Effectiveness of erythrosine-mediated photodynamic antimicrobial chemotherapy on dental plaque aerobic microorganisms: A randomized controlled trial

Manohar Bhat, Swathi Acharya,1 Kakarla Veera Venkata Prasad,2 Raghavendra Kulkarni,3 Anithraj Bhat,4 Devikripa Bhat5

Abstract:
Background: Dental plaque is one of the predominant causes of major oral diseases. Although mechanical and chemical methods are extensively followed to control the development of plaque, plaque-related diseases still persist. Therefore, this necessitates for alternative measures of plaque control, one such alternative is photodynamic antimicrobial chemotherapy (PACT). Materials and Methods: Split mouth randomized clinical trial (CTRI/2017/03/008239) was conducted on 30 participants who reported to the hospital. Participants were asked to rinse their mouth for 1 min using 10 ml of 25 μM erythrosine solutions. Same tooth on both quadrants of the same jaw are selected as the test and control. Intervention used was halogen-based composite curing light with wavelength of 500–590 nm. Plaque sample from the control tooth and test tooth was collected before and after exposure, respectively, and sent to microbiological laboratory for colony count. Results: Logarithmic mean and standard deviation of control group with 105 dilutions of aerobic microbial count were found to be 5.34 ± 0.94, and for experimental group, it was 4.47 ± 1.37. The statistical difference between mean CFU values between aerobic bacterial counts was significant (P = 0.006). Conclusions: Erythrosine-mediated PACT reduces the extent of dental plaque microbial count and has a potential preventive and therapeutic use in day-to-day life and dental clinics.

Key words: Dental plaque, erythrosine, photodynamic antimicrobial chemotherapy

INTRODUCTION

Dental plaque is demarcated clinically as a structured, resilient, yellow-greyish substance that adheres tenaciously to the intraoral hard surfaces, including removable or fixed restorations.1,2 It embraces of microorganisms and intercellular matrix which are allied with a range of oral diseases including dental caries and periodontal diseases, root canal failures, halitosis, denture stomatitis, moniliasis, and dental implant failures.3 Elimination of dental plaque is considered as basic necessity in preventing these diseases.3-6 Existing therapeutic procedures involve either episodic mechanical disruption of oral microbial biofilms as in mechanical plaque control or preserving therapeutic concentrations of antimicrobials in the oral cavity as in chemical plaque control.7 However, mechanical plaque control can be the subject to meager patient compliance7,8 and may be unsuitable for some patients with the mechano-blistering disease, for example, epidermolysis bullosa, where mechanical strength can damage the oral mucosa and cause intolerable pain and distress.9 Antibacterial agents are also extensively used either in local or systemic formats, but it has a risk of development of bacterial resistance on long-time usage.10 Although these plaque control methods are used every day, oral problems related to biofilm still exist. Hence, the development of alternative antibacterial

This is an open access article distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 3.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as the author is credited and the new creations are licensed under the identical terms.

How to cite this article: Bhat M, Acharya S, Prasad KV, Kulkarni R, Bhat A, Bhat D. Effectiveness of erythrosine-mediated photodynamic antimicrobial chemotherapy on dental plaque aerobic microorganisms: A randomized controlled trial. J Indian Soc Periodontol 2017;21:210-5.
therapeutic strategies becomes important in the evolution of methods to control microbial growth in the oral cavity.

The practice of photodynamic therapy for inactivating microorganisms was initially validated more than 100 years ago. Photodynamic antimicrobial chemotherapy (PACT) is a therapeutic procedure that utilizes light energy to activate a photosensitizing agent (photosensitizer) in the presence of oxygen. The principle of the PACT is that the photosensitizer undergoes a transition to a higher energy state, generating an extremely reactive state of oxygen and this singlet oxygen causes a venomous effect on microorganisms. It shows minimum resistance because it inhibits the plasmid interchange involved in the transfer of antibiotic resistance. This gives more impact on the treatment against resistant microorganisms.

