



Methylene blue vs. methyl aminolevulinate photodynamic therapy in the treatment of mild-to-moderate toenail onychomycosis: short and medium-term effects.

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5 **effects.**
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41 ethical evaluation of the study and Dr. Santiago Cano for his assistance with the
42 statistical analysis.
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ABSTRACT

Photodynamic therapy (PDT) has emerged as an interesting alternative option for onychomycosis treatment. The impact of a specific photosensitizer (PS) on the final result is an important factor to consider. We conducted a short- and medium-term controlled trial to compare the effectiveness of PDT in the treatment of mild-to-moderate onychomycosis when it is mediated by two different PSs. Twenty patients were randomized to receive 9 sessions of PDT distributed over 16 weeks mediated either by methylene blue (MB/PDT group) or methyl aminolevulinate (MAL/PDT group). Onychomycosis severity index (OSI) and nail involvement were checked along the study. Complete cure, treatment success and clinical improvement were tabulated at 16 and 40-week follow-ups. OSI scores decreased significantly along the study, from 12.1 ± 5.4 to 3.6 ± 3.2 (MB/PDT group) and from 14.8 ± 6.0 to 5.4 ± 4.4 (MAL/PDT group). At 16-week follow-up, only 20% of the patients in the MB/PDT group reached complete cure and none in the group of MAL/PDT. At 40-week follow-up, complete cure rates were 70% and 40% in the MB/PDT group and MAL/PDT group respectively. Both modalities showed good outcomes in treatment of moderate toenail onychomycosis. MB/PDT showed a faster action but with relapse rates slightly higher than MAL/PDT.

Keywords:

Mild-to-moderate onychomycosis; photodynamic therapy; methylene blue; methyl aminolevulinate.

1. INTRODUCCION

Cutaneous fungal infections are one of the most prevalent skin problems worldwide. Many species of fungi can cause skin infections. Onychomycosis is the most prevalent onychopathy that comprises 50% of nail disorders worldwide.¹ In the USA, 90% of the fungi causing onychomycosis are dermatophytes or yeast such as *Trichophyton rubrum* (71%), *Trichophyton interdigitale* (20%) and *Candida albicans* (5.6%).¹ *T. rubrum* is a representative anthropophilic strain that causes human skin infections including tinea pedis and onychomycosis. *T. interdigitale* is another well-known dermatophytic fungus, frequently observed along with *T. rubrum*.² Longitudinal striae, “jagged edge” with spikes (pattern results from distal-to-proximal invasion of dermatophytes) and “ruin appearance” are the three major onychoscopic features associated with onychomycosis.³ Patients with onychomycosis more frequently showed concomitant tinea pedis and show a clear worsening of nail changes in the last years.⁴

The severity and percentage of nail involvement are usually considered the main prognostic factors for the treatment of onychomycosis.⁵ In most countries, if there is less than 50% involvement of diseased nail plate and less than three nails involved, topical therapy is an appropriate consideration.⁶ Topical treatments should also be used for treatment of white superficial onychomycosis. In these cases, among the innovative physical approaches for moderate onychomycoses are nail peeling,⁷ thermal disruption of fungal biofilm by lasers and photochemical damage by photodynamic therapy (PDT).⁸ Lasers can elicit a fungicidal effect by photothermally heating the fungal mycelium through selective photothermolysis.^{9,10} PDT is a noninvasive therapy that requires light activation of a photosensitizer (PS) in the presence of oxygen which

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3 generates reactive oxygen species leading to selective and highly localized destruction
4 of “abnormal” cells by necrosis or apoptosis. Fungi are able to absorb photosensitizers
5 so PDT can be an alternative way of treating onychomycosis. A few clinical trials have
6 been published on PDT, demonstrating that PDT could be a promising alternative to
7 conventional antifungal therapy.^{11,12}
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15 We conducted a short- and medium-term controlled trial to evaluate the effectiveness of
16 PDT in the treatment of mild-to-moderate onychomycosis. In addition, a comparison
17 between methylene blue (MB) and methyl aminolevulinate (MAL) as PSs was carried
18 out in terms of clinical and mycological cure.
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25 **2. PATIENTS AND METHODS**

