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Effect of photodynamic therapy with two photosensitizers on *Candida albicans*

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ABSTRACT

Background and Objectives: Oral candidiasis (OC) is an opportunistic infection of the oral cavity most commonly caused by *Candida albicans* (*C. albicans*). Considering the drawbacks of standard treatments with antifungal agents, this study sought to assess the efficacy of photodynamic therapy (PDT) with methylene blue (MB) and indocyanine green (ICG) photosensitizers against *C. albicans*.
Materials and Methods: In this in-vitro, experimental study, 130 samples of *C. albicans* standard suspensions were subjected to various combinations of MB and ICG photosensitizers with and without laser irradiation with different exposure parameters, nystatin and chlorhexidine (CHX) in 13 groups of 10. Samples were cultured in microplates containing Sabouraud dextrose agar medium and colony forming units (CFUs) were counted after 24 h of incubation at 37 °C. Data were analyzed by SPSS version 19.0, one-way ANOVA and Tamhane's test.
Results: The maximum number of CFUs was seen in the control group (mean of 214,200 CFUs with a log value of 5.32) while the minimum values were noted in the laser (808 nm and 100 Hz PRR) plus ICG (mean of 13,460 CFUs and log value of 4.12) and nystatin (mean of 13,940 CFUs and log value of 4.14) groups.
Conclusion: Within the limitations of this in vitro study, the results revealed that laser application (808 nm, 100 Hz PRR) plus ICG caused a significant reduction in *C. albicans* CFUs.

1. Introduction

Oral candidiasis is an opportunistic infection of the oral cavity. *Candida albicans* is the causative agent of the most common form of OC accounting for 60–70% of the cases [1]. Non-pathogenic *C. albicans* is found in the normal microbial flora of the mouth. However, under certain conditions, it may cause OC [2]. *C. albicans* may be activated in subjects with impaired cellular immunity and cause oral infections [3]. Nystatin and amphotericin B are prescribed routinely for treatment of OC. However, due to bitter taste, these medications cause nausea and are not well tolerated by patients [4]. Moreover, *Candida* species are becoming increasingly resistant to some antifungal agents such as fluconazole and fluconazole-resistant *Candida* species have been found in 81% of AIDS patients under treatment for oral candidiasis [5]. Treatment of fungal infections, especially the invasive forms, is always challenging due to limited availability of medications and the risk of development of resistant species. These findings highlight the need for developing novel treatment strategies for fungal infections [6–8].

Photodynamic therapy is a new therapeutic strategy based on the interaction of a non-toxic photosensitizer and a harmless light source. Combination of these two factors in presence of oxygen results in creation of reactive oxygen species (ROS) and triggers a cascade of biological events that leads to apoptosis and death of microorganisms [9]. In other words, in this process, cells are treated with photosensitizers, which make them susceptible to killing following light exposure. Photosensitizers often have minimal inherent cytotoxicity but trigger the formation of ROS following excitation with appropriate-wavelength light. Photodynamic therapy has been successfully used for treatment of neoplasms and is believed to be a promising novel treatment strategy for a number of non-neoplastic conditions [10,11]. However, the efficacy of PDT for treatment of fungal infections has yet to be fully elucidated. Considering the advantages of PDT, this study aimed to assess the efficacy of PDT with MB and ICG photosensitizers and variable laser parameters against *C. albicans*.

2. Materials and Methods

In this in-vitro, experimental study, 130 samples of *C. albicans* standard suspensions were prepared and evaluated in 13 groups of 10. Sample size was selected based on previous studies [12].

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A 0.5 McFarland standard suspension of standard strain *C. albicans* (ATCC 10231) was prepared and cultured in the solid Sabouraud dextrose agar. After ensuring the purity of culture, a new 0.5 McFarland standard suspension was prepared. The suspension was transferred to a 96-well microplate (0.1 mL in each well) using a sampler. The same amount of photosensitizer (MB or ICG) or sterile saline was also added. All these steps were performed under a laminar hood to ensure sterility and dark environment. Diode laser (A.R.C. laser GmbH, Nurnberg, Germany) at 606 and 808 nm wavelengths was calibrated and irradiated (Fig. 1).The study groups were as follows: (See Figs. 2,3.)

