

Effect of the 1064 nm Nd: YAG Laser on the MICs of Antifungals Used in Clinical Practice for the Treatment of Fungal Nail Infections



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Abstract

Introduction: The fungal nail infection (onychomycosis) involves 18%-40% of all nail disorders, which, although not fatal, can cause mechanical, aesthetic, occupational, and economic problems. Drug treatments due to prolonged treatment periods, drug interactions, adverse effects, and slow progression may associate with numerous negative outcomes. This study aimed to evaluate the long-pulsed 1064-nm Nd: YAG laser effect on fungal colonies and subsequently possible change in the minimum inhibitory concentrations (MICs) of common antifungals compared with the same non-lasered colonies as a novel way to investigate laser and antifungal interaction.

Methods: Sixty onychomycosis samples consisting of saprophyte (n=20), dermatophyte (n=20), and yeast (n=20) duplicate colonies were isolated. A series was treated by a long-pulsed 1064-nm Nd: YAG laser. Afterward, the MIC (CLSI-M38-A2 and CLSI-M27-A3) of two series against common antifungals were compared.

Results: After 1064-nm Nd: YAG laser irradiation in all 20 tested saprophytes, the MICs of terbinafine (P value<0.035) were changed, and in all 20 tested dermatophytes, the MICs of voriconazole (P value<0.021) were changed. Also, in all 20 tested yeasts, the MICs of caspofungin (P value<0.037) were changed. Moreover, in saprophytes, dermatophytes, and yeasts, significant changes in the MICs of itraconazole (P value<0.032), terbinafine (P value<0.025), and caspofungin (P value<0.037) were detected. Our result showed the GM MICs of the 1064-nm Nd: YAG laser in all saprophyte, dermatophyte, and yeast groups were lower than in the control group.

Conclusion: The present study indicated that the long-pulsed 1064-nm Nd: YAG laser significantly changes the MICs of antifungals in onychomycosis clinical samples.

Keyword: Long-pulsed 1064-nm Nd: YAG laser; Minimum inhibitory concentration; Yeasts; Dermatophytes; Saprophyte.



Introduction

The fungal nail infection is also called onychomycosis. Three groups of saprophytic molds, dermatophytes, and yeasts contain the etiologic agents. Numerous issues affect the frequency rate of onychomycosis, such as age, sex, underlying disease, occupation, diagnostic assessment method, living environment, geographic area, and country.¹ This infection is responsible for 40% of all nail disorders.²⁻⁵ Nail disorders, especially onychomycosis and psoriasis, are difficult to treat.⁶

The current treatment of onychomycosis is divided into three groups: systemic pharmacologic drugs, topical pharmacological agents, and non-pharmacological therapies.¹ There was not very much success in the treatment of nail fungal infections with oral and topical therapy. Oral antifungal treatment involves prolonged use, and notable incidences of side effects and drug-drug interactions have been reported for it.^{7,8} Among non-pharmacological treatments, different lasers at different energy levels and various treatment modalities with or

without concomitant antifungals have been studied.⁹ Some FDA-approved laser systems in a patient with onychomycosis are 532-nm output mode Nd: YAG (neodymium-doped yttrium aluminum garnet), 630-680-nm output mode Nd: YAG, 1064-nm Nd: YAG Q-switched, 1320-nm Nd: YAG, 980 nm diode, and 870/930-nm diode lasers.¹⁰ Lasers likely apply their fungicidal effects through numerous (photothermal, photo-acoustic, thermomechanical, and photochemical) mechanisms toward cell wall structures (chitin, melanin, and xanthomennin).¹¹⁻¹³

Because temperatures more than 50°C are necessary to attain a direct thermal killing effect on fungal mycelia,^{3,4} the use of pulse durations decreases the pain and minimizes complications, containing necrosis.^{3,5} For pulse durations shorter than the thermal relaxation time of the target cell wall structure, maximal efficacy can be obtained.^{5,6} At wavelengths of 750 to 1300 nm, nail plates are penetrated and fungi are targeted.¹⁴ The 1064-nm Nd:YAG laser is a long-pulsed device that emits light with a wavelength of 1064 nm. This means that fungal material gets superheated as it passes through the nail plates and into the bed of nails. It facilitates the transmission of well-controlled energy to a specific target, does not damage tissues other than targeted areas, and can be used safely in patients with onychomycosis.¹⁵ Exposure to high temperatures affects the growth of fungi, causing cell damage and death.¹⁶ Since it is a longer wavelength, it is able to penetrate deeper into the tissue, and it is able to effectively inhibit fungal growth in the nail bed.^{2,17} The pulse length is in the range of milliseconds, and because the laser may produce a high degree of nonspecific heat, a dedicated cooling system may be required for the treatment.¹¹ The long pulse Nd:YAG laser is thought to be more efficient in penetrating tissue, helping to effectively eliminate fungus growth in a nail bed as a result of its longer wavelengths.¹⁸ This study aimed to evaluate the combination therapy of laser and common antifungals in fungal colonies and subsequently possible MIC change of antifungals compared with the same non-lasered colonies. Therefore, the aim of the present study is to survey the possibility of change the treatment protocol of onychomycosis from the systemic antifungals to topical forms through combination therapy with a 1064 nm Nd: YAG laser. Improving clinical management and selecting the best treatment method will be facilitated by clarifying this factor.

Materials and Methods

Patients, Sampling and Data Collection

This was an experimental interventional study of the fungal culture of nail scraping collected from patients with onychomycosis affected by a long-pulsed 1064-nm Nd: YAG laser. The scraping samples were collected from patients admitted to medical mycology laboratories of

Razi hospital and School of Public of Health Department, which were not private and were parts of Tehran University of Medical Sciences located in Tehran, Iran, during a period of 8 months (10 July 2019 to 9 March 2020). In Iran, the TUMS Mycology Laboratory is one of the main referral centres where antifungal susceptibility tests are done for patients with fungal infections not responding to traditional therapy so that a more suitable treatment plan can be selected.

