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Photodynamic Inactivation of *Candida Albicans* Sensitized by Malachite Green

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Abstract: The aim of this study is to investigate the ability of malachite green (MG) combined with 650nm diode laser to kill *Candida albicans* and to spectrally study the MG photodegradation after photodynamic therapy (PDT) spectrally. Cultures of *Candida albicans* were exposed to 40mW, 650 nm diode laser in the absence of MG. In PDT group, the MG was added to the Candida suspension for 5 min then exposed to diode laser for (5, 10, 15, 20) min at power density of 0.59W/cm^2. The absorption spectrum of the photosensitized fungal suspension was obtained. The data were submitted to T-test ($p<0.05$). A 650nm diode laser in the presence of MG reduced the number of CFU/ml in 98.4%. Laser with 650nm alone and MG alone did not reduce significantly the number of CFU/ml of *Candida albicans*. Absorption spectrum showed that MG is photodegraded after irradiation .In conclusions, diode laser with 650nm was effective tool to photoinactivate *Candida albicans in* the presence of MG and that the dye is photodegraded following irradiation.

Introduction

The fungus *Candida albicans* commonly colonizes the epithelial surfaces of the body, with the oropharyngeal cavity and the vaginal tract as primary sites of mucosal colonization. It is an opportunistic pathogen able to produce both superficial and systemic infections, in immunocompromised hosts (Calderone et al., 2002).

In the last decades, Candidiasis became a human disease of increasing importance due to the high number of immunocompromised patients associated to acquired human immunodeficiency virus (HIV), use of immunosuppressants after organ transplantations and antineoplasitc (Allen C.M., 1994).

Resistance of *Candida albicans* is increasing against traditional antifungal, such as fluconazole.Therefore,the search for new therapeutic approaches is stimulated by the fact that standard antifungal treatments are prolonged and expensive and the appearance of drug resistance strains is more frequent in patients (Calzavara P.G., et al., 2005).

Recently treatment modality, known as photodynamic therapy (PDT) has been presented as a potential antimicrobial therapy. PDT is a process in which cells are treated with an agent that makes them susceptible to killing by exposure to light. Photosensitizing agents are generally macrocyclic compounds that exhibit no or minimal inherent toxicity, but result in the generation of cytotoxic reactive oxygen species when optical excitation occurs with light of the

appropriate wavelength (Dougherty T.J., et al., 1998). The first evidence that photosensitization could be lethal for microbial cells was reported in 1900 by Raab and Bertoloni G., et al., (1989) who showed that low concentrations of methylene blue were able to kill *Paramecia* upon exposure to daylight.

The application of PDT to the treatment of microbial infection is also gaining widespread interest as an alternative or adjunct to conventional antimicrobial therapy, including PDT of fungal infections (Jori, G. et al., 2006).

Wilson, et al. (1993) observed that *Candida albicans* yeasts sensitized in vitro by toluidine blue, thionine, and crystal violet associated with He-Ne laser. The higher microbial reduction was observed with toluidine blue. Photodynamic therapy associated with methylene blue was able to eradicate *Candida albicans* from the oral cavity of mice that were previously inoculated with this microorganism (TeichertM.C., et al., 2002).

Malachite green (MG) shows strong absorption at the red end of the visible spectrum and presents an easily transit through the cellular membrane in gram-positive as well as gram-negative bacterial species (Kowaltowski A.J., et al., 1999).

The aim of this study is to investigate in vitro the ability of MG associated with 650nm diode laser to kill *Candida albicans* as well as to investigate MG photodegradation after PDT spectrally.

Materials and Methods

Candida albicans strain was isolated from oral cavity from patient was kindly provided by the Central Public Health laboratory (Baghdad, Iraq).The strain was plated on Sabouraud Dextrose Agar (Himedia laboratories, India)then incubated at 37°C for 48h. After this period, growth was suspended in 5ml of sterile physiological solution (0.85%NaCl) and centrifuged at (3000r.p.m.) for 15min.This procedure was repeated twice and the pellet was resuspended in 5ml of physiological solution.The suspension was diluted in an optical density 0.5 McFarland standard solution ($5x10^6$ CFU/ml).

Malachite green (0.025mg/ml) was used as photosensitizer.The stock solution of M.G was prepared by the dissolution (0.01%) w/v of the powder in physiological solution (o.85%NaCl) then, the solution was filtered through a sterile filter membrane (0.22 µm, Millipore, Sao Paulo, Brazil).