Most of the oral infections are biofilm linked and localized in nature. These diseases are well studied in PACT against systemic antimicrobials. However, more instant advantage could be derived from photosensitizers already permitted for oral use. One such photosensitizer is erythrosine, which is an organic iodine compound that has photodegradable property when introduced to a light of 530–590 nm. It is primarily used for food coloring and also as dental plaque disclosing agent. It is economical and least harmful to the body. Hence, this study was undertaken to evaluate the effect of erythrosine-mediated PACT on plaque microorganisms.

MATERIALS AND METHODS

The present research was two-arm simple randomized controlled trial using split-mouth design conducted over 2 weeks. Before the start of the main study, a pilot study was conducted on 10 participants. Intra-examiner calibration on plaque and gingival score was done among 10 participants before the start of the research; intra-examiner reliability came around 90%. Estimation of sample size was done under 5% error and 95% power of the test. The required sample size came up to 29 which were rounded up to 30 in each group. Ethical clearance was obtained former to the start of the study.

Prospective adult participants who indicated willingness to participate in the study were scheduled for screening visit by the examiner. All enrolled participants were examined by the examiner, who conducted an oral examination. All the participants were asked to rinse their mouth for 1 min using 10 ml of 25 µM erythrosine solution (Alpha Plac red no. 3, DPI). Participants who had similar plaque scores on the contralateral tooth of the same arch were included and participants with orthodontic appliances or more than one incisor, with prosthetic crown, those who required immediate care, destructive periodontal disease, pregnant and breastfeeding women, chronic systemic conditions (heart, kidneys, liver, or infectious diseases including AIDS), those undergoing antibiotic or steroid therapy in the preceding month were excluded from this study.

Clinical procedures

To avoid selection bias, randomization was performed with lottery method. In the present study, standardization of dental plaque was done by considering the same tooth on both quadrants of the same jaw as experimental and control tooth. First, plaque sample from the control tooth was collected separately under sterile condition. After collecting the control sample, a composite curing tungsten filament light source with 500–600 nm wavelength was directed over the experimental tooth for 60 s. Plaque sample from the experimental tooth was collected separately under sterile condition and sent for microbiological analysis. Here, control samples were collected first to eliminate the spill-over effect of light application into the control area.

Microbiological procedure

Collection and transport

The dental plaque samples were collected by a sterile Columbia scaler from the tooth surface and immediately transferred to a sterile plastic tube containing 4 ml transport media (Thioglycollate Broth, TG broth) seeded with a few sterile 0.5-mm glass beads. The plastic tubes were vortexed at high setting to disperse the plaque, so as to obtain homogenous suspension.

Dilution

All the dilutions were performed in a sterile biosafety cabinet; the cabinet was treated with ultraviolet light for 30 min before the beginning of the procedure for the...
sterilization of the cabinet. Dilution of the plaque solutions was done by adding 1 ml plaque solution into a sterile tube containing 9 ml of saline seeded with glass beads. These tubes were vortexed at high settings so that the solutions form a uniform $10^2$ fold dilution. $10^2$ fold dilutions were performed by adding 1 ml of $10^1$ fold diluted solution into 9 ml of saline and vortexed at high settings. Similarly, serial dilutions were performed till $10^5$ dilutions. Based on the pilot study results, the dilutions to be used for aerobic and anaerobic bacterial colony counting was determined. The dilutions found to be $10^1$, $10^2$, and $10^3$ for aerobic bacterial colony culture.

**Inoculation**

The 5% brain–heart infusion (BHI) agar (HiMedia laboratories, Mumbai) plates were kept under sterile condition. The plates were incubated for 24 h before the inoculation procedure to ensure sterility.

About 100 μl from abovementioned dilutions, aerobic, and anaerobic bacterial cultures were inoculated onto 5% BHI agar plates. A sterile "L"-shaped spreader and a turntable were used to ensure the uniform spreading of diluted dental plaque sample. Inoculated plates with aerobic bacterial culture were marked with subject and sample characteristics. Then, the plates were incubated in air at 37° centigrade for 24 h.

**Colony counting**

All plates with well-dispersed colonies were read for the number of colony-forming units using the digital colony counter [Figure 4].

