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Research Article

EFFECTIVE PHOTOSENSITIZING ABILITY OF THREE DYES AT VARYING CONCENTRATIONS USING CULTURING TECHNIQUES – AN IN-VITRO STUDY

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A R T I C L E I N F O

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A B S T R A C T

Introduction: Although different photosensitization agents/dyes have been extensively used in literature, but little is correlated to their actual absorption spectrum or concentrations when analyzing the results obtained from those studies. Also whether the bactericidal effect is due to the agent itself or effect of photo activation is not clear.

Aims and Objectives:
1. To evaluate the absorption and spectrum of range of 3-dyes using Spectrophotometer.
2. To evaluate the bactericidal efficacy of 3-dyes at varying concentrations using culture technique.
3. To evaluate the difference in bactericidal efficiency of each dye on photoactivation.

Methodology: The absorption and the spectrum of range for 3-dyes at varying Concentrations (Methylene blue 1%, 2% ; Erythrosine 2%, 3%; Eosine 1%, 3%) was evaluated using a Spectrophotometer. To determine the photosensitizing effect of the dyes at varying concentrations, two different experiments were performed for each concentration on blood-agar plates. Zone of bacterial inhibition was evaluated on parallel set of dyes with/ without photoactivation using 980nm diode laser.

Results: Photo-activation of the dyes at suitable concentration leads to a decrease in the microbial count.

Conclusion: Greater microbicidal benefits can be achieved if the absorption range and concentration of a dye are correlated to the energy delivered for photo-activation.

INTRODUCTION

Light has always been used for therapeutic purposes. Beneficial as well as adverse reactions of solar light have been known for many centuries. Ultraviolet radiation was applied for therapeutic purposes, e.g., for the treatment of skin tuberculosis or rickets.[1]Numerous intensive studies triggered by Raab *et al*'s (1899) serendipitous observation led to the discovery that many dyes and pigments, such as eosin and chlorophyll, can sensitize various organisms and tissues, including human skin, to light.[2]When reports emerged on light absorbing properties and of fluorescence of various dyes, it became clear that dye excitation by light exerts destructive action in biological systems. This so-called 'photodynamic action' was described as a process in which light, after being absorbed by dyes sensitizes organisms for visible light inducing cell damage.[1]Photodynamic therapy (PDT) was introduced in medical therapy in 1904 as the light-induced inactivation of cells, microorganisms or molecules and is based on the principle that a photosensitizer (i.e. a photoactivatable substance) binds to the target cells and can be activated by light of a suitable wavelength in the presence of oxygen.[3]Photosensitizers used in PDT include: (i) phenothiazine dyes[Methylene Blue (MB) and Toluidine Blue O]; (ii) phthalocyanines [aluminum di sulphonated phthalocyanine and cationic Zn(II)-phthalocyanine]; (iii)chlorines [chlorin e6, Sn(IV)chlorin e6, chlorin e6-2.5 Nmethyl-d-glucamine], and polyethyleneimine conjugates of chlorin e6; (iv) porphyrins (hematoporphyrin HCl, Photofrin®); (v) xanthenes (erythrosine, eosin); and (vi) monoterpene (azulene).[4]

The oral cavity is especially suitable for photodynamic therapy (PDT) because it is relatively accessible to illumination.Local infections such as those that occur within the oral cavity may be potential targets for antibacterial photodynamic therapy. The supra and sub-gingival plaque biofilm on tooth surfaces consists of diverse community of micro-organisms embedded in an extracellular matrix of polymers (EMP) of host and microbial origin. In the biofilm, bacteria exhibit increased

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resistance to antibiotics, environmental stresses and host immune defense mechanisms. Thus, periodontal diseases are promising applications as the biofilms should easily be accessible for flushing with the dye and for activating them by light.[5] Although different photosensitization agents/dyes have been extensively used in literature, but little is correlated to their actual absorption spectrum or concentrations when analyzing the results obtained from those studies. Also whether the bactericidal effect is due to the agent itself or effect of photoactivation is not clear. Hence, the aim of the present study was to evaluate the absorption and spectrum of range of 3-dyes at varying concentrations using Spectrophotometer. The objectives of the study were to: 1) To evaluate the bactericidal efficacy of 3-dyes at varying concentrations using microbial culturing techniques and 2) To evaluate the difference in bactericidal efficiency of each dye on photoactivation using 980 nm diode laser.

