Original Article

Comparative evaluation of photodynamic therapy induced by two different photosensitizers in rat experimental candidiasis

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ABSTRACT

Background: Antimicrobial efficacy of photodynamic therapy (PDT) depends on both the photosensitizer (PS) and laser parameters. In the present study, antimicrobial effectiveness of PDT with different concentrations of two PSs was compared.

Materials and Methods: In this study, we employed fifty-nine 6-week-old male Wistar rats. All except the negative and overall control groups were immunosuppressed and then orally inoculated with a suspension of Candida albicans (9 × 10⁸ CFU/ml). At 4 days after oral inoculation, swabbing of tongue dorsum was performed to recover yeast from the tongue before treatment; on the next day, PDT was carried out on tongue dorsums by use of different concentrations of methylene blue (MB) or poly-L-lysine-chlorine (e6) conjugate (pL-ce6) as PS; followed by a 10 min diode laser illumination at 660 nm (n = 6 each). Then, sampling was again performed. The difference between yeast recovery before and after treatment was compared between the groups by one-way analysis of variance test (α = 0.05). After sacrificing the animals, their tongues were surgically removed and processed for histological evaluation of the presence of yeast and tissue reaction.

Results: PDT mediated by both PSs, regardless of the type and their concentration, resulted in a significant microbiological and histological reduction in C. albicans counts in comparison with positive control group (P < 0.001). There was no difference in epithelial lesions and inflammatory responses between groups.

Conclusion: PDT mediated MB or pL-ce6 is a promising approach for treatment of oral candidiasis.

Key Words: Candida albicans, methylene blue, photodynamic therapy, photosensitizer, poly-L-lysine-chlorine (e6) conjugate

INTRODUCTION

Oral cavity harbors a diverse and complex microbial community, which is relatively innocuous in a healthy individual.[¹] However, under certain circumstances such as impairment of innate and adaptive host defenses, perturbation of normal bacterial flora or an underlying disease, the dynamic equilibrium between its components would alter. This leads to the overgrowth and infection of the opportunistic organisms.[²] Candida species represent one of the opportunistic mycoflora of the oral cavity with special importance to human health.[³] This yeast, particularly its albicans genus,[⁴] is the most often isolated organism from patients,[⁵] which can cause a vast variety of diseases ranging from superficial mucosal infections to systemic life-threatening conditions like candidemia.[⁶] Today with the increasing administration of various immunosuppressive drugs as well as chemo and radiotherapy, which can cause long-term xerostomia and the increased prevalence of human immunodeficiency virus infections, individuals are more susceptible to fungal infections.[⁷] On the other hand, conventional therapy has encountered many challenges; some of which are the appearance of resistant organisms, high rates of infection relapses in
immunosuppressed patients because of the fungistatic rather than fungicidal nature of these treatments.\textsuperscript{[9]} Drug interactions and side-effects including hepatotoxicity in elderly patients\textsuperscript{[3]} and also the commitment for patient compliance with therapy.\textsuperscript{[7]} Hence, it is important to seek for an alternative treatment modality. One such promising modality is photodynamic therapy (PDT). PDT is a safe, local treatment based on the interaction of a photosensitive drug (a photosensitizer [PS]) and visible light.\textsuperscript{[8,10]}

The application of light for targeting pathogenic microbe is known for more than a 100 years.\textsuperscript{[11]} Administration of a PS before light emitting increases its efficacy and selectivity.\textsuperscript{[12]} In PDT, the PS binds to target cell and its synergism with light produces free radicals, which are cytotoxic and perforate cell membrane. This photo damage process induces pore formation on the target cell membrane, allowing the PS dye to penetrate more into the cell. Thereby, the same procedure is followed inside the cell on mitochondria and other organelles’ membrane, which eventually leads to cell death. It is suggested that as PDT exploits different antimicrobial mechanisms than that of traditional treatment, it would be an efficient alternative modality in cases of resistance to conventional treatment.\textsuperscript{[13]}

In vitro investigations have shown inactivation of Candida spp. by photodynamic therapy.\textsuperscript{[14-16]}

Peloi et al.\textsuperscript{[17]} suggested that to achieve the highest efficacy of antimicrobial PDT, there should be a balance between PS concentration in the target tissue and the intensity of photons emitted on it.