Sixty nail scraping samples were gathered from the patients who have shown nail changes which indicate onychomycosis, such as thickening or vitiation of nails, detached nails yellow or white, discoloration of nails, and plate thinness. The following criteria were used for the exclusion: the patients who received topical and systemic antifungal therapy at sampling time or at least < 15 days prior to the sample collection and the patients who did not have sufficient clinical data to complete the analysis. Demographic data which included age and gender were collected. Scrapings were gathered from the nails. The outermost debris gathered was discarded, and the samples were extracted from a place next to the cuticle which contained more fungal elements.

Culture and Phenotypic Examination

Sixty nail clippings were gathered from the patients with onychomycosis. The specimens were screened by direct microscopic examination using 10% potassium hydroxide (KOH) and cultured on sabouraud dextrose agar with chloramphenicol (S/C, Merck, Germany). For up to 30 days, the culture tubes had been in incubation at a temperature of 30 °C. The growth of the fungus was evaluated every day. Its identification characteristics, such as colony size, shape, pigmentation, and texture, were used to identify any growth obtained.

Yeast isolates were identified using CHROMagar™ *Candida* medium (CHROMagar, HiMedia, India). Also, filamentous fungi were identified using Potato-dextrose agar (PDA; Merck, Darmstadt, Germany) and Czapek agar (CZ, Micro media, Hungary).^{19,20} Validation of all isolates was carried out by polymerase chain reaction (PCR) sequencing as below.

Molecular Technique

DNA Extraction

According to the manufacturer's recommended guidelines, fungal genomic DNA was taken out of harvested colonies using a high purity PCR template preparation kit from Roche, Germany.

PCR Conditions and Sequencing

Aspergillus isolates were determined according to the β -tubulin gene sequence using the forward (Bt2a: 5'-GGTAACCAAAT CGGTGCTGCTTTC-3') and reverse (Bt2b: 5-ACCCTC AGTGTAGTG ACCCTTGGC-3)

primers. Also, other fungal species were the use of universal primers: ITS1 (5'TCC GTA GGT GAA CCT GCG G 3') and ITS4 (5'TCC TCC GCT TAT TGA TAT GC 3) (Life Technologies, Barcelona, Spain). The reaction was started with a 5-minute denaturation at 94 °C, followed by 35 cycles of 30 seconds at 94 °C, 60 °C for 45 seconds, 72 °C for 1 minute, and one final extension at 72 °C for 5 minutes.

Single sequencing with the forward direction primer (Bioneer, South Korea) was applied to positive PCR products. The trace files obtained from the sequencer were aligned. The consensus sequences were automatically exported to the WI database, and all ambiguous characters were replaced or removed by a manual edit. After that, they were checked, compared with the existing sequences from GenBank, and validated by the curators (Table S1 in Supplementary file 1). The schematic diagram of the study procedure is described in Figures 1A and 1B.

Antifungal Susceptibility Testing

Based on the documents M38-A2 and M27-A3 presented in the Clinical and Laboratory Standards Institute (CLSI) guidelines, *in vitro* antifungal susceptibility testing was performed against filamentous fungi and yeasts, respectively. AFST included the following antifungal drugs: voriconazole (VOR), caspofungin (CAS), itraconazole (ITR), terbinafine (TRB), fluconazole (FLU), and amphotericin B (AMP B) (all from Sigma-Aldrich Company, St. Louis, MO). We developed the following dilutions: (0.008-8 µg/mL) for TRB, (0.016-16 µg/mL) for VOR, (0.016-16 µg/mL) for ITR, (0.064-64 µg/mL) for FLU, (0.008-8 µg/mL) for CAS, and (0.016-16 µg/mL)

for AMP B (Sigma-Aldrich Company, St. Louis, MO). Reference strains of *Candida parapsilosis* (ATCC 22019) and *Candida krusei* (ATCC 6258) were used as CLSI control strains. The AFST was performed in duplicate.

Laser Irradiation Method

Colonies were grown on an S/C 8 cm polystyrene plate with a 5-7 mm depth of agar after seven days and colonies were grown on an S/C 8 cm polystyrene plate with a 5-7 mm depth of agar, reaching a diameter of 10 mm after seven days of growth. In the present study, the long-pulsed 1064-nm Nd: YAG laser (Clarity™, Long-Pulsed Alexandrite & Nd: YAG Laser, Lutronic, Inc) was administered on the fungal colonies using the selected laser spot size of 3 mm, fluence of 45-55 J/cm², pulse width 30 millisecond (ms), pulse rate: 1 Hz, interval: once, in one session and without a cooling system on for twice. Moreover, the irradiation angle 90 degrees, with 10-cm distance assessed by the company holder, upside of the closed plate was used.

Furthermore, factory long-pulsed 1064-nm Nd: YAG laser adjustment for the treatment of the fungal nail infection in clinic were spot size: 2 mm, pulse width: 0.1 ms, fluence: 26 J/cm², pulse rate :2 Hz, interval: 1-2 weeks, on nail scrapings in 4 sessions, or spot size: 3 mm, pulse width: 30 ms, fluence: 45 J/cm², pulse rate: 1 Hz, interval: 1 week on nail scrapings in 4 sessions.

