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Photodynamic therapy for dental plaque treatment

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Abstract

The anatomical complexity of tooth roots provides place for bacterial deposits, making eradication of periodonto pathogens difficult. The conventional methods for the removal of dental plaque like mechanical treatment and antimicrobial therapy have their own limitations. To overcome the limitations, a novel non-invasive photochemical approach called Photodynamic Therapy (PDT) was applied which is based on the principle that a visible range of light activates a photosensitizer (PS), leading to the formation of reactive oxygen species which induce phototoxicity to the bacterial cell. In this project, a light source was designed using red Light Emitting Diode (LED) to illuminate the biofilm formed by Streptococcus viridians group. The biofilm was formed in 96 well microtiter plates and was sensitized with Toluidine Blue O (TBO) of two concentrations (500mg/l, 100mg/l). The viability of cells when evaluated using MTT ((3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay was found to be decreased significantly. It was also found that high concentration of TBO (HTBO-500mg/l) was more effective assuming that the biofilms were exposed with equal intensity of light.

Keywords: Dental plaque, Photodynamic therapy, Photosensitizer, Light emitting diode, Oral bacteria, Biofilm.

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1. Introduction

Microbial biofilms in the oral cavity are involved in the etiology of various oral conditions, including caries, periodontal and endodontic diseases, oral malodor, denture stomatitis, candidiasis and dental implant failures. Conventional mechanical plaque control to remove bacterial deposits, calculus, and cementum contaminated by bacteria and endotoxins through mechanical scaling and root planing is often difficult for elder and compromised patients because of the pain or the risk of aspiration. Similarly, removal of the plaque and the reduction of the number of infectious cells can be difficult to access by mechanical scaling and root planing. Some therapeutic alternatives, such as systemic and local antibiotics, have been used in cases not responding to conventional treatments, although this therapy brings undesirable side effects like development of bacterial resistance due to their inadequate and excessive use [1,2] as well as difficulties concerning the access of topical agents with effectiveness against the biofilm [3]. The limitation of these methods has pressurized on the development of a novel antimicrobial concept with fewer complications where Photodynamic Therapy (PDT) can be a useful adjunct to mechanical as well as antibiotics in eliminating oral bacteria. In PDT, a photosensitizing agent is used which is activated by light of specific wavelength, resulting in the production of free radicals, singlet oxygen, peroxides and other reactive oxygen species, which have a toxic effect on bacterial cells, leading to cell death without causing harm to the host [4]. This minimally invasive method is effective against resistant bacteria and has a rapid effect on the target organisms and does not lead to the development of resistance mechanisms [5]. Moreover, antimicrobial PDT is selective and painless and does not affect the patient's sense of taste. PDT has been found as a very potent method of treating dental

plaque which has not been clinically applied yet. In this study, an effective illumination system of RED LED array for 96 well culture plate was designed and the effect of photosensitizer called TBO along with RED LED light source on bacterial biofilm was determined. The bacterial sample of *S. virdians* group was collected and was cultured in Brain Heart Infusion (BHI) broth media which was then added to 96 well culture plate for biofilm formation. The biofilm formed was then quantified using Crystal Violet (CV) assay. The biofilm was then exposed with HTBO (500mg/l) with RED LED and LTBO (100mg/l) with RED LED light source. Other experimental groups like LED only, TBO only and control were also included. The results were analyzed using MTT assay. It was found that the viability of bacteria was significantly decreased in both the groups of TBO concentration but the higher reduction was obtained in HTBO and LED group. No significant reduction was observed in group exposed with LED only. However, the viability of bacteria increased to negligible amount when exposed to TBO only. Therefore, it was found that application of PDT significantly suppressed dental biofilm and was TBO dose dependent at equal intensity of light.

2. Material and Methods

A compact red LED (633 nm) array-based illumination system with a homogeneous illumination area was specifically designed for in vitro PDT to expose the biofilm grown in the standard 96 well plate. The illumination system included a 12*8 LED array which maintained constant current across each array. The heights of the LEDs were adjusted in order to achieve equal distance of illumination from each LED to the each well of 96 well plate which provided the desired irradiance and homogeneity. The distance of 8mm was maintained between the LED array and the culture plate vertically.