The light source used was diode laser In-GaAIP (Dream lasers, China) with output power of 40mW and wavelength of 650 nm. The laser beam illuminated an area of (3mm) and the irradiation time was (5, 10, 15,20)min at power density of $0.59W/cm^2$.

Laser Irradiation

Suspensions of *Candida albicans* ($5x10^6$cell/ml) were divided into 4 groups of sterile ependrof tubes. The control group (L-P-) was untreated neither by laser nor by photosensitizer. In the laser group (L+P-) the suspension was irradiated for (5, 10, 15, and 20) min in the absence of the photosensitizer the tubes contain 1ml of candida suspension.

In the MG group (L-p+), malachite green was added to the suspension for (5) min in dark conditions. In the group (L+P+) which contain o.5ml of candida suspension and o.5ml MG .MG was added to the Candida suspension for (5) min and treated with laser for (5, 10, 15, 20) min respectively at power density of 0.59 W/cm^2.After irradiation procedure, serial dilutions of 10^{-2} and 10^{-3} in physiological solution were obtained from each group and aliquots of 0.1 ml were plated in triplicate on Sabouraud dextrose agar .After the incubation at 37^o C for 48h the number of colony forming units per milliliter (CFU/ml) was obtained.

MG Photodegradation

For understanding the processes that occur during photoinactivation of *Candida albicans* combined with MG and changes in MG solution before and after the irradiation, UV-VIS optical absorption spectrum was performed using a spectrophotometer (UV-VIS, SP3000, Optima, Taiwan) at fixed wavelength 650nm for group (L+P+) before and after laser irradiation .

Statistical Analysis

The variation between groups was evaluated using the student t – test, with a confidence level of 95% ($P < 0.05$) that is considered statistically significant.

Results

Figure 1 represents the viable count of *Candida albicans* for groups (L-P-), (L-P+) and (L+P-). It is clear from the result that neither the MG (0.025 mg/ml) in the absence of laser light nor the laser light in the absence of MG had a statistically significant effect on the viable count of the *Candida* suspension.

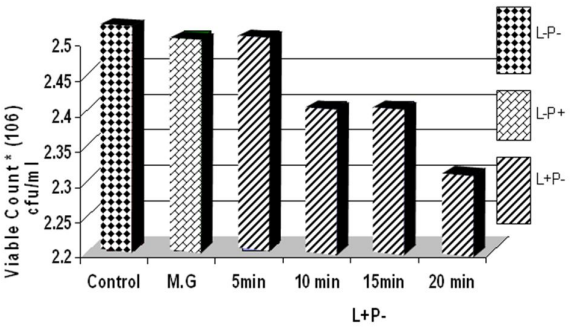

Fig. (1): Viable count of *Candida albicans* for group (L-P-) = group was untreated neither with laser nor with photosensitizer, group (L-P+) = group was treated only with photosensitizer, group (L+P-) = group was treated only with laser.

Figure 2 shows the viable count of *Candida albicans* for group (L-P-) which is the group that was untreated neither with laser nor with photosensitizer, group (L-P+) is the group that was treated only with photosensitizer, group (L+P+) is the group that was irradiated with laser in presence of the photosensitizer.

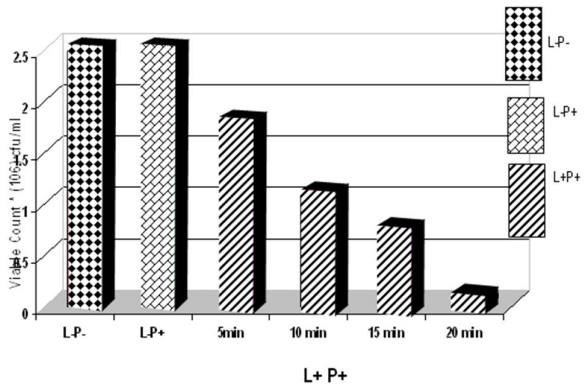

Fig. (2): Demonstrates that *Candida albicans* was eradicated to some extent by laser light in the presence of MG. The degree of photo inactivation was dependent upon the irradiation exposure time.

As can be seen from Figure 2 that the rate of killing reached 98.4 % of the original suspensions in group (L+P+) at 20min exposure time. The statistical analysis showed significant differences between PDT group (L+P+) and other groups (L-P-), (L-P+) and (L+P-).