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28 A controlled trial of 40-week duration was designed. Patients with mild-moderate signs
29 of distal and lateral subungual onychomycosis (DLSO) in the first toe, (infection
30 confirmed by PAS stain and microbiological culture to identify only dermatophytes as
31 causative agents), were randomly allocated into two different treatment groups by
32 means of a computer-generated random list: group 1: MB/ PDT or group 2: MAL/ PDT
33 (Figure 1). Characteristics of the recruited patients or inclusion criteria are shown in
34 Table 1. Inclusion criteria: OSI ~13; ~35% nail involvement. Exclusion criteria were as
35 follows: less than 18 years of age, glucose-6-phosphate dehydrogenase deficiency
36 (which contraindicates the use of MB), porphyria diagnosed (which contraindicates the
37 use of MAL), suffering from any disease that can cause nail abnormalities and can
38 interfere with evaluation (e.g. tinea pedis, psoriasis or lichen planus), internal diseases,
39 use of topical or systemic antifungals for at least 6 months before enrollment in the trial
40 and pregnancy (Figure 1).
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3 A local Ethics Committee approved the study (Hospital Clínico San Carlos in Madrid,
4 Spain; Internal code: 17/501-E) in accordance with the Declaration of Helsinki, the
5 principles of Good Clinical Practice and all applicable regulatory requirements. All
6 subjects provided written informed consent form before their inclusion in the study.
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13 Two weeks before the experimental assessment of treatment started, subjects received
14 instruction of the pretreatment with 40% urea to be performed regularly during research
15 monitoring and 3-7 days before a PDT session. The treatment consisted in nine PDT
16 sessions distributed over 16 weeks. In parallel, clinical photographs, microbiological
17 samples for fungal cultures and nail samples for histology (PAS stain) were taken at
18 different time-points in order to assess the efficacy of each treatment (Figure 1).
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28 **2.1. Interventions**

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31 Plates of the affected nails were softened with 40% urea ointment. Periungual skin was
32 protected with vaseline to avoid any lesion produced by the urea. Upon application of
33 the urea, the nail was covered with occlusive dressing for 12-24 h. In order not to
34 excessively macerate the periungual skin the daily softening treatment with urea was
35 limited to 3 days in toenails with hyperkeratosis less than 2 mm, whereas it was
36 maintained during 7 days in toenails with hyperkeratosis greater than 2 mm.
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45 *Photodynamic therapy*

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48 MB/PDT protocol: In each PDT session, MB (at a concentration of 2% in aqueous
49 solution) was applied to the nail and surrounding area, leaving the solution in contact
50 with the nail for 3 min.
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3 MAL/PDT protocol: In each PDT session, MAL (Metvix®, Galderma, La Defense
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5 Cedex, France) was applied to the nail plate under occlusion and protected from light
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7 for 3h.
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11 In both groups, after incubation period, irradiation was carried out with a 635nm LEDs
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13 lamp (Aktelite® CL128, Photocure ASA, Oslo, Norway) using a fluence of 37J/cm².
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15 The lamp was placed at 10 cm from the skin and the illumination time was calculated
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17 automatically (10 min.). The same protocol was repeated nine times at two-week
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19 intervals. Side-effects were recorded during each session of PDT.
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23 **2.2. Outcomes**