2.1 Bacteria culture:

The isolated bacterial sample of *S. Viridans* was collected from National Reference Laboratory (Kathmandu, Nepal). The bacterial sample was routinely cultured in Blood Agar Media (Himedia laboratories, India) following the manufacturer's instructions and the cultures were incubated under the standard conditions at 37°C in aerobic environment.

2.2 Growth of biofilm:

Following the bacteria culture, bacterial suspension was prepared by adding a loop full of bacteria in 10 ml of Brain Hearth Infusion broth (Himedia laboratories, India). The bacterial suspension was then incubated at 37°C for 24 hours. After the completion of incubation period the suspension was centrifuged at 3000 rpm for 5 minutes for three times and the supernatant was discarded. The bacterial suspension was visually adjusted at 4 McFarland equivalent solutions and 75 µl of this equivalent solution was added to each well using micropipette. The plate was shaken gently in VDRL shaker (Accumax, India) for 8 minutes before adding 125 µl of BHI broth in each well. The different plates with sample bacteria were then incubated at 37°C under aerobic environment. The biofilm formation was observed at 2, 3, 5 & 10 days of incubation period using Crystal Violet (Himedia laboratories, India) assay following the standard protocols. Media was changed at every two days interval and was replaced with fresh media by gently removing the previous media and washing each well with PBS in order to provide fresh nourishment to the bacteria in the microtiter plates. The CV assay determined maximum biomass at 5 days incubation period for this bacterial species and then same optimum condition was maintained for the biofilm formation in five days' time and experimental procedure of PDT was carried out on the samples of biofilm.

2.3 Study Design:

The experimental groups were categorized and each of the groups was incubated for five days biofilm formation by changing media at two days and four days' time. For the effective study on the experimental groups, two different concentrations (100mg/l, 500mg/l) of TBO were prepared. At the end of 5 days the media from each well was gently pipetted out, washed with PBS and air dried. 20µl of respective concentration of TBO was added to each well of microtitter plate 5 minutes prior to the exposure by RED LED. The study design included following groups:

- RED LED + HTBO (500mg/l).
- RED LED + LTBO (100mg/l).
- TBO only (100mg/l).
- RED LED only.
- NO LED and NO TBO (Control)

*The groups (1, 2, 4) containing RED LED exposure were irradiated for 7 minutes

2.4 MTT assay:

MTT assay was performed to determine the viability of cells. Firstly, 20µl of freshly prepared MTT solution (Himedia laboratories, India) was added to each well and the plates were rotated in a rotator for 20 minutes at 180 rpm. The plates were then incubated at 37°C for two hours. After incubation time, 100µl of isopropanol (Fisher scientific, UK) (100%) was then added to each well to solubilize the formazan for 30 minutes with gentle tapping from sides in each 5 minutes. The plates were then read using Elisa plate reader (Stat fax, USA) at the wavelength of 570nm.

2.5 Statistical analysis:

First, the data were evaluated to check the equality of variances and normal distribution of errors. To determine the significance of the irradiation alone, the presence of

sensitizer alone and the combination of sensitizer and light, the data were analysed by two way ANOVA model. The t-test was chosen for evaluating the significance of all pair wise comparisons with a significance limit of 5%.

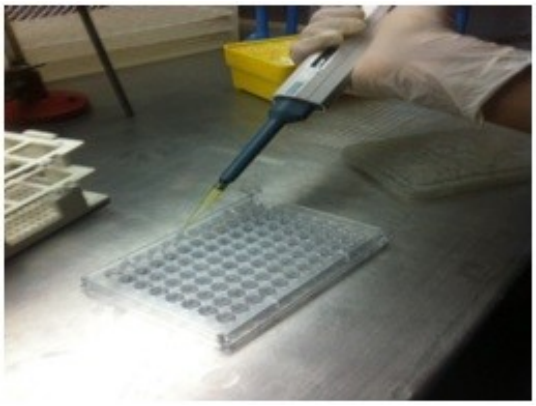

Figure 1*: in-vitro* biofilm formation on 96 well microtiter plate

3. Results

The results obtained after MTT assay of the experimental group were analysed to determine effectiveness of PDT. Among the experimental groups, the control group (no treatment) showed higher optical density, which provided an approximation on the total viable bacteria on each group before the experiment.

