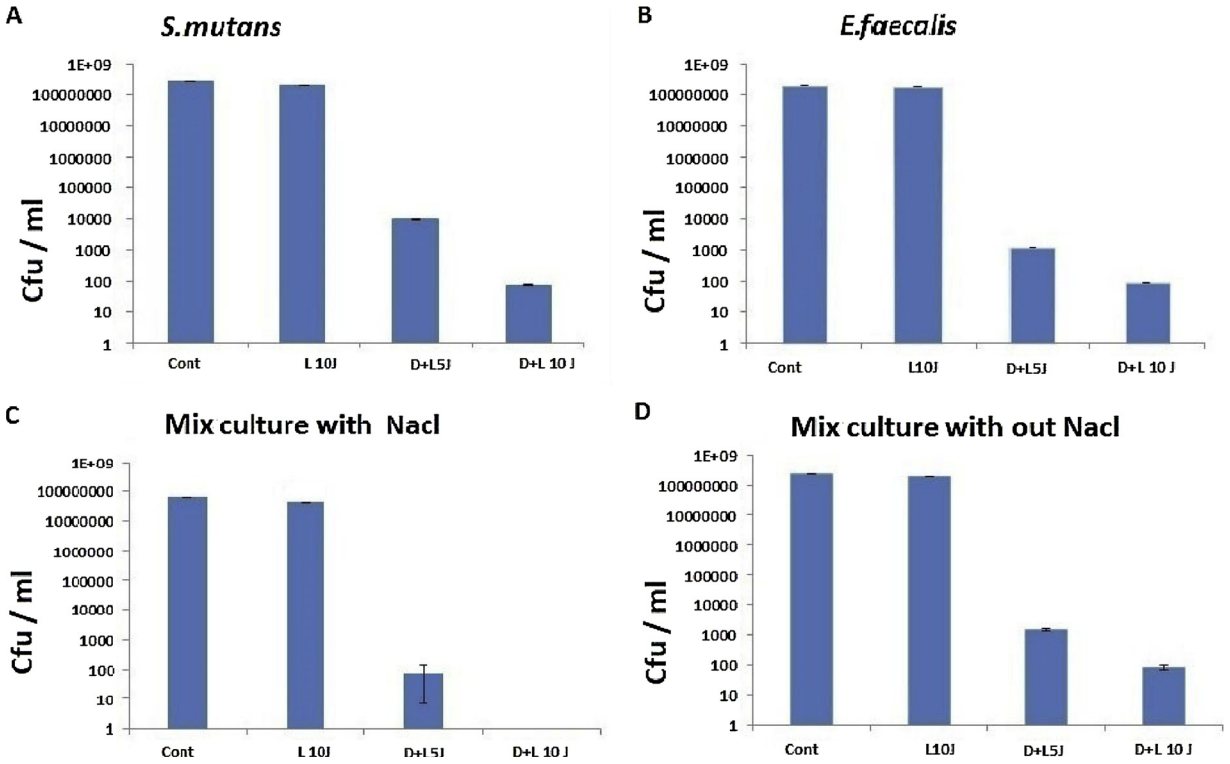

Fig. 1. Effect of aPDT on planktonic cells of (A) *S. mutans,* (B) *E.faecalis,* (C) Mix culture with NaCl and (D) Mix culture without NaCl: bacteria was incubated with TBO (10 μM) followed by exposure to 5 J/cm^2 and 10 J/cm^2 of light. **Control**- without PS and light; L10 J -only light without PS; **D + L5 J and D + L10 J**- Toluidine blue O + light 5 J/cm^2 and 10 J/cm^2 respectively.

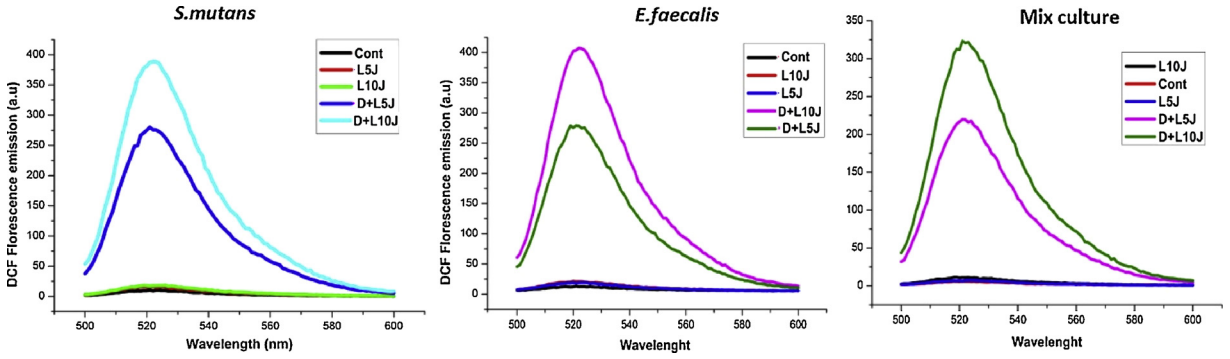

Fig. 2. Intracellular reactive oxygen species (ROS) detection in (A) *S. mutans* (B) *E. faecalis* and (C) Mix culture by fluorescence intensity of DCF with TBO after irradiation of 5 J/cm^2 and 10 J/cm^{2}of 630 nm light.