**Statistical analysis**

The collected data were entered into the computer (MS-Office, Excel 2010) and subjected to statistical analysis using the statistical package – SPSS version 20 (IBM Statistical Package for Social Sciences Version 20; IBM corporation). Normal distribution was determined by Kolmogorov–Smirnov and Shapiro-Wilk test after logarithmic transformation of the mean values of colony forming units. Independent sample $t$-test was used to know the statistical significant differences between the two groups.

**RESULTS**

A total of 30 participants with 22 males of mean age 31.9 years and 8 females with mean age of 29.75 years were included in the present study.

**Aerobic colony counts**

Control and experimental group with dilution $10^2$ showed $290.50 \pm 216.53$. After logarithmic transformation, the mean and standard deviation of control group of the same dilution showed $5.34 \pm 0.94$. Similarly, mean and standard deviation of experimental group with $10^2$ dilutions and its logarithmic transformation showed $155.9 \pm 141.69$ and $4.47 \pm 1.37$, respectively [Table 1 and Figure 5].
Comparison of mean difference between control and experimental groups using independent sample t-test for aerobic bacterial count with $10^5$ dilutions were performed. Levene’s test of equality of variance in different samples were used to determine the assumption of equal variances or not. Here, t-test assumes the variance of the population from which different samples were drawn were equal. It tests the null hypothesis that the population variances were equal. The $P$ value of Levene’s test for Equality of Variances here is 0.123 which was more than that of 0.05. Hence, the null hypothesis of equal variances was accepted. Based on this, the $P$ value of independent sample t-test with equal variance was assumed. Independent sample t-test with equal variance showed that there was highly statistical significant difference among the control and experimental group ($P = 0.006$) for aerobic bacterial count with $10^5$ dilutions [Table 2].

**DISCUSSION**

Oral health is an essential portion of overall health; it has been rightly said that one is not healthy without good oral health.$^{[11,12]}$ One of the key factors which play a major role in the oral health is dental plaque. Controlling the dental plaque will help us in controlling most of the oral diseases including caries, endodontic and periodontal diseases, halitosis, candidiasis, and dental implant failures.$^{[13]}$ Present therapeutic techniques comprise either intermittent mechanical elimination of oral microbial biofilms or maintaining the antimicrobial concentrations in the oral cavity, both of which are with limitations.$^{[13]}$ The problem here is the progress of bacterial resistance to antimicrobial agents. It is commonly recognized that the development of bacteria in biofilms indicates a substantial decrease in susceptibility to antimicrobial agents matched with cultures grown in suspension.$^{[13,14]}$ The development of alternative antibacterial therapeutic strategies which do not develop bacterial resistance, therefore, becomes important in the development of methods to regulate microbial growth in the oral cavity.$^{[13]}$

In the present study, we targeted plaque microorganisms using the photodynamic antimicrobial chemotherapeutic principle. The singlet oxygen (Nascent oxygen) used in PACT, which has a diffusion distance of approximately 100 nm (137) and a half-life of $<0.04$ s$^{[13,14]}$ shows no resistance observed by any bacteria as it directly targets on cell wall and DNA of the bacteria.$^{[13,15,17]}$

Erythrosine is an organoiodine compound also known as Red No. 3. It is fluorine derivative, cherry-pink in color, chiefly used for food coloring. Its maximum light-absorbance is at 530 nm in an aqueous solution, and it is subjected to photodegradation. Dentists currently use erythrosine as dental plaque disclosing solution or tablets. Erythrosine retains a positively charged surface, thereby it can directly aim on both Gram-positive and Gram-negative bacteria.$^{[10,13,18-20]}$ The positive charge of photosensitizer shows high affinity toward the negatively charged outer bacterial membrane. This primarily induces localized damage, which helps its penetration$^{[21,22]}$ and the ability of erythrosine to initiate photochemical degradation reactions is well explained.$^{[17,22,23]}$ The killing efficacy of photoactivated erythrosine is also well documented.$^{[7,24]}$ Evidently, erythrosine has a gain over other photosensitizers, as it already aims dental plaque and has full agreement for use in the oral cavity. In the present study, we used erythrosine of 25 µM concentrations which is 1000 folds less concentrated than that of plaque disclosing solution. In many studies, this concentration showed effective antimicrobial property.$^{[7,25]}$

The light source utilized in the present research was Quartz–halogen-based composite curing unit with wavelength of light ranging from 500 to 600 nm which is parallel to the range of light source required for activation of Erythrosine.