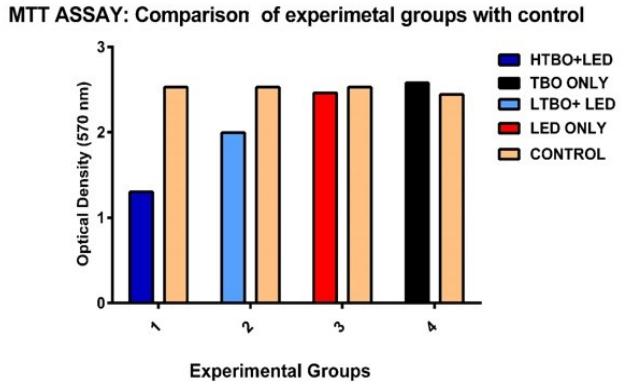

Figure 2: Comparison of viability of cells using MTT of different experimental groups

3.1 Effect of LED only:

The experimental group exposed with LED only for 7 minutes showed no statistical significant ($p>0.05$) bacterial reduction figure (2), indicating that the LED alone seems ineffective to reduce bacterial viability.

3.2 Effect of TBO only:

The experimental group exposed with TBO only for 5 minutes showed increase in viability to a little amount compared to the control. But the result was not statistically significant ($p>0.05$) figure (2), indicating that TBO alone seems ineffective in PDT.

3.3 Effect of TBO + LED:

The result obtained for two groups with different concentration of TBO showed varying results.

3.4 Effect of HTBO (500mg/l) + LED:

The experimental group exposed with HTBO for 5 minutes and irradiated with Red LED for 7 minutes showed decrease in viability to 54% compared to the control. The result was statistically significant ($p<0.05$) figure (3), indicating that TBO of concentration 500mg/l along with the exposure of light for 7 minutes is effective to reduce the bacterial viability.

3.5 Effect of LTBO (100mg/l) +LED:

The experimental group exposed with LTBO for 5 minutes and irradiated with Red LED for 7 minutes showed decrease in viability to 23% compared to the control. The result was statistically significant ($p<0.05$) figure (3), but the reduction was less compared to high concentration of TBO maintaining other factors similar.

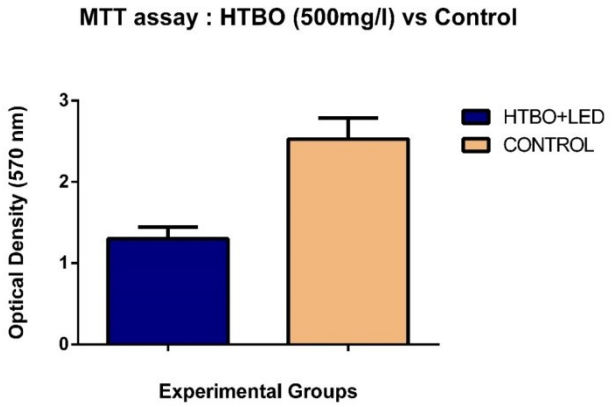

Figure 3: Comparison of viability of cells using MTT assay exposed to HTBO+LED group

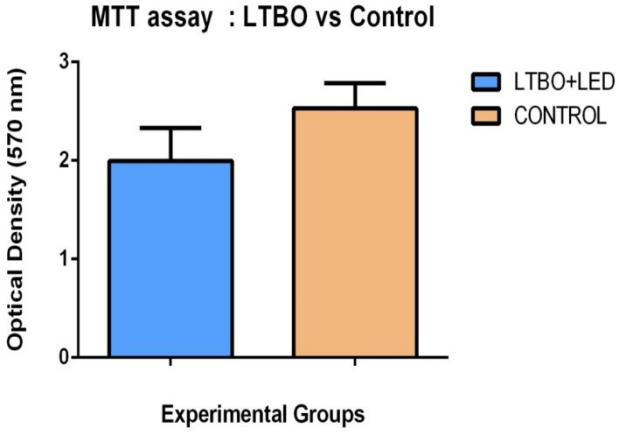

Figure 4: Comparison of viability of cells using MTT assay exposed to HTBO+LED group

4. Discussion

The result obtained in this study demonstrated that there was no noticeable decrease in viability of bacterial cell with the application of light only which was found to be 3%

and agrees with previous studies performed by Burns *et al*. [6], Williams *et al*. [7], Zanin *et al*. [8,9] and Qin *et al*. [10].