Teichert et al.\textsuperscript{[13]} examined photodynamic effect of different concentrations of methylene blue (MB) in a murine model of oral candidiasis and demonstrated a dose dependent effect of PDT mediated MB against Candida albicans. Junqueira et al.\textsuperscript{[18]} observed a considerable reduction in viability of C. albicans by employment of MB mediated low power laser of gallium aluminum arsenide at total 10 J energy. Furthermore, poly-L-lysine-chlorine (e6) conjugate (pL-ce6) exhibited a powerful antimicrobial effect in previous in vitro studies.\textsuperscript{[15,19-21]} The antimicrobial mechanism of this poly cationic molecule is obtained by replacing the cations in the lipopolysaccharide component of microorganism cell wall and distorting the outer membrane structure.\textsuperscript{[15]} However, in vivo application of antimicrobial PDT, using pL-ce6 and red laser light has not yet been well established. Thus, in the present study, the photo-inactivation of C. albicans using different concentrations of pL-ce6 and MB are compared in an immunodeficient rat model.

**MATERIALS AND METHODS**

**Experimental animals**

The study design and animal experimental procedures were approved by Ethics Committee for Animal Investigations (Torabinejad Dental Research Center, Esfahan University of Medical Science). For animal experiments, fifty-nine 6-week-old male Wistar rats, weighing approximately 250 g, were included. The animals were kept in clean cages in a temperature of 23 ± 2°C, separated from other animals, to avoid cross contamination due to their immunosuppressed condition in the experimental period. The time set of events is shown in Table 1. Initially, the rats were randomly assigned to four groups including experimental groups (n = 51), positive control group (n = 3), which corresponded to immunosuppression and Candida inoculation, but were not treated, negative control (n = 3) which only received

| Group        | Day 0                  | Day 1       | Day 4       | Day 5                     | Day 6                      |
|--------------|------------------------|-------------|-------------|---------------------------|----------------------------|
| PS+/L+       | First immnosuppression | Candida     | Second      | Candida recovery          | PDT and recovery of C. albicans; sacrifice |
|              |                        | inoculation | immunosuppresion |                           |                             |
| PS−/L+       | First immnosuppression | Candida     | Second      | Candida recovery          | LLLT and recovery of C. albicans; sacrifice |
|              |                        | inoculation | immunosuppresion |                           |                             |
| PS+/L−       | First immnosuppression | Candida     | Second      | Candida recovery          | Topical administration of PS alone and recovery of C. albicans; sacrifice |
|              |                        | inoculation | immunosuppresion |                           |                             |
| Positive control | First immnosuppression | Candida     | Second      | Candida recovery          | Recovery of C. albicans; sacrifice |
|              |                        | inoculation | immunosuppresion |                           |                             |
| Negative control | First immnosuppression | —           | Second      | Candida recovery          | Sacrifice                  |
|              |                        |             | immunosuppresion |                           |                             |
| Overall control | —                      | —           | —           | Candida recovery          | Sacrifice                  |

C. albicans: Candida albicans; LLLT: Low level laser therapy; PDT: Photodynamic therapy; PS: Photosensitizer
immunosuppressant without any infection inoculation or treatment and the overall control (n = 2) group, which were not immunosuppressed and did not receive Candida inoculation or treatment procedures [Table 2]. The animals in all groups except overall control rats were immunosuppressed with two intramuscular injections of methyl prednisolone (Damloran, Iran) in each femur at a dose of 100 mg/kg body weight 1 day before and 3 days after induction of candidiasis (days 0 and 4).

In regard to previous studies, which found that a tetracycline-laced diet is necessary to have a prolonged oral infection with adequate size in rat experimental candidiasis, in the current study this drug was added to drinking water of rats at the concentration of 0.4 mg/ml 1 day before immunosuppressant injection until the end of the experiment.