The power output from 75 watts halogen dental curing unit was 663.72 mW/cm² which was calculated with the formula $I = P/A$, where $I$ is intensity of light, $P$ is power of the light in Watts, $A$ is surface area of the globe of light distribution, and $A = 4πr^2$ where $r$ is the radius in meter.

The present study is two-arm simple randomized controlled trial using split mouth designs. Because the patients serve as their own control, which can intensify the statistical efficiency, on an average, fewer patients are needed.$^{[26]}$ Carryover effect was eliminated by collecting the control sample before the light exposure (intervention) on the experimental group.

Light application was performed only for 60 s on the test side. Even though approximately 98% of the photocytolysis action was revealed by erythrosine in 5 min,$^{[7,27]}$ in the present study, we exposed for only 1 min (60 s). The time limitation of 60 s was maintained for two reasons – Manufacturers of curing gun have mentioned to use 1 min in each 5 min gap, and beyond 60 s the lamp would get heated up, causing discomfort to both subject and operator.

Table 1: Mean and standard deviation and their logarithmic transformation of aerobic colony counts

| Colony | Dilution | Group | $n$ | Mean±SD | Mean after log transformation | SD after log transformation |
|--------|----------|-------|-----|---------|-------------------------------|-----------------------------|
| Aerobic | Hundred  | Control | 30  | 290.50±216.53 | 5.34                          | 0.94                        |
| Aerobic | Experimental |          | 30  | 155.90±141.69 | 4.47                          | 1.37                        |

$n$ – Sample size, SD – Standard deviation

Table 2: Comparison of mean difference between control and test groups for aerobic bacterial colony count

| Colony | Dilution | Levene’s test for equality of variances | $F$ | $P$ | t-test for equality of means | $P$ |
|--------|----------|----------------------------------------|-----|-----|-----------------------------|-----|
| Aerobic| Log hundred | Equal variances assumed                | 2.450 | 0.123 | 2.854                       | 0.86 | 0.006* |
| Aerobic| Log hundred | Equal variances not assumed            | 2.854 | 0.86  | 2.854                       | 0.86 | 0.006* |

*Significant association set at ≤0.05; $F$ value and $T$ value are the part of Independent sample t test; $P$ – Probability Value (p value)
Light dose was calculated by multiplying the output power by the irradiation time as given in the succeeding equation:

\[
\text{Light dose (J/cm}^2) = \frac{\text{Output power (mW / cm}^2 \times \text{irradiation time (s)}}{1000}
\]

On calculation, the light dose or fluence in the present study is around 39.82 J/cm² which is very less compared to the light dose used for the killing of premalignant and malignant cells. The light dosage in the present study was determined because of the following reason – it should not cause any collateral damage as the photosensitizing concentrations and light energy doses needed to kill the infecting microorganism have little effect on adjacent host tissues. There are still concerns about short-term and long-term effect of PACT on biological tissues. Luan et al. conducted a research on safety of toluidine blue facilitated photosensitization to periodontal tissues in mice and stated that no necrotic or inflammatory changes were found in periodontal tissues following photodynamic therapy. This shows that PACT is a harmless treatment, that does not damage the adjacent normal tissues.

It was observed that there was highly significant difference \((P = 0.006)\) among control and experimental group for aerobic bacterial count with \(10^3\) dilutions. It showed statistical and laboratorial significant reduction in microbial count in the experimental group. This reduction in bacterial count was in line with the other studies done in vivo and in situ. Some research has shown the utilization of PACT in the therapy of a number of pathogenic bacterial, fungal, and viral infections.

**CONCLUSION**

Therefore, it can be concluded that erythrosine-mediated PACT reduces the extent of dental plaque microbial count and might have a potential preventive and therapeutic use in day-to-day life and in dental clinics.