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26 Clinical clearance was assessed by a dermatologist by scoring the photographs taken
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28 over the study by using the onychomycosis severity index (OSI) (mild ≤ 5 , moderate: 6-
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30 15 and severe: 16-35) and percentage of nail involvement. Complete clinical clearance
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32 was defined by a completely normal appearance of the nail or the presence of $\leq 5\%$ nail
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34 plate involvement in onychomycosis. Mycological clearance was assessed after the
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36 results obtained after fungal culture and by biopsy of the nail affected by PAS stain
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38 (Figure 2). The biopsy was carried out by cutting a fragment of the distal portion of the
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40 nail plate at least 3 mm wide together with the attached subungueal keratosis. The
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42 extracted samples were then stained with periodic acid Schiff (PAS) and their
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44 subsequent histological study allowed detecting the presence of fungi in the lower
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46 portion of the nail plate (Figure 2). Complete cure was defined as the addition of clinical
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48 and mycological cure.
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55 The difference between baseline OSI score and those obtained at 16 and 40-week
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57 follow-up was transformed in percentage of change. Values obtained were classified as
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3 follows: i) 0-25% (no improvement), ii) 26-50% (mild improvement), iii) 51-75%
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5 (moderate improvement) and iv) 76-100% (outstanding improvement).¹³
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8 As reported by Shemer et al.,¹⁴ treatment success and clinical improvement can also be
9 tabulated to achieve a more complete analysis of the results. In this way, treatment
10 success was defined as mycological cure + less than 10% of nail affected. Clinical
11 improvement was defined as mycological cure + less than 25% of nail affected.
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18 In order to assess the degree of satisfaction of the patient with the applied treatment, in
19 each PDT session, the patient was asked whether they noticed pain or discomfort in the
20 session itself or during the time elapsed between sessions.
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26 **2.3. Sample size**

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28 As a change of 10 points or more in OSI scores is considered significant,¹⁵ by using
29 IBM SPSS Sample Power 3.0 software it was estimated that a sample size of 9 patients
30 per group was required to detect a 10-point change in OSI scores with 90% power,
31 $\alpha=0.05$ and standard deviation of 6. A total sample of 24 patients with only one toenail
32 affected by DLSO (12 per group) were randomly allocated anticipating in this way
33 possible dropouts (Figure 1).
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44 **2.4. Statistical analysis**

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46 Statistical analysis was performed with a SPSS software package for Windows (version
47 25.0; SPSS, Chicago, III). Results were expressed as arithmetic mean and standard
48 deviation. The Shappiro-Wilk test was used to verify the normality of distribution of
49 quantitative variables, and then they were analyzed by bifactorial repeated measures
50 ANOVA test (considering an intra-subject factor (time) and an inter-subject factor
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(treatment)) with the post hoc Bonferroni correction for identification of intra-group differences. Significance of the obtained results (p value) was judged at the 5% level.

3. RESULTS

After the application of restrictive inclusion and exclusion criteria and taking into account dropouts and exclusion of patients with incompletely recorded histories, the data of final 20 subjects (10 patients Group 1: MB/PDT; 10 patients Group 2: MAL/PDT) were analyzed (Figure 1). The groups were divided obtaining similar distributions of OSI and percentage of disease involvement (Table 1). Baseline clinical and laboratory data of included patients are summarized in Table 1.

Complications or side effects were not observed during or after application of either PDT modality. In the case of MAL/PDT, some patients referred pain or itching sensation, but only during irradiation time. MB application resulted in temporary discoloration of the nail. In any case, the level of patient satisfaction was high.

Figure 3 and Table 2 show the evolution of the clinical clearance during and after treatment. A progressive reduction in the OSI values was observed after treatment. Intragroup significant differences were observed in both treatment modalities at 16- and 40-week follow-up from baseline. Intergroup significant differences attending OSI values were not observed. Regarding the degree of improvement, significant differences between 16-week follow-up and 40-week follow-up were detected with MAL/PDT, while after MB/PDT the degree of improvement at 16- and 40-week follow-up was similar. Significant differences between groups at 16-week follow-up were observed, indicating that the healing process is significantly faster with MB/PDT. Finally, the degree of disease involvement follows a trend similar to OSI scores, significant

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3 intragroup differences at 16- and 40-week follow-ups from baseline and between 16-
4 and 40-week follow-up in both treatment modalities and no point of significant
5 differences between them.
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10 Evolution of the mycological clearance is also shown in Table 2. Complete mycological
11 cure rates were 70% and 60% at 40-week follow-up in MB/PDT and MAL/PDT groups
12 respectively. The fraction of patients who reached mycological cure at 40-week follow-
13 up was significant in both groups respect to baseline and 16-week follow-up. The
14 evolution in mycological cure showed no significant intergroup difference. All
15 MB/PDT patients that reached mycological cure reached complete cure as well (Table
16 3). Among MAL/PDT patients that reached mycological cure, 67% reached complete
17 cure, 17% reached treatment success and 17% reached clinical improvement (Table 3).
18 It is important to point out that during the time interval studied two MB/PDT patients
19 suffered relapses despite the faster healing rate.
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34 **4. DISCUSSION**