**Induction of candidiasis**

A strain of *C. albicans* isolated from an azole-resistant patient was used to induce Candida infection in rats. All steps of preservation and reactivation were carried out according to Mima et al. study. Then, suspension of *C. albicans* at a McFarland standard of 3 (corresponding to 9 × 10⁸ viable cells/ml) was prepared, which was used both in Candida inoculation step and also as the drinking water of experimental and positive control groups with a renewing program of every other day.

*Candida* inoculation was accomplished 1 day after immunosuppresion (on day 1). In this stage, after inducing short anesthesia, the tongue dorsum of the rats were swabbed with a small cotton pad previously soaked in the already prepared suspension of *C. albicans* at McFarland standard of 3.

**PSs and light source**

**MB**

Stock solutions of MB (Merck, Germany) were dissolved in sterile saline to give three concentrations of 450, 500 and 550 mg/L.

**pL-ce6**

According to Soukos et al., study pL-ce6 was prepared. Subsequently, stock solutions in 3 concentrations (500, 1000 and 1500 mg/L) of this compound were provided by dissolving it in sterile saline.

All these solutions were kept in dark before use.

**Light source**

The light source used in this study was a low-level diode laser (Azor Ltd. Laser Medical Equipment, Moscow, Russia) at a wavelength of 660 nm, 25 mW power, 7.5 J energy and a 1 cm cylindrical diffuser.

**Photodynamic therapy**

At 2 days after the last prednisolone injection, on day 6, the rats in the experimental group were randomly divided to 3 major subgroups as shown in Table 2 based on the treatment stages they would receive. The PS+/L+ category consisted of the groups, which received both PS and laser light (six animals in each group). The PS+/L− was only corresponded to PS at the same concentrations and for the same period of pre-irradiation and irradiation time of PS+/L+ groups. Animals in PS−/L+ group were exposed to the same laser dose of the experimental group with no previous application of any PS.

The rats were immobilized via deep ether inhalation anesthesia. Next, each animal was placed in a supine position on a pad and fitted with two sheets, which were located in both lateral sides of its head to hold the device on the tongue. With mandible and cheek retracted, the tongue was gently taken out of the mouth, as far as it would go, to expose it without causing any injury to the tissue. Then, PS was applied topically to the dorsum of the tongue by a sterile swab. Before irradiation, the tongue was released to return into the mouth to prevent the PS’s exposure to undesired environmental light. After 5 min (pre-irradiation time), the tongue was again gently taken out of the mouth to expose it for illumination. For illumination, the laser device was placed onto the

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Table 2: Number of rats in each group

| Major category | Group | Subgroup based on PS concentration (mg/L) | N |
|----------------|-------|------------------------------------------|---|
| PS+/L+         | PL-ce6| 500                                      | 6 |
|                |       | 1000                                     | 6 |
|                |       | 1500                                     | 6 |
|                | MB    | 450                                      | 6 |
|                |       | 500                                      | 6 |
|                |       | 550                                      | 6 |
| PS-/L+         |       |                                          | 3 |
| PS+/L−         | PL-ce6| 500                                      | 2 |
|                |       | 1000                                     | 2 |
|                |       | 1500                                     | 2 |
|                | MB    | 450                                      | 2 |
|                |       | 500                                      | 2 |
|                |       | 550                                      | 2 |
| PS-/L−         | Positive control | 3 | |
|                | Negative control | 3 | |
|                | Overall control | 2 | |

PS: Photosensitizer; PL-ce6: Poly-L-lysine-chlorine (e6) conjugate; MB: Methylene blue
dorsum of the tongue, which was illuminated for 10 min (PS+/L+ group).

Microbiological evaluation/quantification of tongue infection with Candida spp.

