Sensitivity of Four Various Candida Species to Photodynamic Therapy Mediated by Indocyanine Green, an in vitro Study

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KEY WORDS
Candida; Indocyanine green; Laser; Nystatin; Photodynamic therapy; Photosensitizer;

ABSTRACT
Statement of the Problem: Various species of candida contribute to oral candidiasis. It is the time to shift therapies from conventional rigid antimicrobial therapies to more patient specific and safer ones.
Purpose: The present study aimed to investigate antifungal effects of photodynamic therapy (PDT) using Indocyanine green as photosensitizer and low-power laser irradiation on the viability of candida albicans, candida tropicalis, candida glabrata and candida krusei, and to compare it with Nystatin as the conventional treatment.
Materials and Method: In this in vitro study, 0.5 McFarland suspensions of candida's species were prepared (n=50, each). Each strain was then divided into five groups of 10 samples each, according to the following experimental interventions: (1) Nystatin, (2) photodynamic therapy; laser irradiation (wavelength= 808 nm, power= 100 mW, energy density= 10 J/cm2, exposure duration= 100 s) in the presence of the photosensitizer, (3) laser irradiation alone, (4) treatment with the PS alone and (5-control: no exposure to laser light or photosensitizer.
Next, serial dilutions were prepared and seeded onto Sabouraud dextrose agar. The colonies were counted, and the values of log (CFU/ml) were analyzed by variance and the Tamhane test (p< 0.05).
Results: Photodynamic therapy mediated indocyanine green is significantly effective in reducing the number of CFU/ml of all species of candida tested, compared to control group (p< .001). Nystatin, laser irradiation and photodynamic therapy, with respectively decreasing potency, have considerably reduced the number of candida's colonies in all four bacterial strains (p< .05). Photosensitizer alone, showed impressive antimicrobial potency against all species of candida except candida albicans, in comparison to control group.
Conclusion: It seems that laser therapy alone is more powerful than photodynamic therapy mediated indocyanine green; however, conventional treatment is still at the top of the table of antimicrobial efficacy in relation to all candida species.

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Introduction
Oral candidiasis is an opportunistic infection in the oral cavity. Candida albicans (C.albicans) is the most prevalent cause of oral mucosa candidiasis, which reaches to 60-70 percent of cases [1]. C.albicans the form of “non-pathogen,” role as the normal flora of mouth, though under special conditions, it may lead to candidiasis [2].
Today, there are various antifungal medications introduced in this regard; such as ionophores (Nystatin and Amphotericin B) and azole drugs. However, there are still some limitations in facile prescription of these drugs such as the bitter taste of Nystatin, which leads to
nausea and patient intolerance [3], or the occurrence of
drug resistance, especially to azoles, as was described in
81 percent of patients with HIV infection, who were
under oral C.albicans therapy [4]. Moreover, oral candi-
didiasis may occur iatrogenic as an adverse effect of
other treatments such as chemotherapy for cancer sup-
pression; in these patients, drug resistance was also
abundantly observed [5]. These findings and coinfection
with microbial flora such as Pseudomonas Aeruginosa
necessitate a powerful, universal attempt to develop a
novel strategy to remove fungal infections, without
causing harmful effects or inducing resistance reactions
[6-8].

Photodynamic therapy (PDT) assisted laser is a new
modality, based on non-toxic photosensitizer (PS) and
safe light source. The combination of these items can
counter a biological cascade in the presence of radical
oxygen for apoptosis and annihilation of microorgan-
isms as long as malignant cells [9]. Different PSs (such
as methylene blue, indocyanine green and toluidine
blue) as well as diverse laser parameters were examined
in this regard to achieve the best result in elimination of
microbes and malignant cells. The indocyanine green as
a new PS which followed by laser illumination, has
shown promising effects against periodontal and peri-
implant pathogens and malignant cells of melanoma [10
-12]. The present study was to compare the susceptibil-
ity of four various species of candida to PDT, PS, laser
irradiation, and to conventional treatment of Nystatin.

Materials and Method

Study design

This in vitro investigation was employed for a total of
200 samples in four main categories of reference strains
of candida species. Including C.albicans ATCC 10231,
Candida tropicalis (C.tropicalis) PFCC 89-1456, Can-
dida glabrata (C.glabrata) ATCC 90030 and Candida
krusei (C.krusei) DSM 70079; 50 samples each. Four
treatment modalities (including PDT, PS only, laser
only, and Nystatin) were examined on each sub-
category of any strain of candida (N=10). Additionally,
10 samples in each group were selected randomly as
control (N=10), which did not receive any treatment or
intervention.