MATERIALS AND METHODS

This in-vitro study population was carried in the department of Periodontology and department of Microbiology of M.G.Vs K.B.H Dental College and Hospital and the department of Pharmacology of M.G.Vs College of Pharmacy. Ethical clearance was obtained from Institutional Ethical Committee (IEC) and the guidelines of declaration of Helsinki were strictly followed. The absorption and the spectrum of range for 3-dyes at varying concentrations (Methylene blue 1%, 2% ; Erythrosine 2%, 3%; Eosin 1%, 3%) was evaluated using a Spectrophotometer(Figure 1). The dyes at varying concentrations were placed in the transport vessels of the spectrophotometer and the readings were noted on the computer. To determine the photosensitizing effect of the dyes at varying concentrations, two different experiments were performed for each concentration on a two-section 2% blood-agar plate. The blood agar plates were smeared with plaque samples(supra and sub-gingival plaque collected with the help of Gracey Curette, later dipped in 1 ml saline and incubated for 24 hrs) with the spreader (Figure 2).The plaque samples were taken from a periodontal pocket (>5mm)from a single site. Later the blood agar plates were punched with a cork borer (8mm), three puches on each side of the plate were created and the prepared dyes were inserted into the wells. Care was taken to follow the same sequence of concentration and types in both sectins of the agar plate. Photoactivation using diode laser (Biolase® USA, 980nm, 0.5 V, 30 s, non-contact mode)[6]was done on one side of the plate whereas other side was left without photoactivation (Figure 3).The agar plates were then incubated for 24 hours and a zone of bacterial inhibition was evaluated if present.Unpaired t-test was performed to determine the difference between the zone of inhibition between theparallel set of dyes with/ without photoactivation. The value of $p < 0.05$ was considered significant.

Figure 1 Spectrophotometer used in the study to determine the wavelengths of three dyes at varying concentrations.

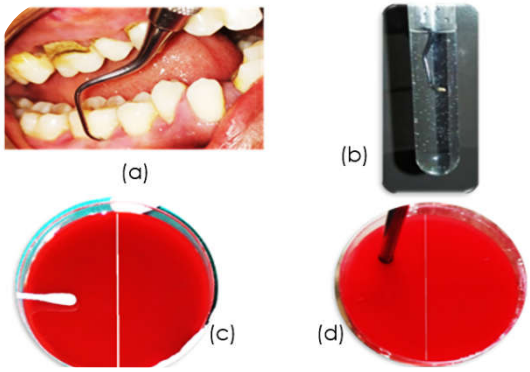

Figure 2 Figure showing sample collection and the preparation of agar plates. (a) Collection of supra and subgingival plaque from pocket (>5mm) with gracey curette. (b) Plaque sample incubated in 10 ml saline for 24 hrs. (c) Plaque sample smeared on an agar plate. (d) Punches created on the agar plate, 3 punches on each side.

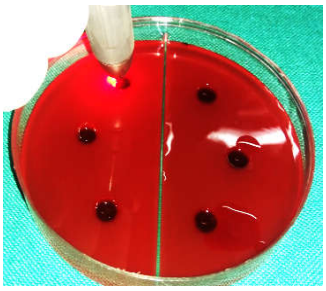

Figure 3 Photoactivation done on one side, using diode laser 980 nm, 0.5 V, 30 sec andthe other side left without photoactivation.

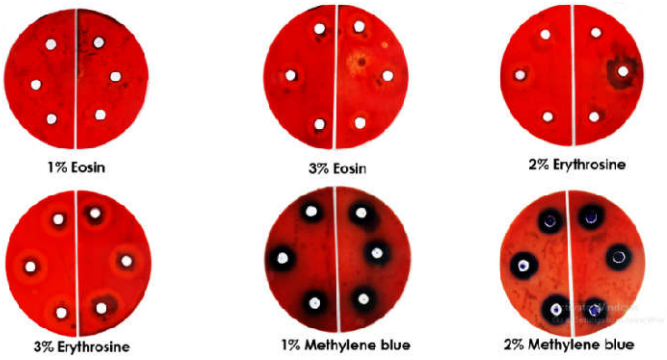

Figure 3 Zone of inhibition around dyes.

RESULTS

Thewavelengths of different dyes at varyingconcentrations was evaluated using spectrophotometer (Table 1). It was observed that highest wavelength was seen with 1% methylene blue (661 nm) followed by 2% methylene blue (656 nm). The lowest wavelength was seen with 3% eosin (416 nm).

Table 1 Table showing wavelengths of the dyes obtained with Spectrophotometer.

Dyes	Wavelength (λ_{max})
1% methylene blue	661 nm
2% methylene blue	656 nm
1% eosin	423 nm
3% eosin	416 nm
2% erythrosine	525 nm
3% erythrosine	536 nm

To determine the photosensitizing effect of the dyes at varying concentrations, photoactivation was done on one side of the

agar plate and the other side was left without photoactivation. The agar plates were incubated and the zone of inhibition was evaluated. It was seen that the zone of inhibition was increased on the photoactivated side and this difference was statistically significant (Table 2). The mean zone of inhibition was the greatest for 3% erythrosine dye with photoactivation and the least for 1% and 2% eosin without photoactivation.