The treatment was composed of irradiation of the whole colonies area twice, about 20-70 pulses. In fact, to resemble the *in vivo* condition of Nd: YAG laser treatment in clinic and patient tolerance, we irradiated the surface and upper area of colonies with the laser regardless of the

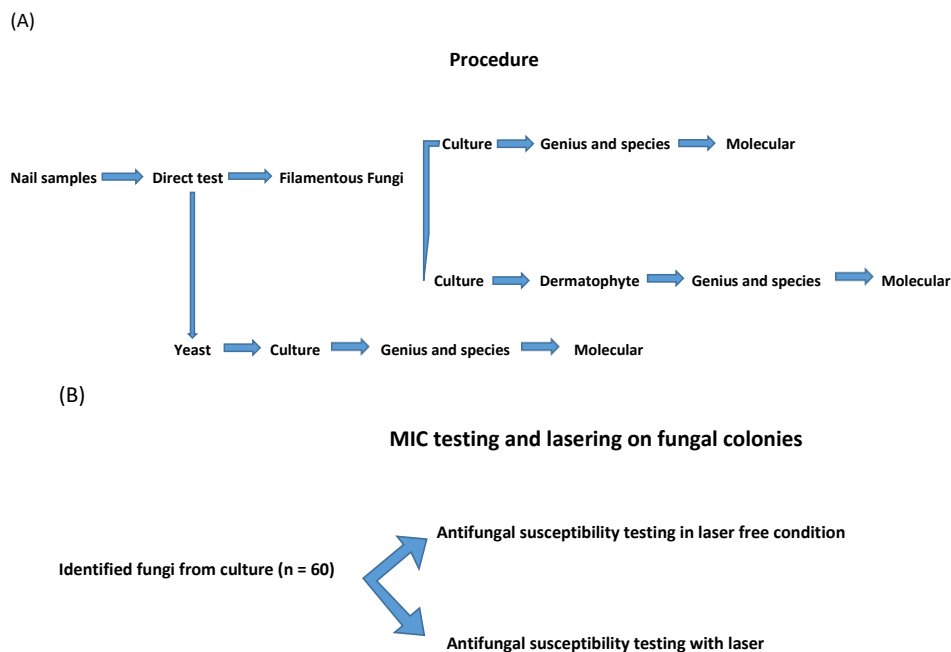


Figure 1. The Schematic Diagram of the Study Procedure. (A) Direct examination, culture, and molecular assessments. (B) Determination of minimum inhibitory concentrations with and without laser irradiation

depth in the manner of all around it with overpassing in common areas for covering the entire area. Due to heat and patient discomfort with laser irradiation during laser therapy, the local temperature during 1064 Nd: YAG irradiation was measured by an infrared thermometer. To evaluate the temperature changes during laser therapy, the temperatures of lasered colonies and approved positive nail clippings fixed in S/C medium plates, 107 times and in 4 conditions (backward and forward with a close and open plate door) were measured by the “distance temperature evaluator” (VisioFocus 06400, TECNIMED srl. P.le Cocchi, 12 -21040 Vedano O. (VA) Italy, <http://www.tecnimed.com/>). The laser treatment was a colony procedure. The schematic diagram of the study procedure is described in Figures 1A and 1B.

Determination of Minimum Inhibitory Concentrations

Sixty nail clipping samples of saprophytic molds ($n=20$), dermatophytes ($n=20$), and yeasts ($n=20$) were cultured in two series (one series remained as the control and another as the response treated by the laser). After the laser treatment, the AFST was done for two series according to CLSI M38-A2 and CLSI M27-A3 protocols. To prevent probable fungi epigenetic change after laser therapy, the minimum inhibitory concentration (MIC) was done within 8 hours.

Statistical Analysis

With the aim of measuring and analyzing the data collected from this study, we used SPSS software version 11 (SPSS Inc., Chicago, IL, USA). MIC ($\mu\text{g/mL}$) results of non-lasered colonies and lasered colonies for saprophytic molds, dermatophytes, and yeasts against related antifungals were compared. Student's *t* test for one paired sample was then performed. We set our significance level at $\alpha \leq 0.05$.

Results

Patient Characteristics

Between October and March 2019-2020, 60 patients met the inclusion criteria of onychomycosis in the present study. The mean age of admission was 54.23 years (range 2-83 years), and 33.34% of the patients were male and 66.66% of them were female. Big toenails were affected more than other nails with onychomycosis (50%), followed by hand nails (40%), and other foot nails (10%) (Figure S1, Supplementary file 1).

Antifungal Susceptibility Testing

The long pulsed 1064 nm Nd: YAG laser caused significant changes in the MICs of saprophytic fungi for TRB (P value = 0.035), dermatophytes for VOR (P value = 0.021), and yeasts for CAS (P value = 0.037); also, overall, in saprophytes, dermatophytes, and yeasts, significant changes for ITR (P value = 0.032), TRB (P

value = 0.025) and CAS (P value = 0.037) were detected (Table 1). In the saprophytic fungi group except for VOR, all the response GM MICs were lower than the GM MICs of control ones.

The concentration of GM MICs for the saprophyte response group treated with the 1064 nm Nd: YAG laser in order of low to high was TRB response, VOR response, ITR response, and AMP B response (0.06, 0.22, 0.76, and 5.03 $\mu\text{g/mL}$, respectively). The results indicated that the best antifungal for saprophytic onychomycosis was TRB because the GM in the TRB response group was lower than that in the TRB control group, and the GMs of both were the lowest antifungal concentration among the others (0.060 and 0.096 $\mu\text{g/mL}$, respectively). The result is presented in Table 2.