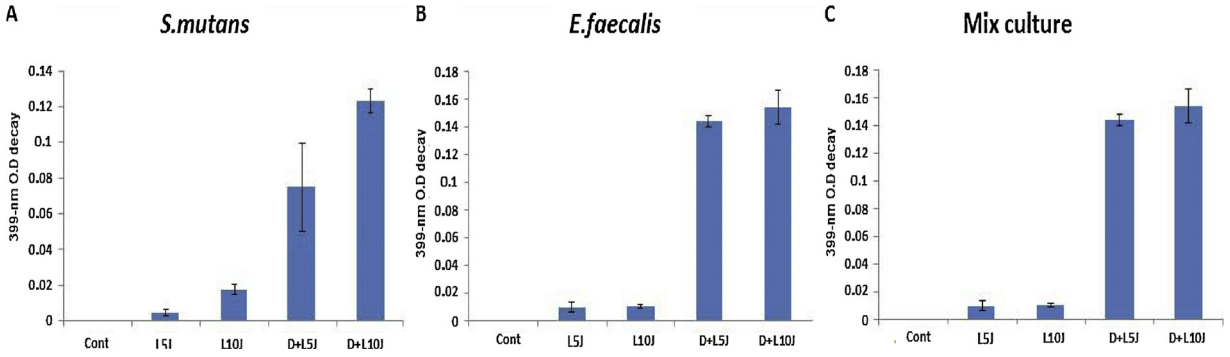

Fig. 3. Detection of singlet oxygen production by TBO after irradiation with 5 J/cm^2 and 10 J/cm^2 of 630 nm laser on (A) *S. mutans* (B) *E. faecalis* and (C) Mix culture irradiation.

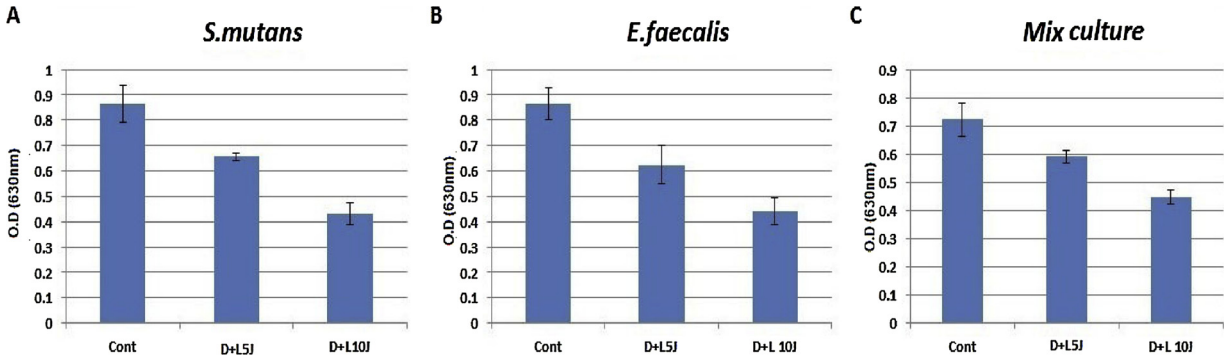

Fig. 4. Inhibition of biofilm formation: (A) *S. mutans* (B) *E. faecalis* and (C) mix culture was treated with 10 μM of TBO and were irradiated with 5 J/cm^2 and 10 J/cm^2 of 630 light.