1 day before and immediately after treatment, the recovery of C. albicans was carried out in all groups under short ether anesthesia. The sampling procedure was accomplished via a sterile cotton wool swab by rubbing it on the dorsal surface of rats’ tongues with a rotating maneuver for 5 s each. Then, the swab ends were cut off and placed in a 1 ml sterile saline tube. All procedures of Candida transformation to a Sabouraud’s dextrose agar medium to determine the number of CFU/ml was performed according to Mima et al. study.\(^{26}\) The mediums were incubated for 48 h at 37°C. After that, the yeast colony counts (CFU/ml) were quantified by a digital colony counter (CP 600 Plus, Phoenix Ind Com Equipamentos Cientificos Ltda., Araraquara, SP, Brazil). In positive control group, the same procedures of Candida recovery and plating were done, both on day 5 and 6 (treatment day), to rule out infection resolution in experimental groups before treatment by the remnant immunologic defenses of rats or due to the sampling procedure on day 5.

Histopathological study

The rats were killed with an over dose of ether immediately after their respective interventions on day 5. Subsequently, tongues were surgically removed and fixed in a 10% formalin fixative solution at pH 7 for 24 h. Then, the samples were mounted on glass slides and stained with H and E and periodic acid-Schiff for histopathological examination and fungal detection by a light microscope (Olympus CX21, Japan). Tissue reaction, caused by C. albicans, was examined by a pathologist blinded to all groups of rats. The presence of five epithelial alterations (including epithelial hyperplasia, disorganization of the basal layer, exocytosis, spongiosis and loss of filiform papilla) was assessed in each case and scored as 0 for the absence of respective alteration and 1 for its presence. With calculating the sum of the scores, each rat tongue tissue received an overall score varying from 0 to 5 points.

Moreover, a semi-quantitative analysis on the presence of an inflammatory response of the conjunctive tissue was carried out. In this regard, score 0 was attributed to cases without any inflammatory responses and one to ones with chronic inflammatory infiltrate in conjunctive tissue.

Statistical package for social sciences (SPSS 13, SPSS Inc., Chicago, IL, USA) was used for statistical analysis of data. The differences between the log\(_{10}\) (CFU/ml) data of C. albicans isolated from the tongues of rats before and after intervention and Candida counts in microscopic view of tongue tissues were compared in the different groups by use of one way analysis of variance , with consideration of significance level as 5%. If a significant difference was found between the groups, comparisons of individual groups were carried out with post hoc analysis to determine where these differences occurred. Kruskal-Wallis test was applied to analyze tissue alterations between the different groups. Comparison of the mean inflammatory response of conjunctive tissue was performed by Fisher’s exact test with consideration of significance level as 5% (\(P < 0.05\)).

RESULTS

A well-established oral candidiasis with a strong positive Candida culture was achieved 4 days after Candida inoculation. In all groups submitted to immunosuppression and Candida inoculation, red and white lesions, respective to atrophic and pseudomembranous candidiasis were observed [Figure 1]. The clinical lesions were present before and after treatment in the experimental period.

Regardless of the type and concentration of PS used in PDT, a significant reduction in Candida counts was seen after PDT when compared with positive

Figure 1: Red and white patches observed on the tongue dorsum of all groups except for negative and overall control groups
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control group ($P < .001$). Figure 2 shows that the most effective PS was MB in concentration of 450 mg/L, which resulted in a reduction from $2.8 \log_{10}$ to $0.9 \log_{10}$.

Total eradication was seen in all PS+/L+ groups except those treated with pL-ce6 in concentration of 1500 mg/L [Table 3]. Moreover, a reduction from $3.43 \log_{10}$ to 0 was the highest $C. albicans$ eradication record among all groups, which was attributed to the cases treated with PDT mediated MB in concentration of 450 mg/L.

PS alone, in the absence of light, decreased the count of $Candida$ but without a significant difference with the control group ($P > 0.05$). In addition, laser illumination alone did not cause a significant reduction in viability of $Candida$ compared with other groups ($P > 0.05$).