In the dermatophyte group, all the response GM MICs were lower than the GM MICs of the control ones. The concentration of GM MICs for the dermatophyte group treated with the 1064 nm Nd: YAG laser in order of low to high were TRB response, VOR response, ITR response, and FLU response (0.031, 0.09, 0.52, and 1.74 $\mu\text{g/mL}$, respectively). The results indicated that the best antifungal for onychomycosis caused by dermatophytes was TRB because the GM MIC in the TRB response group was lower than that in the TRB control group, and the GMs of both were the lowest antifungal concentration among the others (0.031 and 0.033 $\mu\text{g/mL}$ respectively).

Table 1. The Changes in the MICs of Saprophytic Fungi, Dermatophytes, and Yeasts After Long Pulsed ND: YAG 1064 nm Laser Irradiation

	Medication Name	Mean Difference of Medication Concentration ($\mu\text{g/mL}$) (Laser – Control)	Confidence Interval ^a	P Value
All	FLC	-1.784	–Inf to 0.520	0.098
	VRC	-0.182	–Inf to 0.020	0.068
	ITC	-0.17	–Inf to -0.02	0.032
	TRB	-0.022	–Inf to -0.001	0.025
	CAS	-0.51	–Inf to -0.04	0.037
	AMP B	-0.615	–Inf to 0.480	0.169
Dermatophytes	FLC	-0.812	–Inf to 0.150	0.078
	VRC	-0.12	–Inf to -0.03	0.021
	ITC	-0.333	–Inf to 0.120	0.105
	TRB	-0.004	–Inf to 0.000	0.162
Saprophytes	VRC	-0.404	–Inf to 0.150	0.11
	ITC	-0.058	–Inf to 0.020	0.095
	TRB	-0.038	–Inf to -0	0.035
	AMP B	-0.615	–Inf to 0.480	0.169
Yeast	FLC	-2.755	–Inf to 2.020	0.161
	VRC	0.011	–Inf to 0.210	0.541
	ITC	-0.125	–Inf to 0.050	0.106
	CAS	-0.51	–Inf to -0.04	0.037

^aOne-sided paired *t* test.

Table 2. The Geometric Mean of MIC, MIC Ranges, MIC50, and MIC90 Values Obtained by Testing the Susceptibility of 20 Saprophytic isolates Obtained from Onychomycosis Patients to the Antifungal Agents with and without 1064 nm YAG laser therapy

Antifungal	MIC Range (µg/mL)	MIC50 (µg/mL)	MIC90 (µg/mL)	GM (µg/mL)	Mean	Variance	SD	95% CI
TER res (n=20)	0.016-1	0.32	0.125	0.060053	0.135	0.064244	0.253465	-0.01817, 0.28817
TER con (n=20)	0.063-1	0.63	0.25	0.096567	0.173385	0.062210	0.249419	0.02266261, 0.32410739
ITR res (n=20)	0.25-2	0.5	2	0.765983	0.942308	0.386095	0.621365	0.56682089, 1.31779511
ITR con(n=20)	0.5-2	1	2	0.85218	1	0.346154	0.588348	0.64, 1.36
AMP B res (n=20)	1-16	4	16	5.039684	6.75	23.1875	4.81534	3.8401, 9.6599
AMP B con (n=20)	1-16	4	16	5.339	7.416667	29.743056	5.339359	4.19012465, 10.64320935
VOR res (n=20)	0.125-0.5	0.125	0.5	0.229251	0.25	0.011719	0.108253	0.1595, 0.3405
VOR con (n=20)	0.063-0.5	0.25	0.5	0.192968	0.226625	0.015543	0.124674	0.12239493, 0.33085507

MIC: Minimum inhibitory concentration; MIC50: minimal concentration that inhibits 50% of isolates; MIC90: minimal concentration that inhibits 90% of isolates; GM: Geometric mean; VSD: standard deviation; CI: confidence interval; TER: terbinafine; ITR: itraconazole; AMP B: amphotericin B; VOR: voriconazole; res: response of MIC after 1064nm ND YAG laser treatment; con: control MICs without 1064nm ND YAG laser treatment.

The result is presented in Table 3.

In the yeast group, all the response GM MICs were lower than the GM MICs of control ones. The concentration of GM MICs for the yeast group treated with the 1064 nm Nd: YAG laser in order of low to high be VOR response, ITR response, CAS response, FLU response (0.153, 0.435, 0.567 and 1.88 µg/mL, respectively). The best antifungal for onychomycosis caused by yeasts was VOR because the GM MIC in the VOR response group was lower than that in the VOR control group, and the GMs of both were the lowest antifungal concentration among the others (0.153 and 0.186 µg/mL respectively). The result is presented in Table 4. In the present study, one FLU, ITR, and VOR (64, 32 and 2 µg/mL, respectively) resistance *C. albicans* isolate was detected.

The MIC breakpoints for FLU of 16 to 32 µg/mL and ≥64 µg/mL were used to respectively characterize susceptibility dose-dependent (SDD) and resistant (R) categories. For ITR, MIC breakpoints of 0.25 to 0.5 µg/mL and ≥1 µg/mL were respectively considered SDD and R.^{14,15}

Overall, in all (Saprophyte, dermatophyte, and yeast) tested isolates, the concentration of GM MICs with 1064 nm Nd: YAG laser treatment in order of low to high were TRB response, VOR response, ITR response, CAS response, CAS control, FLU response, and AMP B response (0.044, 0.158, 0.561, 0.567, 1.694, and 5.039, respectively). All the response GM MICs in the saprophyte, dermatophyte, and yeast-tested groups were lower than GM MICs in the control ones.

In all three groups of etiologic agents of onychomycoses (saprophytes, dermatophytes, and yeasts), significant changes in the MICs of ITR (0.032 µg/mL), TRB (0.025 µg/mL), and CAS (0.037 µg/mL) were seen.