Group one or control group: No photosensitizer, laser or routine medications were used in this group.

Group two: 0.1 mL of ICG photosensitizer was added to the suspension. Laser was not irradiated.

Group three: Sterile saline solution was added instead of photosensitizer and samples were subjected to diode laser irradiation at a wavelength of 808 nm (continuous wave).

Group four: ICG photosensitizer and 808 nm laser (continuous wave) were used.

Group five: Laser at 808 nm wavelength with 100 Hz pulse repetition rate (PRR) without photosensitizer was used in this group. Sterile saline solution was added instead of photosensitizer.

Group six: 0.1 mL of the ICG was added to the suspensions and followed by laser irradiation at 808 nm wavelength with 100 Hz PRR.

Group seven: 660 nm laser (continuous wave) without photosensitizer was used. Sterile saline was added instead of photosensitizer.

Group eight: Laser (continuous wave) at a wavelength of 660 nm was used with MB photosensitizer.

Group nine: Laser (continuous wave) at a wavelength of 660 nm with 100 Hz PRR was used with MB photosensitizer.

Group 10: Laser (continuous wave) at a wavelength of 660 nm with 100 Hz PRR without photosensitizer was used. Sterile saline solution was added instead of photosensitizer.

Group 11: MB photosensitizer without laser was used.

Group 12: 0.1 mL nystatin (100,000 units, Jaber-ebn-Hayan Pharmaceuticals, Tehran, Iran) was added to the samples in this group.

Group 13: 0.1 mL of 0.2% CHX was added to the suspension.

Diode laser exposure setting in groups with ICG included continuous wave mode, 808 nm wavelength, 10 J/cm^2 radiation dose, 100 mW output power, 100% duty cycle and 100 s of radiation time. The same exposure settings were used in the laser groups with 100 Hz PRR along with ICG except that 50% duty cycle was selected with 200 s of radiation time. In groups using MB, exposure settings included continuous wave mode, 660 nm wavelength, 40 mW output power and 100 s of radiation time. In laser groups with 100 Hz PRR and MB photosensitizer, the same parameters were used except that 50% duty cycle was selected and the radiation time was 200 s [13,14].

Next, the suspensions in each well were cultured in microplates containing Sabouraud dextrose agar medium and the results were reported after 24 h of incubation at 37 °C [12]. (Fig. 2,3). In the nystatin and CHX groups, the pour plate technique was used to count the *C. albicans* colonies in the diluted suspension. For this purpose, 0.1 mL of the suspension was poured into a #8 empty sterile plate by a sampler; 25 mL of the cooled sterile Sabouraud dextrose agar medium was added and after closing the lid, the plate was gently whirled to mix the sample and the medium. The plate was then transferred to an incubator. The CFUs were counted after 24 h of incubation at 37 °C [15].(Fig. 4).

Data analysis:

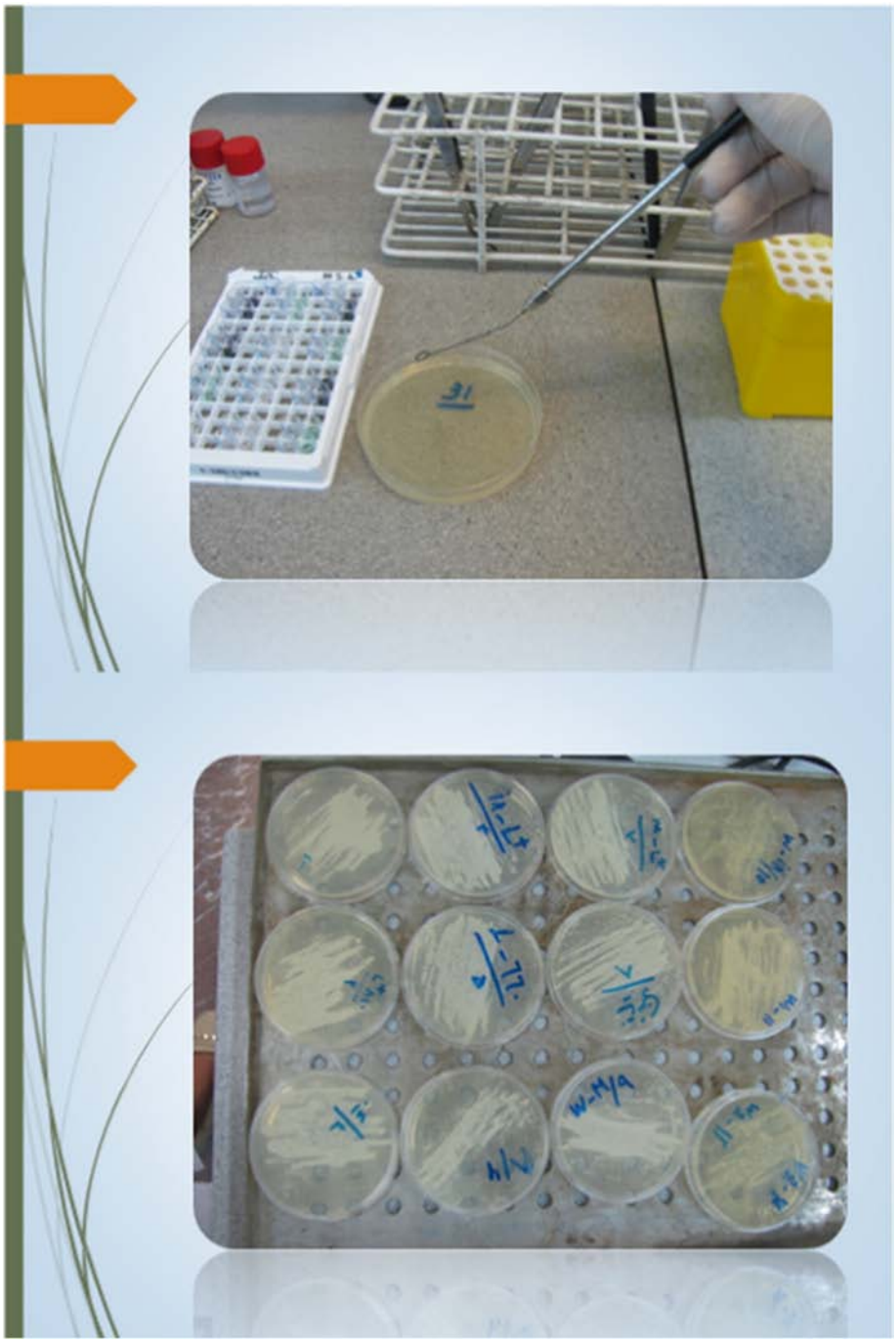
Data were analyzed by SPSS version 19.0 (Microsoft, IL, USA). The mean and standard deviation (SD) of *C. albicans* CFUs as well as the log values were reported for different treatment groups. The difference in the number of *C. albicans* CFUs among groups was analyzed using one-way ANOVA. Pairwise comparison of groups in terms of the number of CFUs was performed using Tamhane's test due to unequal group variances. Type 1 error was considered as 0.05 and $P < 0.05$ was considered statistically significant.

3. Results

The mean and SD of *C. albicans* CFUs and the log values are shown in Tables 1 and 2.

The maximum number of CFUs was seen in the control group (mean of 214,200 CFUs with a log value of 5.32) while the minimum values were noted in the laser (808 nm and 100 Hz PRR) plus ICG (mean of

Fig. 1. Diode laser.



Figs. 2,3. Culture of the suspensions in microplates containing Sabouraud dextrose agar medium.

13,460 CFUs and log value of 4.12) and nystatin (mean of 13,940 CFUs and log value of 4.14) groups. In other words, the highest treatment efficacy belonged to laser irradiation at 808 nm and 100 Hz PRR plus ICG photosensitizer; the efficacy of this treatment modality was slightly higher that that of treatment with nystatin.