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36 Onychomycosis is a chronic, hard-to-treat infection of the nails that can have a
37 significant impact on patients' quality of life. Therapeutic guidelines for treatment do
38 exist,¹⁶ but they are not yet up to date on the latest scientific knowledge.⁵ Novel
39 therapies based on light have been explored as a possible and good alternative treatment
40 for onychomycosis. Selective destruction of fungal structures seems to be the
41 mechanism of action of these novel therapies.¹⁷ PDT has been shown to be effective on
42 photoinactivation of multiple species of fungi including the most common cause of
43 onychomycosis, *T. rubrum*.¹⁸ Moreover, PDT does not induce systemic side effects and
44 has the advantage of a low probability of selection of resistant microorganism, since the
45 resistance to ROS is virtually impossible.¹⁹
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3 Recently a review study concludes that topical therapy for toenail onychomycosis
4 produces clinical and mycological cure but only in patients with mild to moderate
5 toenail onychomycosis (< 50% nail involvement).²⁰ We propose PDT treatment as an
6 alternative to topical treatment in these cases with mild-moderate disease involvement.
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10 For this reason, good candidates for receiving these PDT modalities are patients with
11 superficial onychomycosis or DLSO that involves less than 50% of the nail plate
12 surface area (without matrix involvement and a nail plate thickness less than 2 mm).
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14 This study evaluated the efficacy and safety of MB/PDT vs. MAL/PDT. At 40 week
15 follow-up, complete cure rates were 70% and 40% in the MB/PDT group and
16 MAL/PDT group respectively. These complete cure rates are higher than those obtained
17 with topical agents on toenail DLSO with 20-50% clinical involvement. Compare for
18 example the 17.8% and 15.2% of complete cure rates reported at week 52 with
19 efinaconazole 10%.²¹ In addition, the results here showed present higher cure rates than
20 the 28.6% of complete cure obtained at week 60 after 48 weeks of treatment with once-
21 daily 5% tavaborole solution,²² and the 5.8% of the complete cure at week 60 after
22 treatment with standard 8% ciclopirox nail lacquer⁵ or the 20% of negative mycological
23 culture at 9 months following 5% amorolfine nail lacquer.²³
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43 Urea pretreatment could boost the difference as for the degree of improvement between
44 MB/PDT and MAL/PDT at 16-week follow-up, thus the similar degree of improvement
45 at 16 and 40-week follow-ups for MB/PDT, as urea exerts an emollient/keratolytic
46 action and therefore it allows a better MB/MAL and LED light penetration across the
47 nail plate.¹⁵ The hydrophilic MB diffuses better than MAL across the nail previously
48 treated with urea and thus its action is faster than that of MAL. Recently, there are more
49 and more studies which propose a previous pretreatment of the affected nail with
50 fractionated CO₂ or Er:YAG lasers, enhancing the absorption of PS without the needed
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3 of the use of urea 40% and increasing the aesthetic outcome. This combination has as a
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5 counterpart a greater sensation of pain.^{24, 25}
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8 The common protocol for onychomycosis treatment carried out in the studies published
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10 using MAL as PS consisted of 3 PDT sessions, separated by an interval of 1 or 2 weeks,
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12 using MAL 16%, 3 h as incubation time and red light ($\lambda=630\text{nm}$, 37 J/cm^2).^{15,26} In
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14 contrast, studies using MB consisted of 12 sessions with an interval of 15 days between
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16 them, 3 min as incubation time, and red light ($\lambda=630\text{nm}$, $36\text{-}18 \text{ J/cm}^2$). using MB
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18 (2%).^{27,28}
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23 In order to unify protocols and for an easier PS comparison, in the present study with
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25 patients affected by mild-moderate onychomycosis, nine sessions of PDT were applied,
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27 separated by an interval of 2 weeks, using the same light system operating at $\lambda=630\text{nm}$,
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29 37 J/cm^2 . The final aim was to both ensure total healing and avoid the manifestation of
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31 the recurrence. Success results were obtained after the application of a similar protocol
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33 (8 PDT/light sessions instead of 9) into a clinical trial with 40 patients randomly
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35 assigned to MB/PDT or IPL in the treatment of mild-moderate toenail onychomycosis,
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37 showing clinical improvement rates of 70% and 80%, respectively at 28 weeks from
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39 baseline.¹¹
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44 The fact that onychomycosis is usually not an immediate threat to their health may lead
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46 patients to delay treatment due to the high cost of antifungal medications and their
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48 potentially harmful side effects. Patients that have had onychomycosis for shorter
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50 periods have generally better cure rates than those who have had the disease for longer
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52 periods, hence, the most prudent decision is to initiate a topical therapy as early as
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54 possible.²⁹ Some of our patients did not experience complete cure at this medium-term
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56 period of evaluation of 40 weeks (30% in the MB/PDT group and 60% in the
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3 MAL/PDT group). Among the factors that could predispose to not obtaining complete
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5 cure are slow nail growth, nail abnormalities and poor circulation, all of which may
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7 increase with age.³⁰ Subungueal dermatophytoma that is more common in older people
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9 due to a longer duration of onychomycosis is another predictor of poorer response to
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11 therapy.³¹ In the present study the same sequencing of PDT sessions was applied for the
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13 two modalities of PS in order to achieve a better comparison between the procedures.
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15 Moreover, most of the subjects with similar age were diagnosed with onychomycosis
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17 caused only by dermatophytes, which is easier to eradicate by systemic and topical
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19 drugs.³² In the present study, although both modalities reach similar mycological cure
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21 rates at 40-week follow-up, the clinical cure rates were slower after MAL/PDT than
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23 after MB/PDT. After randomization process, dropouts (exclusion of patients for not
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25 attending sessions/follow-up), some discrepancy regarding the chronicity of the disease
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27 was evidenced between both groups. The delay in healing of patients treated with
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29 MAL/PDT compared to those treated with MB/PDT may be due to the longer duration
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31 of the disease in the former group.
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39 Further studies are needed to define optimal PDT protocol (light source (laser/LED),
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41 fluence/radiance, light exposure time, number and frequency of treatments required,
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43 optimal PS -optimal concentration and incubation time-, combination with topical or
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45 systemic antifungal agents...), to adjust the therapeutic outcome in accordance with the
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47 onychomycosis involvement degree.
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50 51 **5. CONCLUSIONS**