**Table 3: Total eradication percentage among PS+/L+ subgroups**

| PS+/L+ subgroups | PS concentration | Total eradication (%) |
|------------------|------------------|-----------------------|
| pL-ce6/L+        | 500              | 33.3                  |
|                  | 1000             | 16.4                  |
|                  | 1500             | 0                     |
| MB/L+            | 450              | 50                    |
|                  | 500              | 50                    |
|                  | 550              | 16.6                  |

PS: Photosensitizer; PL-ce6: Poly-L-lysine-chlorine (e6) conjugate; MB: Methylene blue

Histopathologic results

In PS+/L+ group, there was no mucosal infection, while in other groups subjected to $Candida$ inoculation, discrete yeasts and pseudohyphae were seen limited to the keratinized epithelial layer of tongue [Figure 3].

In microscopic view, subepithelial infiltrated inflammatory cells dominantly consisted of monocytes; this finding was adhered to only a few exceptions in which a polynuclear cell infiltration was observed.

There was no significant difference in epithelial alterations and inflammatory infiltrate between groups corresponded to $Candida$ inoculation ($P > 0.05$) [Figure 4].

**DISCUSSION**

PDT has shown a promising efficacy in $C. albicans$ eradication in laboratory conditions. [12,16,27] Bliss et al. [28] and Carvalho et al. [29] suggested that the medium conditions influence the PDT results; therefore, it is important to investigate these results in animal models. Rat model is known as the most animal employed in previous studies to mimic oral candidiasis. [30]

It is believed that some forms of immunosuppression are required to predispose the animal to a persistent infection in the oral cavity. [30] However, in a part of the study of Samaranayake et al. [30] and Martins et al. [31] the investigation was conducted while the animals were not immunosuppressed. Nevertheless,
in these studies, in spite of Candida isolation from the samples of the oral cavity, no Candida infection was observed in gross and microscopic examinations. Currently, there is not any known way for oral carriage of C. albicans in microscopic and macroscopic view in an immunocompetant animal model. In the present study, experimental candidiasis was induced by administration of prednisolone and C. albicans inoculation according to study of Mima et al. [26] On day 5 of the experiment, in all groups corresponded to Candida inoculation, white pseudomembranous and red atrophic lesions were observed on the dorsum of tongue; this finding was in line with that of Mima et al. [26]

The experimental candidiasis model, used in the present study, made the recovery of 2-3 CFU/ml log10 of C. albicans possible, which was similar to the findings of Mima et al. [26] and in higher numbers, compared to Teichert et al. [13]

The Candida counts before treatment is an important factor in PS efficacy. Demidova and Hamblin [15] showed that there would be a pronounced cell-density-dependent effect in PS on killing of C. albicans. In this sense, the basement count of Candida should be considered when comparing literature reports of antimicrobial PDT.

In Mima et al. investigation [26] the recovery of C. albicans before treatment was only accomplished in positive control group to avoid bias of Candida reduction resulted from sampling rather than treatment intervention. In the present study, to eschew such a mistake, the recovery of C. albicans was done a day before treatment, giving opportunity for re-growth of the remnant fungi. On treatment day, again positive group was corresponded to the same recovery and plating procedures. The results showed there was no difference in CFU/ml numbers between the two consecutive days. This confirmed that the CFU/ml values, obtained on the previous day from the experimental group, were also as a reference of “Candida counts before treatment stage.”

In the present study, CFU/ml reduction was significantly higher in PS+/L+ subgroups compared to positive control group, regardless of the type and concentration of PS. This result was in agreement with that of Teichert et al. [13] who observed a significant CFU reduction with PDT mediated MB. It is in contrast with Martins et al. study [31] in which no significant reduction in CFU values was observed after PDT, although epithelial lesions were significantly fewer in PDT corresponded group compared to controls. This finding was also reported in a histopathologic investigation by Junqueira et al., [18] which showed no statistical difference in Candida counts between experimental and control groups. These controversial results may be attributed to differences in immunologic state of animals during the investigation period; as it is demonstrated that over the development of an experimental candidiasis, the yeasts and hyphae would be eliminated from the tissues by immune defenses in immunocompetent hosts.

There are several factors to be considered in PDT application to have the maximum efficacy including the type, dose, incubation time and localization of the PS, availability of oxygen, the wave length of light, the light power density and its energy fluence. [9] Although there are a significant number of compounds that may act as PS, only a few are commercially available and had been examined for safety issues.