Tables 1 and 2 show the 95% confidence interval (95% CI) of the mean number of *C. albicans* CFUs and the log values. One-way ANOVA revealed significant differences among groups in terms of the number of *C. albicans* CFUs ($P < 0.0001$). On the other hand, in most cases, significant differences were noted in pairwise comparison of groups by Tamhane's test with regard to the changes in the number of CFUs (Table 3). Only a few groups in pairwise comparisons were not significantly different in this regard including: laser (808 nm and 100 Hz PRR) + ICG and nystatin, laser (660 nm and 100 Hz PRR) + MB and 660 nm laser (CW) + MB, laser (660 nm and 100 Hz PRR) + MB and 808 nm laser (CW) + ICG, 660 nm laser (CW) + MB and 808 nm laser (CW) + ICG, 660 nm laser (CW) + MB and 660 nm laser, 808 nm laser (CW) + ICG and 660 nm laser, 808 nm laser (100 Hz PRR) and 660 nm laser (100 Hz PRR), and ICG and MB. The abovementioned groups showed similar efficacy in terms of decreasing the *C. albicans* CFUs. Pairwise comparison of groups and the related P values are shown in Table 3.

4. Discussion

This in-vitro experimental study evaluated the susceptibility of *C. albicans* to PDT using ICG and MB photosensitizers along with different diode laser parameters. The results of these treatment modalities

Fig. 4. The CFUs were counted after 24 h of incubation at 37 °C.

were compared with those of conventional treatment (nystatin). The highest number of CFUs was found in the control group (mean of 214,200 CFUs and log value of 5.32) and the lowest in laser (808 nm, 100 Hz PRR) + ICG (mean of 13,460 CFUs and log value of 4.12) and nystatin (mean of 13,940 CFUs and log value of 4.14) groups. In other words, the highest efficacy belonged to treatment with diode laser irradiation at a wavelength of 808 nm and 100 Hz PRR along with ICG photosensitizer, which showed an efficacy slightly superior to that of conventional treatment with nystatin. On the other hand, pairwise comparisons also yielded significant differences among groups in terms of the number of *C. albicans* CFUs.

Many previous studies have recommended PDT as an alternative to antifungal medications with positive results. De Souza et al., in 2006 reported that irradiation of laser at a wavelength of 685 nm along with the use of MB photosensitizer had efficient fungicidal effects on *C. albicans*; these results are in line with our findings [12]. Souza et al., in 2010 evaluated the efficacy of PDT with variable energy densities and MB, toluidine blue (TB) and malachite green photosensitizers. They confirmed the optimal efficacy of photosensitizers for use in PDT against *C. albicans* and showed that these effects depended on the laser energy [16]. In our study, the effect of PDT on *C. albicans* was found to be related to the type of photosensitizer used and the energy and wavelength of laser. However, Pupo et al., in 2011 used MB and TB photosensitizers along with Indium Gallium aluminum phosphide (InGaAlP) laser irradiation with an energy density of 53 J/cm^2 and reported that number of viable *C. albicans* cells significantly decreased after PDT and this reduction was irrespective of the type of photosensitizer used [17]. Fekrazad in 2015 confirmed the efficacy of PDT with the use of new methylene blue against *C. albicans* [18].

Paz-Cristobal et al., in 2014 reported that PDT with the use of hypericin and dimethyl MB photosensitizers effectively eliminated *C. albicans* strains resistant to azole [19].Silva et al., in 2014 reported that PDT was effective in disinfecting root canals [20]. The efficacy of laser irradiation along with the use of photosensitizers has been confirmed for elimination of *C. albicans* strains [21,22]. In general, several studies have shown that *C. albicans* strains in different growth phases and morphological states in a suspension or in an adherent biofilm are sensitive to PDT [23–26]. However, *C. albicans* similar to other fungi is more resistant to PDT than Gram-positive bacteria, which is due to the presence of nuclear membrane, larger cell size and decreased number of areas targeted by singlet oxygen per volume unit of cell [27,28].

On the other hand, some parameters such as the type and concentration of dye, physiological status of the target microorganism, irradiation intervals and the output energy of the device all affect the outcome of

Table 1
The mean and standard deviation of *C. albicans* CFUs in the study groups.