Microorganisms and culture conditions

Freeze-dried candida species have been subjected to re-
vive the process. In order to prepare.5 McFarland, tur-
bidity standard, the isolates were passage two times on
Sabouraud dextrose agar plates 24 hours before provid-
ing the suspension of candida. Suspensions of candida’s
strains containing 10 (6) cells/ml were standardized in a
spectrophotometer in 530 nm wavelength (Biochrom
WPA Lightwave II UV, Cambridge, UK).

In order to prevent microbial contamination and also the
irradiation of unwanted environmental lights, all steps
were performed under a darkened biosafety hood at 28-
dergrees centigrade temperature.

Nystatin application

0.1 mL Nystatin (Jaber Ebne Hayyan Pharmaceutical
Company, Tehran, Iran) (100,000 units per ml) was
added to 0.1 ml suspensions of candida in microplates.

Photodynamic therapy

PS application: Solutions of 5mg/ml of indocyanine
green were used according to the manufacturer’s manual
(Diagnostic Green GmbH, Germany). Then, 0.1 ml of
this solution was added to micro plates containing 0.1
ml suspensions of candida.

Laser illumination: Laser was illuminated to micro
plates containing a homogenized solution of 0.1 ml ster-
ile saline and 0.1 ml candida. Laser parameters are
summarized in Table 1.

PDT: Laser was illuminated to micro plates contain-
ing a homogenized solution of 0.1 ml Indocyanine green
(Sigma, New York, York, USA) and 0.1 ml candida
with the same parameters illustrated for laser treatment.

The mixing process of candida suspension with sa-
line, PS or Nystatin was done on a shaker (Behdad
company, Tehran, Iran), for 5 min to achieve a well
homogenized solution.

Eventually, using pour plate method 0.1 ml of each
sample was added to 25 ml Sabouraud dextrose agar
medium, and then incubated at 37°C for 48 hours.

Table 1: Laser parameters and specifications

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laser power</td>
<td>100 mW</td>
</tr>
<tr>
<td>Radiation duration</td>
<td>100 sec</td>
</tr>
<tr>
<td>Dose</td>
<td>10 J/cm²</td>
</tr>
<tr>
<td>wavelength</td>
<td>808 nm</td>
</tr>
<tr>
<td>Probe status</td>
<td>No contact</td>
</tr>
<tr>
<td>Radiation mode</td>
<td>Continuous</td>
</tr>
<tr>
<td>Probecross section diameter</td>
<td>1 cm</td>
</tr>
<tr>
<td>Model</td>
<td>Polaris 2/SW PM2-25/M1/APver 4/0, (Star company, Bielsko-Biata, Poland)</td>
</tr>
</tbody>
</table>
Serial dilutions were then made from the samples contained in the tubes, and plated in duplicate onto Sabouraud dextrose agar culture medium. After 48 hours of incubation at 37°C, the viable colonies were counted and the values of CFU/ml were determined by colony count set (HYC-560 Digital Colony Counter, Hanyang Scientific Equipment Company-HYSC, Korea) (Figure 1).

**Data analysis**

The data have been statistically analyzed by SPSS23 software One-way ANOVA test and Tamhan test have been used to detect any significant difference between groups ($p<0.05$).

**Results**

*C.albicans* counts (CFU/ml) were transformed into base-10 logarithms. So, one-way ANOVA with followed Tamhan test for multiple comparisons was performed (Table 2). The results of dual comparison of the study groups for each candida by "P value", are summarized in Table 3.

All interventions showed anti-fungal efficacy when compared to control, although species of *C.albicans* did not reduce effectively in comparison to control group when subjected to PS alone. In total, the highest number of candida's colonies was seen in a control group, while the least was achieved in Nystatin group.

*C.glabrata* showed the most sensitivity to Nystatin and was eliminated totally. *C.krusei* showed the least sensitivity to Nystatin. Additionally, this trend was also found with PDT employment. *C.tropicalis* was recognized as the most sensitive strain to laser irradiation; inversely, *C.glabrata* was the most resistant to this. Surprisingly, PS only, also showed anti-fungal efficacy with the most promising effects on *C.tropicalis* and the least on *C.glabrata* strain.