**Declaration of patient consent**

The authors certify that they have obtained all appropriate patient consent forms. In the form the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

**Acknowledgement**

We wish to thank Department of Microbiology for useful comments and laboratory assistance.

**Financial support and sponsorship**

Nil.

**Conflicts of interest**

There are no conflicts of interest.

**REFERENCES**

1. Teughels W, Quirynen M, Jakubovics N. Periodontal microbiology. In: Takei N, Carranza K. Carranza’s Clinical Periodontology. 11th ed. St. Louis: Saunders, 2011. p. 232-70.
2. Bhat MA, Prasad KV, Trivedi D, Acharya AB. Dental plaque dissolving agents: An in vitro study. Int J Adv Health Sci 2014;1:1-7.
3. van der Weijden GA, Hioe KP. A systematic review of the effectiveness of self-performed mechanical plaque removal in adults with gingivitis using a manual toothbrush. J Clin Periodontol 2005;32 Suppl 6:214-28.
4. Prasad KV, Sreenivasan PK, Patil S, Chhabra KG, Javalib SB, DeVizio W, et al. Removal of dental plaque from different regions of the mouth after a 1-minute episode of mechanical oral hygiene. Am J Dent 2011;24:60-4.
5. Bergrström J, Batva S, Al-Otaibi BM. The effectiveness of chewing stick miswak on plaque removal. Saudi Dent J 2006;18: 125-33.
6. Terézhalm GY, Biesbrock AR, Walters PA, Grender JM, Bartízek RD. Clinical evaluation of brushing time and plaque removal potential of two manual toothbrushes. Int J Dent Hyg 2008;6:321-7.
7. Loe H. Oral hygiene in the prevention of caries and periodontal disease. Int Dent J 2000;50:129-39.
8. Sreenivasan P, Gaffar A. Antiplaque biocides and bacterial resistance: A review. J Clin Periodontol 2002;29:965-74.
9. Konopka K, Gosliński T. Photodynamic therapy in dentistry. J Dent Res 2007;86:964-707.
10. Meisel P, Kocher T. Photodynamic therapy for periodontal diseases: State of the art. J Photochem Photobiol B 2005;79:159-70.
11. Petersen PE. The world oral health report 2003: Continuous improvement of oral health in the 21st century – The approach of the WHO Global Oral Health Programme. Community Dent Oral Epidemiol 2003;31 Suppl 1:1-23.
12. Evans CA, Kleinman DV. The Surgeon General’s report on America’s oral health: Opportunities for the dental profession. J Am Dent Assoc 2000;131:1721-8.
13. Soukos NS, Goodson JM. Photodynamic therapy in the control of oral biofilms. Periodontol 2000 2011;55:143-66.
14. Moan J, Berg K. The photodegradation of porphyrins in cells can be used to estimate the lifetime of singlet oxygen. Photochem Photobiol 1991;53:549-53.
15. Bertolini G, Lauro FM, Cortella G, Merchàt M. Photosensitizing activity of hematoporphyrin on Staphylococcus aureus cells. Biochim Biophys Acta 2000;1475:69-74.
16. Romanova NA, Brovko LY, Moore L, Pometun E, Savitsky AP, Ugarova NN, et al. Assessment of photodynamic destruction of Escherichia coli O157:H7 and Listeria monocytogenes by using ATP bioluminescence. Appl Environ Microbiol 2003;69:6393-8.
17. Schäfer M, Schmitz C, Horneck G. High sensitivity of Deinococcus radiodurans to photodynamically-produced singlet oxygen. Int J Radiat Biol 1998;74:249-53.
18. Merchàt M, Bertolini G, Giacomini P, Villanueva A, Jori G. Meso-substituted cationic porphyrins as efficient photosensitizers of gram-positive and gram-negative bacteria. J Photochem Photobiol B 1996;32:153-7.
19. Minnock A, Vernon DI, Schofield J, Griffiths J, Parish JH, Brown ST, et al. Photoinactivation of bacteria. Use of a cationic water-soluble zinc phthalocyanine to photoactivate both gram-negative and gram-positive bacteria. J Photochem Photobiol B 1996;32:139-64.
20. Wilson M, Burns T, Pratten J, Pearson GJ. Bacteria in supragingival plaque samples can be killed by low-power laser light in the presence of a photosensitizer. J Appl Bacteriol 1995;78:569-74.