Group	Mean	Standard deviation	Standard error	95% CI Lower	95% CI Upper	Minimum	Maximum
Laser (808 nm, 100 Hz PRR) + ICG	13,460	1895.1	599.3	12,104.3	14,815.7	10,000	15,800
Nystatin	13,940	1240.3	392.2	13,052.8	14,827.2	12,400	16,400
Laser (660 nm, 100 Hz PRR) + MB	19,500	1455.3	460.2	18,458.9	20,541.1	16,800	21,600
Laser 660 nm (CW) + MB	20,400	1232.9	389.9	19,518.1	21,281.9	19,000	22,000
Laser 808 nm (CW) + ICG	22,590	2821.1	892.1	20,571.9	24,608.1	19,300	28,000
Laser 660 nm	26,320	4166.8	1317.6	23,339.3	29,300.7	18,600	29,800
Laser 808 nm	30,700	1538.4	486.5	29,599.5	31,800.5	28,600	32,600
Laser 808 nm (100 Hz PRR)	34,940	1943.8	614.7	33,549.5	36,330.5	31,200	38,400
Laser 660 nm (100 Hz PRR)	35,410	3063.2	968.7	33,218.7	37,601.3	29,800	38,600
CHX	41,060	1532.0	484.5	39,964.1	42,155.9	38,600	43,200
ICG	75,660	3600	1138.4	73,084.7	78,235.3	69,800	79,800
MB	80,900	2433.6	769.6	79,159.1	82,640.9	78,400	84,600
Control	214,200	51,787.6	16,376.7	177,153.4	251,246.6	150,000	286,000

Table 2
The man and standard deviation of log values of *C. albicans* CFUs in the study groups.

Group	Mean	Standard deviation	Standard error	95% CI Lower	95% CI Upper	Minimum	Maximum
Laser (808 nm, PRR = 100 Hz) + ICG	4.12	0.06	0.02	4.08	4.17	4	4.2
Nystatin	4.14	0.04	0.01	4.12	4.17	4.09	4.21
Laser (660 nm, 100 Hz PRR) + MB	4.29	0.03	0.01	4.27	4.31	4.23	4.33
Laser 660 nm (CW) + MB	4.31	0.03	0.01	4.29	4.33	4.28	4.34
Laser 808 nm (CW) + ICG	4.35	0.05	0.02	4.31	4.39	4.29	4.45
Laser 660 nm	4.41	0.08	0.02	4.36	4.47	4.27	4.47
Laser 808 nm	4.49	0.02	0.01	4.47	4.5	4.46	4.51
Laser 808 nm (PRR = 100 Hz)	4.54	0.02	0.01	4.53	4.56	4.49	4.58
Laser 660 nm (PRR = 100 Hz)	4.56	0.06	0.02	4.52	4.6	4.47	4.48
CHX	4.61	0.02	0.01	4.6	4.62	4.59	4.64
ICG	4.88	0.02	0.01	4.86	4.89	4.84	4.9
MB	4.91	0.01	0.01	4.89	4.92	4.89	4.93
Control	5.32	0.11	0.03	5.24	5.39	5.18	5.46

PDT with laser [29]. In our study, laser irradiation (808 nm, 100 Hz PRR) along with ICG photosensitizer showed the highest antifungal efficacy even superior to that of treatment with nystatin. Indocyanine green is a fluorescent dye, which is used as a photosensitizer in PDT with no toxicity for the healthy tissues. Moreover, its effects on increasing the antimicrobial efficacy of laser irradiation in test tubes have been previously confirmed [20].

In our study, the lowest efficacy belonged to the groups with the use of ICG and MB alone. This indicates that laser irradiation in PDT is necessarily required to decrease the number of *C. albicans* CFUs, and use of photosensitizers alone does not have adequate efficacy for this purpose.

Several parameters in the protocol of PDT may influence the results such as the light parameters, photosensitizers and the method of light irradiation [30]. The peak absorbance of the photosensitizer must match the radiated wavelength to ensure generation of singlet oxygen and ROS, which are responsible for elimination of bacterial strains [31,32]. Methylene blue and TB are among the commonly used photosensitizers; they both belong to the phenothiazine family of dyes with maximum absorbance at 656 nm and 625 nm wavelengths, respectively [29]. On the other hand, antimicrobial efficacy of PDT may also depend on the concentration of photosensitizer [31].