Similarly, use of TBO alone in the study increased the viability of cell at very low level of 2%. This may be due to the fact that with exposure to TBO only for 5 minutes, the bacterial mitochondria gets activated and in addition even some small amount of bacteria in dormant state might get activated after the application of TBO only. In this study, a pre irradiation time of *5*min was chosen prior to exposure to LED light. During this period of time dye had been absorbed by the cells without any detectable cytotoxic effects. Study performed by Redman R.S. *et al*. [11] demonstrated that toluidine blue has no effect as a carcinogen in the cheek pouch and there are no reports of toxicity to oral rinsing or direct topical use of a 1% toluidine blue solution in humans. Therefore TBO can be safely used as a PS within oral cavity.

However, with the use of 500mg/l TBO concentration and irradiation for 7 minutes with red LED, the viability of bacteria was decreased at significant level to approximately 54% as compared to control. Various studies have been performed on the efficacy of TBO with light source in Photodynamic Therapy and most of them have confirmed its effectiveness which supports the result of this study. Research conducted by Zanin *et al*.[12] in 2002 have found bacterial viability reduction greater than 95 % when the experiment was conducted in planktonic bacteria. Various journals N. Araujo *et al*. [13], Rolim, *et al*. [14], Williams *et al*. [7] suggested that the reduction in biofilm is quite less than compared to the bacteria in planktonic stage this is due to the fact that the biofilm structure protects the bacteria, and other microorganisms also the thickness of this structure prevents contact between antimicrobial agents and microorganisms, keeping the chemicals away from the deeper layers of the biofilm. As a result, the biofilm boosts the growth rate of the microorganisms [15-17]. Similarly, Fontana *et al*. [18] analyzed the effect of Photodynamic Therapy on dental bacterial biofilm and after PDT, survival fractions were calculated from colony-forming unit counts. It was found that the PDT produced 32% killing of bacteria which were in agreement with our result.

Another experiment conducted by Ichinose-Tsuno *et al*. [19] with TBO concentration of 100mg/l, 500mg/l, 1000mg/l it was found that the bacterial killing is maximum at 500mg/l concentration which supports to our result. Study conducted by the same group has similar findings on the examination of the effect of TBO and LED on *S. oralis* in which different TBO concentrations of 100mg/l, 500mg/l, and 1000mg/l were used. Highest reduction of bacteria was found on 500mg/l TBO concentration with the log reduction of 1.42 $\log_{10}$. The result obtained from the study demonstrated that with the use of TBO of 100mg/l Concentration, the viability of bacteria was decreased approximately to 23%. Though the reduction in viability was found to be significant, however in comparison to 500mg/l concentration of TBO the reduction was less. As the experiment in this study was performed on biofilm instead of planktonic cell suspension, this might be the reason of less reduction in bacterial viability obtained with 100mg/l TBO concentration.

5. Conclusion

Photodynamic therapy seems to be a unique therapeutic approach towards the treatment of dental plaque. The application of PDT along with photosensitizer significantly suppressed the dental plaque causing bacteria in in-vitro conditions. The results obtained in the study indicate that the red LED when used along with TBO as a photosensitizer, decreases the bacterial viability at significant level. This study showed that neither of the light sources nor photosensitizers alone had any effect on *S. viridians* viability, which is in accordance to other similar articles. This study provides initial data on a potentially new approach of dental plaque treatment by forming biofilm in in-vitro conditions. Further validation to determine optimum condition including dye concentration, current intensity, and the frequency of procedure as well as any potential adverse effects are required in order to establish a safe and effective PDT procedure. Further studies on the effect of PDT, not only on single species of bacterial but on oral biofilms, are still required to obtain more definitive and certain results. Although studies have demonstrated that the combination of light and a dye is an effective approach to inactivate microbials, some variables still influence the outcome, such as the nature and concentration of the dye, the cariogenic microorganism species, the light source, experiment performed with and without continuous supply of saliva substitutes as well as the duration and dose of exposure to light. The LED devices which can be shaped into numerous forms and sizes and are cost-effective, may replace currently used light source and their fibre optics for dental plaque treatment.

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