VOR and ITR were the two common antifungals used in three tested groups. The therapeutic agent of choice for the treatment of onychomycosis caused by all three groups (saprophytes, dermatophytes, and yeasts) was VOR because GM in the VOR response group was lower

than that in the VOR control group, and the GMs of both were the lowest antifungal concentration among the others (0.158 and 0.218 µg/mL, respectively). It was followed by the ITR response group which was lower than the ITR control group, and the GMs of both were the second-lowest antifungal concentration among the others (0.561 and 0.707 µg/mL, respectively) (Table 5).

The lowest GM MIC concentration for saprophytes and dermatophytes was related to the TRB response group, and for yeasts, it was related to the VOR response group (0.044, 0.186, 0.031, and 0.060 µg/mL, respectively) (Table S2 in Supplementary file 1).

The results of temperature monitoring showed averages of 38 for 55 J/cm² and 36 for 45 J/cm². The highest temperatures of 55 J/cm² and 45 J/cm² were >43 °C and 39 °C respectively, and the lowest temperature (23 °C) belonged to the yeasts group. The highest temperature (more than 43 °C) belonged to saprophytes which were related to one *Aspergillus flavus* thick colony in open-petri dish door lasering, especially in yellowish-green pigmentation parts, which occasionally produced sparks and smoke. Not any yeasts, dermatophytes, and nail clippings produce sparks and smoke during irradiation. Mean temperatures in Centigrade degree (°C) during ND-YAG laser (45-55 J/cm²) irradiation in four groups of saprophyte, yeast, dermatophyte colonies, and nail clipping samples were as follows: 39, 32.4, 35.2, and 34.1 °C with ranges of 25-42, 23-41, 24-43 and 24-38 °C respectively. There were not any sparks or smoke observed except for one tick Saprophyte colony (more than 43 °C detected).

Discussion

Antifungal resistance is one of the greatest challenges to clinical success, so finding new and efficient therapies is essential.²¹⁻²⁶ The present study showed that the long-pulsed 1064 nm Nd: YAG laser reduced the GM MICs of antifungals in the *in vitro* condition that may reduce the dose or duration of consumption of the antifungals

Table 3. The Geometric Mean of MIC, MIC Ranges, MIC50, and MIC90 Values Obtained by Testing the Susceptibility of 20 Dermatophytic isolates Obtained from Onychomycosis Patients to the Antifungal Agents with and without 1064 nm YAG laser therapy

Anti-fungal	MIC Range (µg/mL)	MIC50 (µg/mL)	MIC 90 (µg/mL)	GM (µg/mL)	Mean (µg/mL)	Variance	SD	95 % CI
TER res (n=20)	0.008—0.125	0.032	0.063	0.031832	0.044083	0.001488	0.038569	0.01957743, 0.06858857
TER con (n=20)	0.008-0.125	0.032	0.063	0.033636	0.047917	0.001562	0.039519	0.02280783, 0.07302617
ITR res(n=20)	0.25-1	0.5	1	0.529732	0.60416	0.088108	0.2968	0.5896691, 0.6186509
ITR con(n=20)	0.25-4	0.5	1	0.7071070.0	0.9375	0.917969	0.958107	0.03503233, 0.26221767
FLZ res (n=20)	0.5-8	2	4	1.74	2.4	4.39	2.095233	0.901, 3.899
FLZ con(n=20)	0.5-8	2	8	2.297397	3.4	6.99	2.643861	1.509, 5.291
VOR res (n=20)	0.063-0.5	0.063	0.125	0.096869	0.133125	0.019915	0.141119	0.01514656, 0.25110344
VOR con (n=20)	0.063-0.5	0.063	0.125	0.114969	0.148625	0.018461	0.135873	0.03503233, 0.26221767

MIC: Minimum inhibitory concentration; MIC50: minimal concentration that inhibits 50% of isolates; MIC90: minimal concentration that inhibits 90% of isolates; GM: Geometric mean; VSD: standard deviation; CI: confidence interval; TER: terbinafine; ITR: itraconazole; VOR: voriconazole; res: response of MIC after 1064nm ND YAG laser treatment; con: control MICs without 1064nm ND YAG laser treatment.

Table 4. The Geometric Mean of MIC, MIC Ranges, MIC50, and MIC90 Values Obtained by Testing the Susceptibility of 20 Yeast isolates Obtained from Onychomycosis Patients to the Antifungal Agents with and without 1064 nm YAG laser therapy

Anti-fungal	MIC Range (µg/mL)	MIC50 (µg/mL)	MIC90 (µg/mL)	GM (µg/mL)	Mean	Variance	SD	95 % CI
ITR res(n=60)	0.125-1	0.25	1	0.435275	1.9625	21.997031	4.690099	3.28621107, 6.09398693
ITR con(n=60)	0.125-2	0.25	2	0.466516	2.062500	21.89453	4.679159	-1.284769, 5.409769
FLZ res(n=60)	0.063-8	2	16	1.889003	5.880250	80.902157	8.994563	0.1653773, 11.5951227
FLZ con(n=60)	0.125-16	2	16	2.17	8.635417	297.035048	17.234705	-2.31498895, 19.58582295
VOR res(n=60)	0.016-2	0.125	2	0.153587	0.498	0.478192	0.691514	-0.16135178, 1.11773578
VOR con(n=60)	0.032-1	0.125	1	0.186592	0.370714	0.162391	0.402978	-0.00197849, 0.74340649
CAS res(n=60)	0.125-4	1	2	0.567156	1	1.201705	1.096223	0.26, 1.74
CAS con(n=60)	0.125-4	2	4	0.777203	1.545455	1.995093	1.412478	0.59653937, 2.49437063

MIC: Minimum inhibitory concentration; MIC50: minimal concentration that inhibits 50% of isolates; MIC90: minimal concentration that inhibits 90% of isolates; GM: Geometric mean; CI: confidence interval; ITR: itraconazole; CAS: caspofungin; FLZ: fluconazole; VOR: voriconazole; res: response of MIC after 1064 nm Nd:YAG laser treatment; con: control MICs without 1064 nm Nd:YAG laser treatment.