A significant decrease in MG absorption as a function of irradiation time can be observed at wavelength 650 nm in Figure 3.

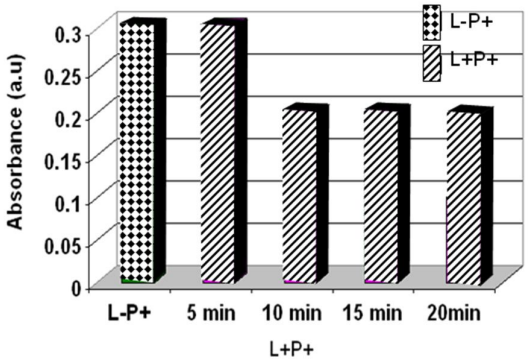

Fig. (3): Absorption spectroscopic measurements for malachite green (0.025mg /ml) after irradiation with 650nm diode laser.

The UV–VIS spectroscopic analysis showed a strong absorption between 550nm and 680nm, as well as at 350-470 nm.

The exposure to laser light showed a clear reduction of absorption peaks caused by malachite green dye, Figure 4.

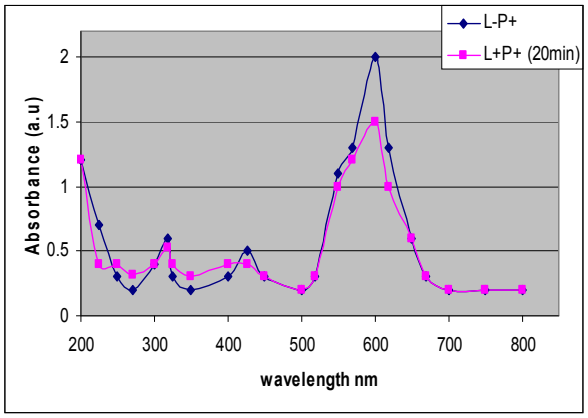

Fig. (4): (200-800nm) range absorption spectra for malachite green before and after irradiated with 650nm diode laser at 20min exposure time.

Discussion

The prevalence of both mucocutaneous and systemic infections in immunocompromised patients by *Candida* species is increasing. However, there is an increment in the number of reports relating the resistance to all the available antifungal agents (Barchiesi F., et al., 1994).

In this context, the development of more effective antifungal therapies is therefore of paramount importance. Photodynamic therapy (PDT) is a process that combines light and a photosensitizing drug, promoting phototoxic response on the treated cells, in general via oxidative damage (Mac Donald I., and Dougherty T.J., 2001).

Several studies have been carried out on the possible action of PDT and different photosensitizing compounds, on bacteria and yeasts (Jori, G. et al., 2006, Chan Y., Lai C., 2003 and Sarkar S., Wilson M., 1993).

The fungicidal activity of PDT, using the photosensitizer Green 2W activated with red light at 630 nm, has been demonstrated in vitro also against *Aspergillus fumigatus* (Friedberg J.S., et al., 2001).

Souza and colleagues (Souza S. C., et al., 2006) demonstrated the phototoxic effects of Methylene blue, a phenothiazinium photosensitizer, on the growth of *C. albicans* after irradiation with a diode laser InGaAlP (683 nm).

In the present study, it is shown that, the photo activation of malachite green at a concentration of 0.025 mg/ml with 650 nm laser light ($0.59W/cm^2$) reduced the number of *Candida albicans* CFU/ml by 98.4%.

Previous studies showed that the use of PDT is very effective against *Candida albicans* (Monfrecola G., et al., 2004, Bliss J.M., et al., 2004, Lambrechts S.A.G., et al., 2005, Munin E., et al., 2007, Senda N., et al., 2000 and Kawamoto K., et al., 2000). However, a great number of variables may influence the number of microorganisms affected by this technique including: the light wavelength, type and concentration of the photosensitizer, microorganism physiologic stage, photosensitizer incubation period, exposure time and laser energy density.

In the present study, UV–VIS absorption spectroscopy showed that after irradiation, the absorption peaks of MG diminished. These findings showed that malachite green photodegraded with an increase in exposure time to the laser light.

These absorption reductions were observed at 550–680 nm, as well as 350–470 and 260–340 nm wavelength bands. The exposure to light reduced the absorption peaks in those bands.