In spite of the vast number of researches on the efficacy of PDT mediated indocyanine green, there are just two papers available about the effects of this modality on *C.albicans*. Also studies on the comparison of susceptibility of different species of candida to introduced remedies are scarce.

Laser irradiation was significantly more effective than

<p>| Table 2: The number of candida colonies according to candida type methods treatment |
|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|</p>
<table>
<thead>
<tr>
<th>Candida strain</th>
<th>Group</th>
<th>Average</th>
<th>Standard deviation</th>
<th>Down limit</th>
<th>Up limit</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C.albicans</em></td>
<td>Control</td>
<td>1064.80</td>
<td>133.042</td>
<td>969.63</td>
<td>1159.97</td>
<td>910</td>
<td>1300</td>
</tr>
<tr>
<td></td>
<td>PS</td>
<td>877.40</td>
<td>146.973</td>
<td>772.26</td>
<td>982.54</td>
<td>620</td>
<td>1094</td>
</tr>
<tr>
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<td>PDT</td>
<td>719.80</td>
<td>96.957</td>
<td>650.44</td>
<td>789.16</td>
<td>600</td>
<td>880</td>
</tr>
<tr>
<td></td>
<td>Laser</td>
<td>614.60</td>
<td>89.399</td>
<td>555.66</td>
<td>673.54</td>
<td>530</td>
<td>730</td>
</tr>
<tr>
<td></td>
<td>Nystatin</td>
<td>43.70</td>
<td>11.738</td>
<td>35.30</td>
<td>52.10</td>
<td>25</td>
<td>64</td>
</tr>
<tr>
<td><em>C.tropicalis</em></td>
<td>Control</td>
<td>925.30</td>
<td>74.005</td>
<td>872.36</td>
<td>978.24</td>
<td>829</td>
<td>1042</td>
</tr>
<tr>
<td></td>
<td>PS</td>
<td>810.90</td>
<td>46.615</td>
<td>777.55</td>
<td>844.25</td>
<td>722</td>
<td>864</td>
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<td>PDT</td>
<td>706.50</td>
<td>49.718</td>
<td>670.93</td>
<td>742.07</td>
<td>640</td>
<td>780</td>
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<td>Laser</td>
<td>505.50</td>
<td>73.686</td>
<td>452.79</td>
<td>558.21</td>
<td>410</td>
<td>620</td>
</tr>
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<td>Nystatin</td>
<td>85.00</td>
<td>19.872</td>
<td>70.78</td>
<td>99.22</td>
<td>48</td>
<td>116</td>
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<tr>
<td><em>C.krusei</em></td>
<td>Control</td>
<td>1279.40</td>
<td>98.067</td>
<td>1209.25</td>
<td>1349.55</td>
<td>1100</td>
<td>1432</td>
</tr>
<tr>
<td></td>
<td>PS</td>
<td>922.50</td>
<td>70.352</td>
<td>872.17</td>
<td>972.83</td>
<td>800</td>
<td>1000</td>
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<td>PDT</td>
<td>742.10</td>
<td>102.499</td>
<td>677.78</td>
<td>814.42</td>
<td>600</td>
<td>949</td>
</tr>
<tr>
<td></td>
<td>Laser</td>
<td>533.60</td>
<td>87.297</td>
<td>471.15</td>
<td>596.05</td>
<td>414</td>
<td>657</td>
</tr>
<tr>
<td></td>
<td>Nystatin</td>
<td>218.40</td>
<td>35.211</td>
<td>193.21</td>
<td>243.59</td>
<td>176</td>
<td>276</td>
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<tr>
<td><em>C.glabrata</em></td>
<td>Control</td>
<td>1100.20</td>
<td>137.056</td>
<td>1002.16</td>
<td>1198.24</td>
<td>900</td>
<td>1300</td>
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<tr>
<td></td>
<td>PS</td>
<td>846.40</td>
<td>105.833</td>
<td>770.69</td>
<td>922.11</td>
<td>730</td>
<td>1000</td>
</tr>
<tr>
<td></td>
<td>PDT</td>
<td>675.30</td>
<td>104.368</td>
<td>600.64</td>
<td>749.96</td>
<td>514</td>
<td>810</td>
</tr>
<tr>
<td></td>
<td>Laser</td>
<td>659.40</td>
<td>62.717</td>
<td>614.54</td>
<td>704.26</td>
<td>575</td>
<td>749</td>
</tr>
<tr>
<td></td>
<td>Nystatin</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
PDT against C.krusei and C.tropicalis; however, when the laser employed on C.glabrata and C.albicans strains, it showed a statistically equal anti-fungal power. PDT reduced candida colonies more effective than PS alone except in samples of C.albicans, which showed no statistically significant difference (p= 0.13).