Table 5. The Geometric Mean of MIC, MIC Ranges, MIC50, and MIC90 Values Obtained by Testing the Susceptibility of all 60 isolates Obtained from Onychomycosis Patients to the Antifungal Agents with and without 1064 nm YAG laser therapy

Antifungal	MIC range (µg/mL)	MIC50 (µg/mL)	MIC90 (µg/mL)	GM (µg/mL)	Mean	Variance	SD	95 % CI
TER res(n=60)	0.008-1	0.032	0.125	0.044885	0.090269	0.034822	0.186607	0.02713027, 0.15340773
TER con(n=60)	0.008-1	0.063	0.25	0.059926	0.112423	0.035543	0.18853	0.04863363, 0.17621237
ITR res(n=60)	0.125-16	0.063	1	0.561231	1.072917	6.563694	2.561971	0.03811452, 2.10771948]
ITR con(n=60)	0.125-16	0.5	2	0.707107	1.284722	6.732301	2.594668	0.23671291, 2.33273109
FLZ res(n=60)	0.063-32	2	8	1.694030	3.95252	44.190244	6.647574	1.2085363, 6.6965037
FLZ con(n=60)	0.125-64	2	8	2.17347	5.665	153.9384	12.407191	0.54356, 10.78644
VOR res(n=60)	0.0078-0.5	0.125	1	0.158	0.370833	0.514477	0.717271	0.12814341, 0.61352259
VOR con(n=60)	0.0039-0.25	0.25	1	0.218	0.553	1.743433	1.320391	0.10624, 0.99976
CAS res(n=60)	0.125-4	1	2	0.567156	1	1.201705	1.096223	0.26, 1.74
CAS con(n=60)	0.125-4	2	4	0.777203	1.545455	1.995093	1.412478	0.59653937, 2.49437063
AMP B res(n=60)	1-16(12)	4	16	5.039684	6.75	23.1875	4.81534	3.6905, 9.8095
AMP B con(n=60)	1-16	4	16	5.3394	7.41667	29.743056	5.453719	3.95153926, 10.88179474

MIC: Minimum inhibitory concentration; MIC50: minimal concentration that inhibits 50% of isolates; MIC90: minimal concentration that inhibits 90% of isolates; GM: Geometric mean; SD: standard deviation; CI: confidence interval; TER: terbinafine; ITR: itraconazole; FLZ: fluconazole; VOR: voriconazole; CAS: caspofungin; AMP B: amphotericin; res: response of MIC after 1064 nm Nd:YAG laser treatment; con: control MICs without 1064 nm Nd:YAG laser treatment.

prescribed by clinicians. This finding may be an additional reason for the effectiveness of the laser in enhancing drug penetration through the nail plate by localized increases in temperature, subsequent fungal destruction, and increased effects of laser therapy.^{24,25}

The lowest GM MIC in dermatophytes belonged to TRB (a cost-effective allylamine) and its response group. These findings may be a suitable suggestion for the outstanding clinical trial in onychomycosis caused by dermatophytes as a significant etiologic agent.

ITR, as a triazole, besides TRB was tried in all three etiologic agents of onychomycosis and showed changes in GM by the 1064 ND YAG laser and a significant change in MIC.²⁶ This result may improve various usages of it in future clinical trials as novel laser combined studies.

VOR as the second generation of triazoles, in either topical or systemic form, showed low GM MIC and also reduced GM MIC by the laser (except in the saprophyte group). Therefore, VOR with the 1064 nm Nd: YAG laser can be a novel and suitable reserve antifungal in challenging circumstances like resistance in treatments for onychomycosis.

For the treatment of onychomycosis caused by molds, the use of AMP B as a topically applied agent is an advantageous and relatively cost-efficient option.^{27,28} We showed that the GM of AMP B for the saprophyte response group was lower than the control group. This suggests the effectiveness of topical AMP B with the laser in molds onychomycosis.

The Nd: YAG laser is an available instrument in dermatology centers for medical or aesthetic facilities. The present study helps clinicians combine these findings through routine dermatology treatments for those with onychomycosis, for either cosmetic or medical aims.

In the survey of the topical or systemic antifungal effect of the laser in our trial, it seems that this study can improve the reduction in drug concentration in the topical usage of antifungal if we prescribe the nail bed as a fungi culture medium.

Heat in a laser may be a discomfort situation for the patient. We evaluated that it can be tolerable with changes in temperature. In rare spark conditions, the temperature may increase, which can be controlled by the change in laser energy, sessions, or by using precise cooling devices.

In a meta-analysis on laser therapy as a novel alternative treatment for onychomycosis, the overall rate of mycological cure achieved by the 1064 nm Nd:YAG laser was 63.0%,²⁴ and the results showed that between two kinds of short and long pulse, the long-pulsed 1064 nm Nd: YAG laser showed better efficacy.²⁸ This is due to the fact that melanocytes can absorb the longer wavelength of the 1064 nm laser easily, which will lead to better treatment results.²⁹

In a previous investigation, the clinical efficacy and fungal clearance of the long-pulsed 1064 nm Nd:

YAG laser (with a pulse width of 35 ms, spot size of 3 mm, and energy of 40 J/cm²) on *Trichophyton rubrum* and *T. mentagrophytes*-induced onychomycosis were analyzed. The results showed that irradiation leads to a change in hyphae from smooth to slightly rough and a different degree of cytoplasm damage in *T. rubrum* and *T. mentagrophytes*.³⁰ The results of the present study were similar to this finding because of the similarity of the lasers.