Wainwright in 1998 showed that photodynamic inactivation of microorganisms depended on the chemical formulation of the photosensitizer and time of incubation of drug with bacterial cells [33]. Penetration of photosensitizers into cells is not a passive phenomenon; because the cell membrane serves as a selective barrier against free diffusion. Also, diffusion of photosensitizer molecules depends on their size and degree of solubility and they can also accumulate in specific sub-cellular locations and relate to hydrophobic areas in membrane organelles [34]. Damage to the bacterial cell wall, increased permeability of cytoplasmic membrane and DNA cleavage are also probable following PDT [33]. Considering the above-mentioned advantages and the confirmed efficacy of PDT for decreasing oral bacterial counts, PDT may be suitable for use in the clinical setting [35,36].

In contrast to MB and TB, number of studies on the efficacy of ICG photosensitizer is small. Positive efficacy of ICG in periodontal treatments for decreasing the count of periodontal pathogens has been confirmed in vitro [37]. In combination with ICG, efficacy of low-level laser has been confirmed for elimination of bacteria in the biofilm and periodontal pockets [37]. Tuchin et al., in 2003 demonstrated the optimal efficacy of diode laser irradiation (803 or 809 nm wavelength) along with ICG for treatment of acne vulgaris [14].

Peak activity of ICG is at 778 and 708 nm wavelengths. The balance between the monomeric and oligomeric forms of ICG depends on factors such as dye concentration, type of solvent, anionic strength, its pH and temperature [38]. By increasing the concentration of ICG, its monomeric form decreases and its peak absorbance shifts towards the short wavelength spectrum, indicating an increase in its oligomeric form in the solution. However, ICG solutions are not stable [39] and after a couple of days, its peak absorbance shifts towards 900 nm wavelength, which is probably due to higher polymerization at this wavelength [38]. ICG mainly absorbs near infrared wavelengths and this mechanism affects its function [38].

In the current study, CHX, after the photosensitizers alone and the control (no treatment) groups, showed the lowest efficacy for elimination of *C. albicans*. Chlorhexidine is a cationic broad-spectrum antimicrobial agent belonging to the bisbiguanide family. It is effective against both Gram-positive and Gram-negative bacteria [39]. Chlorhexidine electrostatically bonds to negatively charged areas in the bacteria and changes their osmotic balance; consequently, the intracellular components leak out [39]. At low concentrations, CHX is bacteriostatic but in high concentrations, it exerts bactericidal effects by causing the coagulation of intracellular constituents [40].

One concern related to laser irradiation is heat generation and its adverse effects on the healthy tissues in the clinical setting. However, diode laser has reported to cause the least increase in temperature among the available types of lasers [41]. In a study by Silva et al., in 2014, diode laser irradiation for 30, 60, and 120 s in presence of photosensitizer resulted in 2° increase in temperature around the lesion, which was negligible [20]. Also, it was demonstrated that irradiation of 0.5 to 5 W diode laser for two minutes had no effect on proliferation of mammalian cells in vitro [42]. Thus, diode laser irradiation for elimination of *C. albicans* probably causes no thermal damage to the healthy tissues. Indocyanine green has insignificant cytotoxicity as well [37].

Different types of lasers have been used in vitro, but diode laser is the most commonly used laser in clinical studies [43]. Therefore, diode laser at 660 and 808 nm wavelengths and 100 and 40 mW output powers were used in our study for 100 and 200 s (10 J/cm^2 energy). Diode lasers are cost-effective, simple to use and easy to handle and they may be used for their therapeutic effects in the oral cavity [44]. At lower radiation doses, diode lasers have greater antibacterial effects compared to higher radiation doses. Moreover, application of high dose laser may damage the adjacent normal tissues (43,).

Candida albicans was used in our study, since it is the most common microorganism responsible for OC infection [1]. Selection of nystatin as the positive control group was because of its confirmed effects on *Candida* strains.

In general, it seems that diode laser irradiation along with the use of ICG or MB is effective for elimination of *C. albicans*; however, considering the differences between the experimental and clinical conditions, generalization of results to the clinical setting must be done with caution. Further studies are required to find the ideal laser parameters and the most suitable concentration of photosensitizers to achieve the

Table 3
The results of pairwise comparisons of study groups in terms of changes in the mean number of *C. albicans* CFUs.