The photodynamic activity of the photosensitizer is based on photooxidative reactions, which induce multiple consecutive biochemical and morphological reactions. When a photosensitizer molecule absorbs light of a resonant energy, it may undergo an electronic transition to the singlet excited state. Following the absorption of light, the photosensitizer, initially at the ground state, is activated to a short-lived excited state that may be converted to a long-lived triplet state. This triplet state is the photoactive state, which may generate cytotoxic species like singlet oxygen. These reactive oxygen species are responsible for irreversible damage to cell membranes including protein modifications. These observations are very similar to those obtained for the PDI of *Candida albicans* with methylene blue (Konan Y.N., et al., 2002 and Wainwright M., 1998).

However, a very inefficient and almost undetectable intersystem crossing to the MG triplet state was observed in bovine serum albumin (BSA) bound to MG at 90 K (Leaver I.H., 1974).

Consequently, when MG is bound to biopolymers under ordinary biological conditions, the electronic excitation of MG is not expected to produce substantial populations of triplets. A significant contribution of this excited state to the overall photobleaching of this dye is not expected. This low triplet yield of MG implies that this dye is not expected to sensitize singlet oxygen to any significant extent in biological systems (Baptista M.S., 1998).

Therefore, MG can be expected to develop its photo damaging effects towards biopolymers primarily via the classical photosensitization mechanism type I (initiated by superoxide, hydroxyl anion and other oxygen radicals), with very little contribution from the type II mechanism (initiated by singlet oxygen) (Bartlett J.A., and Indig G.L., 1999).

This study showed that *Candida albicans* can be photoinactivated by laser light and malachite green. This dye may be beneficial as a photosensitizer in Candidosis treatment. However, clinical assays and double studies are still required to improve our understanding of lethal photosensitization as an adjunct to periodontal therapy.

Conclusions

In conclusion, diode laser with 650nm was an effective tool to photoinactivate *Candida albicans* in combining with malachite green as photosensitizer. The photosensitizer was

degraded after the irradiation and new photoproducts were formed in suspension.

These findings encourage further in vivo studies, to explore the potential of this protocol for oral candidosis treatment in immunocompromised patients.

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الإخماد الديناميكي الضوئي لخميرة المبيضات البيضاء المتحسسة بواسطة صبغة Malachite Green

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الخلاصة

كان الهدف من هذه الدراسة هو للتحري من قدرة صبغة (Malachite Green) مرتبطة مع ليزر الدايود ذو الطول ألموجي 650نانومتر لقتل خميرة المبيضات البيضاء ، بالإضافة إلى التحري عن التحلل الضوئي لصبغة (Malachite Green) بعد العلاج الديناميكي الضوئي طيفياً . شملت الدراسة أربعة مجاميع :مجموعةالسيطرة ، و هي المجموعة غير المعالجة لا بالصبغة ولا بالليزر. عولجت مجموعة اخرى من مزارع المبيضات البيضاء فقط بالصبغة بتركيز (0.025ملیغرام /مل) لمدة 5 دقائق في الظروف المظلمة.ُرضت مزار ع من المبيضات البيضاء ل40ملي واط و 650 نانومتر دايود ليزر في غياب الصبغة . في مجموعة العلاج الديناميكي الضوئي ، تم إضافة الصبغة (Malachite Green) إلى عالق المبيضات لمدة 5 دقائق ثمُ رضت لليزردايود لمدة (5 ، 10 ، 15 ، 20) دقيقة بكثافة طاقة (0.59 واط /سنتيمتر2).خ ّففت العينات و تم حساب عدد مستعمرات المبيضات وتحويلها إلى الوحدة المكونة للمستعمرة لكل مل. تم الحصول على طيف الامتصاص للعالق الفطري المتحسس ضوئيا. وقدمت البيانات لاختبار T بفرق معنوي (أقل من 0.05).أظهرت النتائج ان ليزر الدايود ذو الطول ألموجي 650نانومتر بوجود صبغة (Malachite Green) خفض عدد المستعمرات الى 98.4% .كما وأظهرت ان ليزر الدايود ذو الطول الموجي 650نانومترفقط ، وصبغةMalachite Green فقط لم تقلل معنويا عدد المستعمرات المبيضات البيضاء. طيف الامتصاص اظهر ان صبغة Malachite Greenتحللت ضوئياً بعد التشعيع. في الاستنتاجات ليزر الدايودذو الطول الموجي 650نانومتر كان أداة فعالة لاخماد المبيضات البيضاء في وجود صبغة Malachite Green و أن الصبغة تحللت ضوئياً بعد التشعيع.