Table 2 Mean zone of inhibition of the dyes with/without photoactivation.

Dyes	Mean zone of inhibition (cm)		P value
	With photoactivation	Without photoactivation	
1% Methylene blue	5.66	3.33	0.0078
2% Methylene blue	6.33	3.33	0.0335
1% Eosin	6	3	0.0213
3% Eosin	5.66	3	0.0161
2% Erythrosine	6	3.33	0.0161
3% Erythrosine	7.33	3.33	0.0011

DISCUSSION

Photodynamic therapy is arelatively new type of noninvasive phototherapy for bacterial elimination which uses low-level laser light.[7] Unlike high-level lasers, photodynamic therapy can selectively target the bacteria without potentially damaging the host tissues.[8]

The photosensitizer is placed directly in the periodontal and peri-implant pocket and the liquid agent can easily access the whole root or implant surface before activation by the laser light through placement of the optical fiber directly in the pocket. As a result of the technical simplicity of the method and the high effectiveness of bacterial killing, the application of antimicrobial photodynamic therapy in the treatment of periodontal and peri-implant diseases has been studied extensively.[9] In implantology, PDT has been reported to reduce the occurrence of peri-implantitis, an inflammatory process that results in infection of the surrounding tissues and leads to bone loss. As a secondary effect of its antimicrobial action, PDT can promote bone formation leading to osteointegration.They are also used in the treatment of oral cancer, bacterial and fungal infections, and in the photodynamic diagnosis of the malignant transformation of oral lesions. Photodynamic therapy has been suggested as an alternative to chemical antimicrobial agents to eliminate subgingival species and treat periodontitis.[10] Antimicrobial PDT not only kills the bacteria, but may also lead to the detoxification of endotoxins such as lipopolysaccharides. These lipopolysaccharides treated by PDT do not stimulate the production of pro-inflammatory cytokines by mononuclear cells. Thus, PDT inactivate endotoxins by decreasing their biological activity.[11]The advent of subablative forms of laser photonic energy along withthe application of a photosensitizer agent has shownto reduce the bacterial burden and alleviates clinicalinflammation.

An ideal PDT photosensitizer should absorb light of wavelength that falls within the visible‐red and near‐infrared region of the electromagnetic spectrum (approximately 650–900 nm), known as "the therapeutic window," where maximum penetration of light into the tissues is observed.[12]The absorbancy of light should be in the red range of the spectrum. The longer the wavelength of the incident light the deeper the penetration into the tissue.[13,14] The transmission of light decreases exponentially with increasing tissue thickness, absorbance and scattering. Binding to macromolecular structures may induce a red shift in the wavelength of maximum absorbancy.[15]

In the present study, the absorption spectrum and bactericidal efficacy of 3-dyes at varying concentrations (Methylene blue 1%, 2% ; Erythrosine 2%, 3%; Eosin 1%, 3%)was evaluated. These dyes at these concentrations are the most commonly used dyes in dentistry for photodynamic therapy. If the absorption spectrums of the dyes at varying concentrations are known more accurate results can be obtained during its use for photodynamic therapy. Greater microbicidal benefits can be achieved if the absorption range and concentration of a dye are correlated to the energy delivered for photo-activation. The development of photosensitizers with longer wavelengths of activation will allow for deeper tissue penetration.Studies have shown that methylene blue[16], erythrosine[17] and eosin[18] are potent sensitizers. However, what concentration of the dye should be used for which particular type of laser is relatively unclear. In this study, it was observed that maximum wavelength was seen with methylene blue 1%, i.e, 661 nm which is nearer to the wavelengths of the diode lasers (e.g 810nm, 940nm, 980 nm etc). Hence, the chances of the photosensitizer getting absorbed in that particular concentrations are increased. Also, in the present study the bactericidal effect of dyes with/without photoactivation was evaluated. It was observed that the zone of inhibition was more when the dyes were photoactivated. However, a zone of inhibition was also seen around the dyes which were not photoactivated. From this finding it can be stated that the dyes have a bactericidal property of their own as they were able to produce a zone of inhibition around them. Although more studies should be conducted to confirm this finding.

CONCLUSION

UsingSpectrophotometer highest wavelength was observed with 1% methylene blue (661 nm) and lowest with 3% eosin (416 nm). Photoactivation of the dyesproduced statistically significant zone of inhibition which wa maximum for 3% erythrosine.

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