21. Merchat M, Spikes JD, Bertoloni G, Jori G. Studies on the mechanism of bacteria photosensitization by meso-substituted cationic porphyrins. J Photochem Photobiol B 1996;35:149-57.

22. Tran J, Olmsted J. Intramolecular triplet-triplet energy transfer from xanthene dyes to an anthryl substituent. J Photochem Photobiol A Chem 1993;71:45-9.

23. Conlon KA, Berrios M. Light-induced proteolysis of myosin heavy chain by Rose Bengal-conjugated antibody complexes. J Photochem Photobiol B 2001;65:22-8.

24. Krasnoff SB, Faloon D, Williams JE, Gibson DM. Toxicity of xanthene dyes to entomopathogenic fungi. Biocontrol Sci Technol 1999;9:215-25.

25. Lee YH, Park HW, Lee JH, Seo HW, Lee SY. The photodynamic therapy on Streptococcus mutans biofilms using erythrosine and dental halogen curing unit. Int J Oral Sci 2012;4:196-201.

26. Antczak-Bouckoms AA, Tulloch JF, Berkey CS. Split-mouth and cross-over designs in dental research. J Clin Periodontol 1990;17:446-53.

27. Metcalf D, Robinson C, Devine D, Wood S. Enhancement of erythrosine-mediated photodynamic therapy of Streptococcus mutans biofilms by light fractionation. J Antimicrob Chemother 2006;58:190-2.

28. Garg AD, Bose M, Ahmed MI, Bonass WA, Wood SR. In vitro studies on erythrosine-based photodynamic therapy of malignant and pre-malignant oral epithelial cells. PLoS One 2012;7:e34475.

29. Andersen R, Loebel N, Hammond D, Wilson M. Treatment of periodontal disease by photodisinfection compared to scaling and root planing. J Clin Dent 2007;18:34-8.

30. Christodoulides N, Nikolaidakis D, Chondros P, Becker J, Schwarz F, Rössler R, et al. Photodynamic therapy as an adjunct to non-surgical periodontal treatment: A randomized, controlled clinical trial. J Periodontol 2008;79:1638-44.

31. Luan XL, Qin YL, Bi LJ, Hu CY, Zhang ZG, Lin J, et al. Histological evaluation of the safety of toluidine blue-mediated photosensitization to periodontal tissues in mice. Lasers Med Sci 2009;24:162-6.

32. Pe MB, Sano K, Inokuchi T. Effects of photodynamic therapy in the normal mouse tongue. J Oral Maxillofac Surg 1993;51:1129-34.

33. Soukos NS, Wilson M, Burns T, Speight PM. Photodynamic effects of toluidine blue on human oral keratinocytes and fibroblasts and Streptococcus sanguis evaluated in vitro. Lasers Surg Med 1996;18:253-9.

34. Wainwright M. Photodynamic antimicrobial chemotherapy (FACT). J Antimicrob Chemother 1998;42:13-28.

35. de Almeida JM, Theodoro LH, Bosco AF, Nagata MJ, Oshiiwa M, Garcia VG, et al. In vivo effect of photodynamic therapy on periodontal bone loss in dental furcations. J Periodontol 2008;79:1081-8.

36. Bonsor SJ, Nichol R, Reid TM, Pearson GJ. Microbiological evaluation of photo-activated disinfection in endodontics (an in vivo study). Br Dent J 2006;200:337-41.

37. Hayek RR, Araújo NS, Gioso MA, Ferreira J, Baptista-Sobrinho CA, Yamada AM, et al. Comparative study between the effects of photodynamic therapy and conventional therapy on microbial reduction in ligature-induced peri-implantitis in dogs. J Periodontol 2005;76:1275-81.

38. Walsh LJ. The current status of laser applications in dentistry. Aust Dent J 2003;48:146-55.