Discussion

Present investigation evaluated anti-fungal efficacy of PDT mediated indocyanine green on four common strains of candida. The number of colonies in all candida groups decreased significantly when subjected to PDT. In an in vitro study performed by Fekrazad et al. [13], the effects of PDT mediated indocyanine green and new methylene blue against C.albicans was compared with control (no treatment). Similar to the results of present study, they reported a promising anti-fungal effect of PDT [13].

Azizi et al. [14], also investigated into the in vitro effects of PDT induced by indocyanine green and methylene blue on C.albicans and like the present study, compared this method with Nystatin; moreover, they used different laser parameters in laser dose and mode of radiation (pulse or continuous). Overall, in compliance with the current study results, authors reported a significant difference between the two modalities compared to control and as well between the two, Nystatin showed better results in C.albicans eradication than PDT. However, unlike our consequences, the anti-fungal efficacy of Nystatin was statistically equivalent to PDT, when pulse mode laser irradiation was induced by indocyanine green.

Different PSs accompanying light illumination have been examined previously, in literature, as the novel anti-candida modulation such as methylene blue, toluidine blue, 5-aminolevulinic acid, and photofrin. Although there is not a univalent consensus on the results, one theory is nearly accepted by all, which defines that regardless of the type of PS, this modality can be used as an auxiliary treatment to conventional drug therapy [15-18].

The type and concentration of PS, method of laser radiation, laser distance, laser dosimeter, and physiologic status of the aim microorganism, are all effective in the results of PDT by laser [19]. PS alone, in spite of having a light efficacy in candida annihilation has shown the least anti-fungal effects in the present and previous investigations compared to other treatment modalities [13-14]. This conclusion demonstrated that the laser illumination following PS application in PDT is of paramount importance; therefore, it is suggested to make forthcoming investigations with a focus more on selecting the best laser parameters’ layout than the type or characteristics of PS.

The characteristics of the aimed microorganism have a key role in PDT success rate. C.albicans seems to be more resistant than gram-positive bacteria against this treatment modality. It is suggested that perhaps the presence of nuclear membrane, larger cell size, and the fewer target areas for free oxygen radicals per unit of cell volume in C.albicans may play a significant role in

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**Table 3:** The results of dual comparison of the study groups for each candida by “P value”

<table>
<thead>
<tr>
<th>Candida type</th>
<th>P value</th>
<th>Comparison Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>C.albicans</td>
<td>0.088</td>
<td>Control-PS</td>
</tr>
<tr>
<td></td>
<td>0.0</td>
<td>Control-PDT</td>
</tr>
<tr>
<td></td>
<td>0.0</td>
<td>Control-Laser</td>
</tr>
<tr>
<td></td>
<td>0.130</td>
<td>PDT-PS</td>
</tr>
<tr>
<td></td>
<td>0.001</td>
<td>Laser-PS</td>
</tr>
<tr>
<td></td>
<td>0.0</td>
<td>Nystatin-PS</td>
</tr>
<tr>
<td></td>
<td>0.151</td>
<td>PDT-Laser</td>
</tr>
<tr>
<td></td>
<td>0.0</td>
<td>PDT-Nystatin</td>
</tr>
<tr>
<td></td>
<td>0.0</td>
<td>Nystatin-Laser</td>
</tr>
<tr>
<td>C.tropicalis</td>
<td>0.006</td>
<td>Control-PS</td>
</tr>
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<td></td>
<td>0.0</td>
<td>Control-PDT</td>
</tr>
<tr>
<td></td>
<td>0.0</td>
<td>Control-Laser</td>
</tr>
<tr>
<td></td>
<td>0.0</td>
<td>Control-Nystatin</td>
</tr>
<tr>
<td></td>
<td>0.002</td>
<td>PDT-PS</td>
</tr>
<tr>
<td></td>
<td>0.0</td>
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<td></td>
<td>0.0</td>
<td>Nystatin-PS</td>
</tr>
<tr>
<td></td>
<td>0.0</td>
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<td></td>
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<td></td>
<td>0.0</td>
<td>Nystatin-Laser</td>
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<tr>
<td>C.krusei</td>
<td>0.0</td>
<td>Control-Nystatin</td>
</tr>
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<td></td>
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<td>C.glabrata</td>
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</tr>
<tr>
<td></td>
<td>1.000</td>
<td>PDT-Laser</td>
</tr>
</tbody>
</table>
this resistance [20]. In the present study as well, C. albicans was more resistant to PDT compared to other employed species of candida.