In vivo studies comparing different types of PSs are scarce. Demidova and Hamblin [15] compared photoinactivation effect of pL-ce6, MB and toluidine blue and concluded that on medium conditions, pL-ce6 has the most powerful fungicidal effect after light illumination at fluencies of 0-200 J/cm2. In the present study, the fungicidal photodynamic effect of pL-ce6 in all concentrations of 500, 1000 and

**Figure 4:** Histopathological view of the tongue of a rat in PS+/L+ group. Epithelial hyperplasia, basilar hyperplasia, loss of filiform papilla and spongiosis were observed in these animals. Animals corresponded to immunosuppression and Candida inoculation, whether submitted to photodynamic therapy or not, showed a mild inflammatory reaction (arrows) in lamina properia (H and E, ×100)
1500 mg/L was statistically the same as MB and significantly higher than the control group.

MB has been used as a photosensitizing agent since 1920.[9] In many in vitro and in vivo studies, PDT mediated MB has shown promising fungicidal efficacy.[13,16,18]

Teichert et al.[13] observed total elimination of C. albicans with 450 and 500 mg/L of MB after red light laser illumination, though the less concentrations of 250-400 mg/L resulted in a dose dependent reduction of Candida counts without total eradication. In the present study, the concentrations of 450, 500 and 550 mg/L of MB were used with 664 nm diode laser light and a significant CFU/ml reduction was achieved with MB mediated PDT independent of its concentration. This may be attributed to the fact that in high doses of PS, the remaining amount that does not have binding with target cells accumulates in cell and acts as an optical shield by absorbing the light without any killing effect.

In contrast to Teichert et al. results concerning total Candida elimination by use of 450 and 500 mg/L MB mediated PDT,[13] in the present investigation, this finding was merely seen in 50% of animals in the groups which used PDT mediated MB with concentrations of 450 and 500. It is supposed that the laser parameters account for this difference; as in the present study, the laser output power was 25 mW whereas in Teichert et al. investigation[13] was 400 mW. Nevertheless, the lower power output is preferable for PDT approaches as it produces less heat and is closer to clinically safety protocols.[32] Therefore, it is suggested to consider more than one session for PDT to achieve better results without compromising safety issues.

Histopathology results in the present study were consistent with microbiologic observations as the semi-quantitative assays exhibited lower Candida infection in PS+/L+ group compared with positive control group and without any significant difference with PS−/L+ group. Predictably, it is thought that there would be more differences between groups in long-term assays. No evidence of adverse effect on corresponding tissue was detected in histopathological survey. A mild infiltration of mononuclear inflammatory cells was observed in subjacent connective tissue in all groups submitted to Candida inoculation regardless of their treatment stage. This result was reported in several previous studies either in immunocompetent animals[31] or immunocompromised conditions[26] as well as in the present study probably reflecting the exclusion of the immune system role in PDT antimicrobial effect.

The high potency of PDT in eradication of azole resistant species of Candida as well as its simple, nontoxic, repeatable and noninvasive characteristics make this method as a promising alternative choice to traditional antifungal treatments. However, there are yet some shortcomings with this technique, such as the high cost of pL-ce6 preparation or the remaining stain after MB application on tissues. In addition, this method of topical treatment of mucocutaneous candidiasis can be accomplished merely in available and localized sites.

Therefore, more investigations should be developed to determine optimal laser parameters in coordination with an appropriate PS along with considering and improving ergonomic essentials of this new modality to meet the demands of clinical arena.

**CONCLUSION**

The results of this study revealed that MB and pL-ce6 are significantly efficient PSs in photodamage of C. albicans without harming the corresponding tissues. However, further in vivo studies are necessary to investigate the long-term effects of this treatment and to investigate other usable light sources with these photosensitizing materials.

**ACKNOWLEDGMENTS**

This study was supported by a grant from the Vice Chancellor of Research of Isfahan University of Medical Sciences.

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