In another study, in only 12.4% of patients, total clinical clearance of onychomycosis with Nd:YAG laser therapy has been observed. The authors indicated that Nd: YAG laser therapy was not cost-effective in comparison to oral TRB therapy. They determined that restricted effectiveness was observed with Nd: YAG laser therapy for onychomycosis and it might be limited to a select group of patients who did not receive reasonable oral antimycotic treatment. It was not similar to our study because we tried the Nd: YAG laser with the usual antifungals. Also, as a cost-effective solution for the usage of Nd: YAG laser, we used it once.

In a retrospective cohort study from the Netherlands, the authors suggested that the only factor which could lead to greater effectiveness in Nd: YAG laser therapy for onychomycosis might be the power (Joules, the number of passes, and pulse duration).

In the mentioned study, the analyses of the subgroups treated with pulse duration of 30 ms and multiple laser passes led to some improvement. But the effectiveness was only slightly improved. The authors proposed to combine both treatment strategies (oral antifungal medications and laser therapy) in the treatment of onychomycosis.²⁶ In the present study, this combination of the Nd: YAG laser and common standard treatments of onychomycosis like TRB and azole (ITR) was postulated and showed *in vitro* MIC changes.

Yang et al in a study used combined therapy of oral TRB and contact-cooled long-pulsed 1064 nm Nd: YAG laser with a spot size diameter of 6 mm, pulse duration of 12 ms, fluences ranging from 50 to 80 J/cm², and no cooling gel. The treatment was evaluated with the monotherapy of TRB and laser alone. The etiologic agent of onychomycosis was different. Their result showed that mycological and clinical clearance rates significantly improved in combined therapy.¹⁸

This finding was in accordance with the results of the current study that there was a significant change in sensitivity to TRB for saprophytes, dermatophytes, and yeasts, but with different sessions and energy of laser treatment.

In a review article conducted by Gupta et al, it was concluded that a topical 1% TRB on big toes affected with non-dermatophyte molds onychomycosis would have a great benefit if used combined with 1064 nm Nd: YAG laser therapy.³¹ The finding was the same as the result

of the present study and can be a confirmation of the combination therapy of topical TRB and laser.

The results of a clinical trial conducted by Hamed Khater and Khattab in Egypt on the treatment of onychomycosis with oral ITR pulse therapy with and without a long-pulsed Nd:YAG laser (the spot size of 4 mm diameter, pulse duration of 35 ms, fluence from 35 to 40 J/cm², and pulse rate of 1 Hz) showed the best clinical cure for the group under treatment with the combination of laser and ITR.³² The mentioned study was similar to the present study in terms of laser adjustment and the use of ITR for combination therapy, but in terms of the sessions of treatment was different from the present study.

Zhong et al. discussed that for the treatment of toenail onychomycosis, a 1064-nm Nd: YAG laser, once a week for eight times, can effectively improve the appearance of the nails, and in serious onychomycosis cases, combined therapy with the Nd:YAG laser and ITR can be more appropriate.³³ It was similar to the results of the present study regarding the significant change in MIC for ITR in all three groups of the etiologic agents of onychomycosis. The difference in sessions and details in laser adjustments were seen.

In a clinical trial conducted in China, patients with onychomycosis were grouped for treatment scheduled by a combined long-pulsed 1064 nm Nd: YAG laser (3-mm spot size, 0.3–2.0-ms pulse width, 5–15 J/cm² fluence, and 1–10-Hz frequency laser for four sessions at weekly intervals) with oral ITR and oral ITR alone. After 8, 16, and 24 weeks of the follow-up protocol, the result of combined ITR and laser therapy was statistically significantly different from oral ITR therapy alone. Also, laser therapy was recommended as a pure medication or combination treatment for mild and moderate onychomycosis.³⁴ It was similar to the results of the present study regarding the significant change in MIC for ITR in all three groups of the etiologic agents of onychomycosis. The difference in sessions and details in laser adjustments were seen.

In a study by Halvae et al in Tehran, Iran, the GM MIC of saprophytic onychomycosis for TRB was 3.48 µg/mL, and in the present study, the GM MIC in the TRB response group was 0.044 and in the TRB control group was 0.059, which was different from the results of the current study.³⁵ In the current study, TRB in the saprophyte group was more effective in laser and non-laser treatment than ITR in the laser and non-laser group, which was different from the result of Halvae and colleagues' study.³⁵ These may be due to the presence of TRB resistance fungal isolates in the study conducted by Halvae et al. TRB may also fail to treat dermatophytosis due to the point mutations in squalene epoxidase genes.³⁵

In a previous study, ITR GM MIC in yeast onychomycosis was 0.218,²⁰ and in the present study, the GM MIC in the ITR response group was 0.56 and in the ITR control group was 0.7, which were similar to

the results of the current study. Their FLU GM MIC in yeast onychomycosis was 1.74, and ours was 1.88 for the FLU response group and 2.17 for the FLU control group, which was different that may be due to the presence of one *C. albicans* FLU resistance isolate in the current study (MIC response=32, MIC control=64).

In the study conducted by do Espírito Santo and Deps on the treatment of onychomycosis a long-pulsed 1064-nm Nd: YAG laser was applied using a 7-mm diameter spot size; fluence of 20 J/cm² for fingernails, 40 J/cm² for the big toenail, and 50 J/cm² for thicker big toenails; pulse duration of 10 ms; and repetition rate of 2 Hz. Treatment consisted of 4 pulses for fingernails and 6 pulses for big toenails, which covered the entire nail plate. As measured by an Infrared Digital Thermometer, the mean temperature recorded was 40 °C.³⁶ It was similar to the mean temperature (32–39 °C) used in the current study.