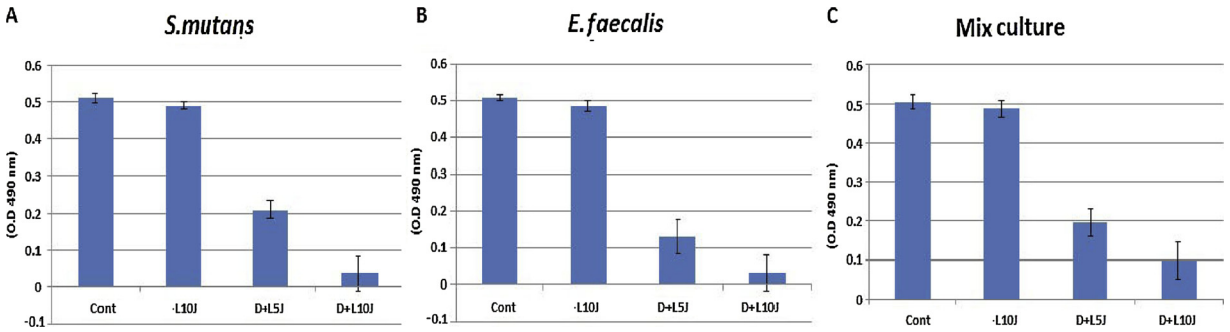

Fig. 5. EPS reduction after aPDT on (A) *S. mutans* (B) *E. faecalis* and (C) Mix culture: 10 μM of TBO were irradiated with 5 J/cm^2 and 10 J/cm^2 of light.

with NaCl shows only *E. faecalis* growth while mix culture without NaCl shows both the bacterial growth (Fig. 1c and d). So we can find out the number of *S. mutans* by subtracting the value of *E. faecalis* (grown on BHI agar plate containing NaCl) from the mix culture (grown on BHI agar plate without NaCl). After photoactivation of mixed culture with 10 J/cm^2 of light was speeded over BHI agar plates containing NaCl does not show any growth it means it eliminates all *E. faecalis* bacteria and *S. mutans* does not grow on this NaCl containing plate. However when same (mixed culture with 10 J/cm^2 of light) was speeded on BHI agar without NaCl then it shows growth, this suggested that the growth which appears is of *S. mutans* only. It is already reported that *S. mutans* tend to dominate oral flora as caries progresses [27]. We are getting the similar result as shown in Fig. 1b and c, we can see the difference that while grow as a monospecies *E. faecalis* shows much growth as compared to mixed culture (Fig. 1c). *S. mutans* suppresses the growth of *E. faecalis* in mixed culture.

The inhibition in biofilm formation was depend upon the light doses and concentration of the PS. Monospecies biofilm of *S. mutans* and *E. faecalis* inhibits biofilm formation to the same extent but dual species biofilm is less susceptible than mono species as shown in Fig. 2. It is already reported that dual species biofilm is more resistant and harder to eliminate than monospecies biofilm [28]. This may be due to the interaction of more than one type of EPS produced by these bacteria might result in a more viscous matrix [29]. This is further validated by congo red experiment we observed the same pattern of reduction as we found in CV. Dual species biofilm shows fewer EPS reduction than monospecies biofilm.

EPS in biofilm resist the entry of conventional antibiotic to their interior which leads to antibiotic-resistant strain. Penetration of PS to the interior of the biofilm is not important in aPDT because ROS is produced to the exterior is as effective in killing bacteria as it is produced inside the cell. Penetration or crossing the cell wall barrier is not necessary for aPDT. We have estimated the total ROS generated by DCFH-DA. DCFH-DA is a nonfluorescent compound which is converted to DCF, a fluorescent compound through a cascade of reaction. DCF attains its excited state of DCF by photo-excitation [30]. We found potentiation of killing is depending upon the ROS generation; it is clearly visible in Fig. 4 that ROS production increases with the increase in light doses. We found 7–7.4 folds increase in ROS generation after irradiation of 5 J/cm of light doses while 12.2–13.3 fold increased ROS production after 10 J/cm^2 of light as compared to control. Total intracellular ROS generation is more or less same in the entire treated group but photoinactivation is more pronounced in mono-species bacterial biofilm than dual species biofilm. Singlet oxygen species is considered as most important and potential ROS among all due to its high oxidative efficiency. It is reported by Nakano et al. and Tatsuzawa et al that singlet oxygen produced high oxidative damage in the prokaryotic cell but it is absolutely safe for eukaryotic cells [31,32]. AMDA was used to evaluate the amount of singlet oxygen produced by TBO and we found that singlet oxygen production is increased as we increased the light doses. Singlet oxygen produced by TBO with 10 J/cm^2 is more phototoxic to the bacterial cell than TBO with 5 J/cm^2. Singlet oxygen is capable of crossing the barrier of the cell membrane and it can also damage nucleic acid [33,34].

5. Conclusion

We have concluded that mono species bacterial biofilm are more susceptible than dual species bacterial biofilm and this may be due to the interaction of different type of EPS produced by different bacteria. *S. mutans* in dual species biofilm has become dominant over *E. faecalis* even after treatment. Antimicrobial photodynamic efficiency is directly related to the ROS production, we found that dual-species biofilm shows more resistance than monospecies biofilm.

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