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53 Results of the present clinical trial suggest that PDT mediated by MB or MAL is a safe
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55 and effective method with satisfactory results in the treatment of mild-to-moderate
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57 toenail onychomycosis. In order to optimize the protocol, further evaluation in larger
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59 patient population would be required and in a long-term interval.
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Declaration of interest

The authors have no other relevant affiliation or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Authors contributions

Both authors had the same contribution in conducting the manuscript. Both authors read and approved the manuscript in its submitted form.

For Review Only

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For Review Only

Table 1.- Baseline demographics of recruited patients. (n = number of patients; (%) = percentage of patients)

	MB/PDT	MAL/PDT
Sex		
Male [n (%)]	4 (40%)	9 (90%)
Female [n (%)]	6 (60%)	1 (10%)
Age [mean±(SD)]; range; median	63.6±6.7; [53,71]; 63	64.7±11.8; [43,78]; 66
Hystological analysis [n (%)]		
PAS stain +	10 (100%)	10 (100%)
PAS stain -	0 (0%)	0 (0%)
Microbiological analysis [n (%)]		
T rubrum	9 (90%)	8 (80%)
T interdigitale	1 (10%)	2 (20%)
Baseline OSI [mean±(SD)]	12.1±5.4	14.8±6.0
Chronicity (years) [mean±(SD)]	1.8±0.9	3.2±2.8
Nail involvement (%) [mean±(SD)]	37.5±20.4	33.0±18.4

Table 2.- Evolution of OSI, percentage of disease involvement and mycological response during the study. (n = number of patients; (%) = percentage of patients)