In a previous study for treating 72 patients (194 nails with onychomycosis) with 1064-nm Nd: YAG, fluence of 35–40 J/cm² was applied (4-mm diameter spot size, pulse duration of 35 ms, and repetition rate of 1 Hz) and the mean temperature reached to 45±5 °C³⁴ and the mean temperature reached 45±5 °C.³⁷ It was similar to our experience.

Limitations

1. It was an *in-vitro* study, and a clinical trial is better to help the clinicians to select the best treatment protocols. We selected the standard laser adjustments in the clinic and detected temperature as being tolerable for humans.
2. Many samples were excluded due to the following reasons: They did not grow enough or they grew too much; both response and control groups did not grow after the laser; they were contaminated with air-born fungi especially *A. flavus* and *A. niger* on dermatophyte and yeast during waiting for enough growth. We will try to repeat with more positive onychomycosis specimens through 169 onychomycosis samples.
3. Sparks in colonies were due to thickness, texture, open door of the plate, and its pigmentation, and in-clinic was due to the thickness or color of the infected nail. The spike was rare in our study and could be decreased by lower adjustment of laser energy (Joules, pulse duration, and the number of passes) alternately.
4. Caspofungin, which showed changes in MICs when used in combination with laser therapy in yeasts and voriconazole in the dermatophytes, is not a standard treatment for onychomycosis. But we tried it as a commonly known antifungal.
5. It is better to try more topical antifungals and new azoles like luliconazole or tavaborole, which have been used more recently.²⁰ We tried itraconazole and

terbinafine as classic and somehow gold standard treatments of onychomycosis, and both of them had topical and systemic drug forms.

6. Fungal growth in the nail differs from culture media regarding the difference in the counts of spores or mycelium in molds or producing biofilm in *Candida* species and other physiological differences. We consider this limitation as a common disability of the *in vitro* antifungal sensitivity test in microbes.
7. Epigenetic changes in fungi after laser irradiation may occur. We are concerned about this phenomenon by doing MIC in a few hours after irradiation.
8. *Cladosporium* sp. and *Rhodotorula* sp. may not be the natural etiology of onychomycosis. However, due to as a result of isolation from both cultures, we tried their MICs as rare etiologic agents of onychomycosis.
9. Temperature detection may be done for more samples by using an adjacent thermometer to the laser instrument. We consider that the in-clinic cooling system of the laser instrument could be the opposite of our adjustment.
10. Laser treatment involves more than one session, but we tried only once for colonies. This can be both a limitation and a strength of this study. The possibility of getting false negative results or achieving to less significant results especially for itraconazole and terbinafine are among the limitations of using laser treatment in one session. Whereas, the use of fewer sessions than routine clinical treatment is its strength.

Strengths

1. Evaluation of the laser effect on the interaction between fungi and antifungals in *in vivo* conditions can show the beneficial effects of the laser in the treatment of onychomycosis for the first time and demonstrate the effect of antifungals on fungal colonies with and without the effect of the laser at the same time considering the fungal growth stage and physiologic condition.
2. We suggest a combination of classic antifungal treatment with laser therapy not only for its aesthetic role but also for reducing the concentration of antifungals.
3. We assessed the MICs in few hours after laser treatment to prevent an epigenetic change in fungi.
4. We used available and typical lasers in dermatology clinics.

Future Directions

1. We suggest an evaluation of the treatment of fungal nail infection caused by azole resistance *T. mentagrophytes* using combination therapy with a 1064 nm Nd: YAG laser or VOR replacement treatment with or without a 1064 nm Nd: YAG laser.
2. We suggest clinical trials on *A. flavus* species isolated

from onychomycosis as a common etiologic agent of toenail onychomycosis with new topical antifungal treatments.

3. We suggest a trial on topical or oral forms of voriconazole on *T. mentagrophytes* and *T. rubrum* as a common dermatophytic agent of onychomycosis with an Nd: YAG laser.
4. We suggest a trial on a group of *C. albicans* and *C. glabrata* fluconazole and caspofungin resistance with an Nd: YAG laser.
5. We suggest a trial with four sessions of an Nd: YAG laser on colonies to better adjust with *in vivo* clinical treatment.
6. We also suggest that different degrees of damage to the lasered fungal colonies should be presented by electron microscopy.

Conclusion

This finding can be a basis for conducting a clinical trial study aimed to change systemic treatment antifungals to topical form through combination therapy with a 1064 nm Nd: YAG laser. Also, oral antifungals combined with an Nd: YAG laser are suggested to reduce their treatment duration and side effects or change the use form to topical antifungal. Moreover, these findings of the present study may cover the following discovery on the combination therapy of a 1064 nm Nd: YAG laser and new topical or systemic antifungals. Furthermore, subsequent studies on azole resistant *T. mentagrophytes*, *A. flavus* species, fluconazole and caspofungin resistant *C. albicans*, and *C. glabrata* species can be conducted. Further clinical investigations could be designed into more 1064 nm Nd: YAG laser sessions on colonies. Besides, different degrees of damage to the lasered fungal colonies can be presented by electron microscopy.

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Competing Interests

The authors have no conflict of interest to declare.

Ethical Approval

This study was approved by the Research Ethics Committee of Tehran University of Medical Sciences (the number of Ethics Committees protocol: IR.TUMS.SPH.REC.1398.197). Ethical principles as well as national standards concerning medical research in Iran have been found to be fulfilled by the project.

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Supplementary Files

Supplementary file 1 contains Tables S1-S2 and Figure S1.

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