Maximum absorption of light by colored PS molecules is also an important issue when applying PDT. The wavelength of radiation must be set where there is the most absorbance by the PS molecules. This will produce a maximum amount of oxygen free radical to eliminate the target microorganism. For example, the most absorbent of the indocyanine green is in the range of 805-810 nm [21-22]. Therefore, it seems that the wave length used in the present study was optimum; however, the authors believe that higher-energy density of the laser was needed to achieve better results. Unfortunately, in previously published studies, different laser parameters with different conditions and methods have been examined, which make the comparison and consensus between various and contradictory results very difficult [14,23]. In the present study laser, the parameters were chosen according to Azizi et al. [14] investigation.

One of the critical concerns about laser application on vital tissues is the laser side effect in increasing the targeted tissue temperature, shifting the tissue healing pathways to unwanted and harmful ones. Nonetheless, it is demonstrated that diode lasers in comparison to other laser types would produce less heat [24]. Silva et al. [25] reported an increase of 2 centigrade in temperature of target tissue when applying PDT (by diode laser) for 30, 60, and 120 seconds, which can be negligible. In another study conducted by Hirata et al. [26], laser application in power ranging 50-500 mW for 2 min duration caused moderator effects on mammalian cell proliferation in vitro [26]. It seems that these side effects could be less prominent when time duration of laser illumination is reduced. The toxicity of indocyanine green has not been demonstrated, up to date [25].

Wainwright et al. [27] demonstrated that microorganism’s eradication by PDT depends on the chemical formula of PS and duration of drug exposure to bacterial cells. The cell membrane acts as a selective barrier against PS diffusion; therefore, the PS’s penetration into cells would be done through an active transfer mechanism. Damaging the cell membrane of bacteria, increasing the permeability of the cytoplasmic membrane, and intervention with DNA replication are probable mechanisms of action of PDT [27].

Previous data confirmed PDT usability in dental practice for anti-microbial purposes [28-29]. The effects of PDT induced by indocyanine green have been demonstrated as an effective treatment modality for periodontal disease [11-12] and also for eradication of melanomas and acne vulgaris [10,30]. Nevertheless, there are still some unknown points, which prevent it to be as a standard modality; it seems that in vitro studies are yet needed to standardize various options and parameters introduced in literature. Furthermore, comparative investigations with conventional therapies should be held to assess the cost benefit of this novel modality. In the present study, Nystatin showed significant better results than PDT, so the authors of this study propose anti-candida PDT application when Nystatin therapy is impossible or tolerable or as a conjunctive therapy to traditional treatments.

Indocyanine green is approved by Food and Drug Administration (FDA) and has been used for diagnostic purposes in the field of medicine, such as detection of capillary roots [31]. Absorption wavelength of indocyanine green is at 805 nm. It is demonstrated that indocyanine green binds to plasma proteins, so does not lead to chemical changes in the body [11].

In the present study, the efficacy of laser with or without PS, application was nearly equal, while Fekrazad et al.’s [13] investigation of laser illumination following indocyanine green application showed considerable better anti-fungal results than laser alone. This may be due to different brands of indocyanine green used in the two investigations, as we used Cardiogreen and they employed Emundo (both by Sigma Aldrich Company). Accordingly, it seems that Emundo has more potency in this field of application than Cardiogreen. Nonetheless, this hypothesis should be examined more specifically, in a matched comparative study. Moreover, laser parameters and mode of PS application as well as the method of PS dilution were different in these two investigations; each one could have a critical role in achieving dissimilar results.

**Conclusion**

Under the conditions of the present study, susceptibility of all species of candida was primarily to Nystatin. PDT is significantly effective in reducing the candida colo-
nies. PS alone, showed sufficient antimicrobial potency against all species of candida except *C. albicans*, in comparison to control group. The outlook for new treatments in candidiasis seems to be very clear. Further studies on the efficacy of these treatments are recommended.

**Conflicts of Interest**

None declared.

**References**