	MB/PDT	MAL/PDT
Baseline OSI [mean±(SD)]	12.1±5.4	14.8±6.0
16-w follow-up OSI [mean±(SD)]	2.6±1.9 ^a	9.2±5.1 ^a
40-w follow-up OSI [mean±(SD)]	3.6±3.2 ^a	5.4±4.4 ^a
Degree of improvement at 16-w follow-up [n (%)]		
No (0-25%)	0 (0)	6 (60)
Mild (26-50%)	1 (10)	1 (10)
Moderate (51-75%)	5 (50)	3 (30)
Outstanding (76-100%)	4 (40)	0 (0)
Mean percentage of improvement degree	77.4%	36.1%
<i>Significance between groups</i>	<i>p</i> <0.05 (0.001)	
Degree of improvement at 40-w follow-up [n (%)]		
No (0-25%)	2 (20)	1 (10)
Mild (26-50%)	0 (0)	3 (30)
Moderate (51-75%)	2 (20)	2 (20)
Outstanding (76-100%)	6 (60)	4 (40)
Mean percentage of improvement degree	76.2%	66.1% ^b
<i>Significance between groups</i>	<i>p</i> >0.05	
Baseline nail involvement (%) [mean±(SD)]	37.5±20.4	33.0±18.4
16-w follow-up nail involvement (%) [mean±(SD)]	11.0±7.7 ^a	19.0±9.7 ^a
40-w follow-up nail involvement (%) [mean±(SD)]	4.0±3.6 ^{a,b}	14.5±12.8 ^{a,b}
Histological analysis at baseline [n (%)]		
PAS stain (+)	10 (100)	10 (100)
PAS stain (-)	0 (0)	0 (0)
<i>Significance between groups</i>	<i>p</i> >0.05	
Histological analysis at 16-w follow-up [n (%)]		
PAS stain (+) culture (+)	8 (80)	10 (100)
PAS stain (-) culture (-)	2 (20)	0 (0)
<i>Significance between groups</i>	<i>p</i> >0.05	
Histological analysis at 40-w follow-up [n (%)]		
PAS stain (+) culture (+)	3 (30) ^{a,b}	4 (40) ^{a,b}
PAS stain (-) culture (-)	7 (70) ^{a,b}	6 (60) ^{a,b}
<i>Significance between groups</i>	<i>p</i> >0.05	

(a) Intragroup significance between the baseline and follow-up values

(b) Intragroup significance between the 16-week follow-up and 40-week follow-up values

Table 3.- Cure rates at 16 and 40-week follow-ups. ((n = number of patients; (%) = percentage of patients)

	MB/PDT		MAL/PDT	
	16 weeks n (%)	40 weeks n (%)	16 weeks n (%)	40 weeks n (%)
Mycological cure	2 (20)	7 (70)	0 (0)	6 (60)
Clinical cure	2 (20)	7 (70)	0 (0)	4 (40)
Complete cure	2 (20)	7 (70)	0 (0)	4 (40)
Treatment success	0 (0)	0 (0)	0 (0)	1 (10)
Clinical improvement	0 (0)	0 (0)	0 (0)	1 (10)

Figure captions

Figure 1.- Participant flow diagram for clinical trial.

Figure 2.- Histological analysis of a nail of one patient through PAS stain: (A) before the study, where the presence of fungal elements can be seen; (B) after the study, without presence of fungal elements.

Figure 3.- Clinical evolution of two toenail treated for onychomycosis with MB/PDT (A,B) and MAL/PDT (C,D), respectively: (A) before treatment (30% of nail involvement), (B) at 24 week after MB/PDT (40-week follow-up) (2% of nail involvement) (C) before treatment (40% of nail involvement) and (D) at 24 week after MAL/PDT (40-week follow-up) (5% of nail involvement).