Group 1	Group 2	Mean difference in CFUs	P value
Laser 808 nm, (PRR = 100HZ) + ICG	Laser 660 nm (PRR = 100 Hz) + MB	6040	0.0001
	Laser 660 nm (CW) + MB	6940	0.0001
	Laser 808 nm (CW) + ICG	9130	0.0001
	Laser 660 nm	12,860	0.0001
	Laser 808 nm	17,240	0.0001
	Laser 808 nm (PRR = 100 Hz)	21,480	0.0001
	CHX	27,600	0.0001
	ICG	62,200	0.0001
	MB	67,440	0.0001
	Control	200,740	0.0001
	Nystatin	480	0.1
	Laser 660 nm (PRR = 100HZ)	21,950	0.0001
Laser 660 nm (100 Hz PRR) + MB	Laser 660 nm (CW) + MB	900	0.1
	Laser 808 nm (CW) + ICG	3090	0.49
	Laser 660 nm	6820	0.04
	Laser 808 nm	11,200	0.0001
	Laser 808 nm (100 Hz PRR)	15,440	0.0001
	CHX	21,560	0.0001
	ICG	56,160	0.0001
	MB	61,400	0.0001
	Control	194,700	0.0001
	Nystatin	5560	0.0001
	Laser 660 nm (PRR = 100 Hz)	15,910	0.0001
Laser 660 nm (CW) + MB	Laser 808 nm (CW) + ICG	2190	0.97
	Laser 660 nm	5920	0.1
	Laser 808 nm	10,300	0.0001
	Laser 808 nm (PRR = 100 Hz)	14,540	0.0001
	CHX	20,660	0.0001
	ICG	55,260	0.0001
	MB	60,500	0.0001
	Control	193,800	0.0001
	Nystatin	6460	0.0001
	Laser 660 nm (100 Hz PRR)	15,010	0.0001
Laser 808 nm (CW) + ICG	Laser 660 nm	3730	0.92
	Laser 808 nm	8110	0.0001
	Laser 808 nm (PRR = 100 Hz)	12,350	0.0001
	CHX	18,470	0.0001
	ICG	53,070	0.0001
	MB	58,310	0.0001
	Control	191,610	0.0001
	Nystatin	8650	0.0001
	Laser 660 nm (PRR = 100 Hz)	12,820	0.0001
Laser 660 nm	Laser 808 nm	4380	0.52
	Laser 808 nm (PRR = 100 Hz)	8620	0.004
	CHX	14,740	0.0001
	ICG	49,340	0.0001
	MB	54,580	0.0001
	Control	187,880	0.0001
	Nystatin	12,380	0.0001
	Laser 660 nm (PRR = 100 Hz)	9090	0.0001
Laser 808 nm	Laser 808 nm (PRR = 100 Hz)	4240	0.004
	CHX	10,360	0.0001
	ICG	44,960	0.0001
	MB	50,200	0.0001
	Control	183,500	0.0001
	Nystatin	16,760	0.0001
	Laser 660 nm (PRR = 100 Hz)	4710	0.06
Laser 808 nm (PRR = 100HZ)	CHX	6120	0.0001
	ICG	40,720	0.0001
	MB	45,960	0.0001
	Control	179,260	0.0001
	Nystatin	21,000	0.0001
	Laser 660 nm (PRR = 100 Hz)	470	0.1
CHX	ICG	34,600	0.0001
	MB	39,840	0.0001
	Control	173,140	0.0001
	Nystatin	27,120	0.0001
	Laser 660 nm (PRR = 100HZ)	5650	0.01
ICG	MB	5240	0.12
	Control	138,540	0.001
	Nystatin	61,720	0.0001
	Laser 660 nm (PRR = 100 Hz)	40,250	0.0001
MB	Control	133,300	0.001
	Nystatin	66,960	0.0001
	Laser 660 nm (PRR = 100 Hz)	45,490	0.0001
Control	Nystatin	200,260	0.0001
	Laser 660 nm (PRR = 100 Hz)	178,790	0.0001
Nystatin	Laser 660 nm (PRR = 100 Hz)	21,470	0.0001

best antifungal efficacy by PDT in the clinical setting. Moreover, future studies are required on pure bacterial samples in vitro or in situ and also on bacterial samples extracted from dental biofilm.

5. Conclusion

Within the limitations of this in vitro study, the results revealed that laser application (808 nm, 100 Hz PRR) plus ICG caused a significant reduction in *C. albicans* CFUs.

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