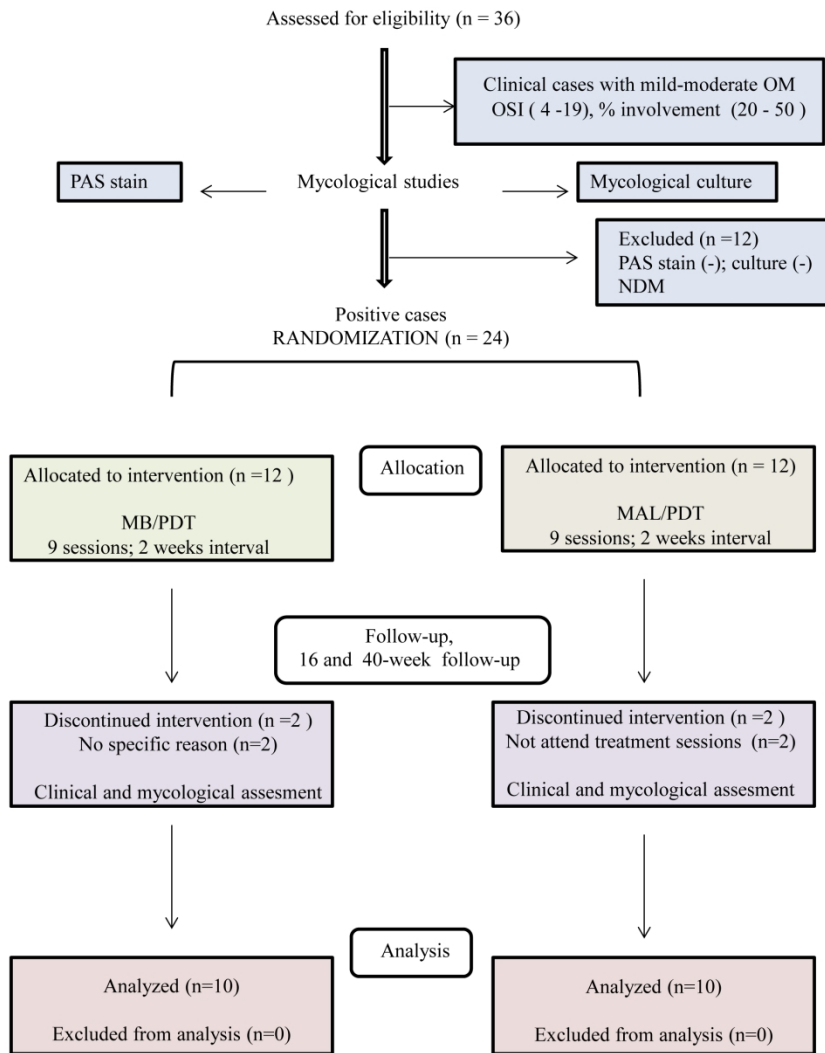


Figure 1

Figure 1.- Participant flow diagram for clinical trial.

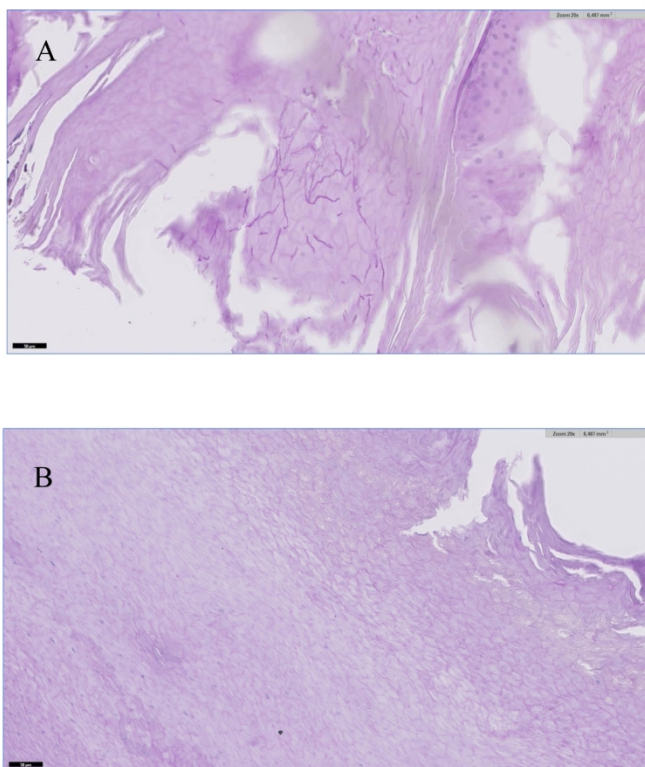


Figure 2

Figure 2.- Histological analysis of a nail of one patient through PAS stain: (A) before the study, where the presence of fungal elements can be seen; (B) after the study, without presence of fungal elements.

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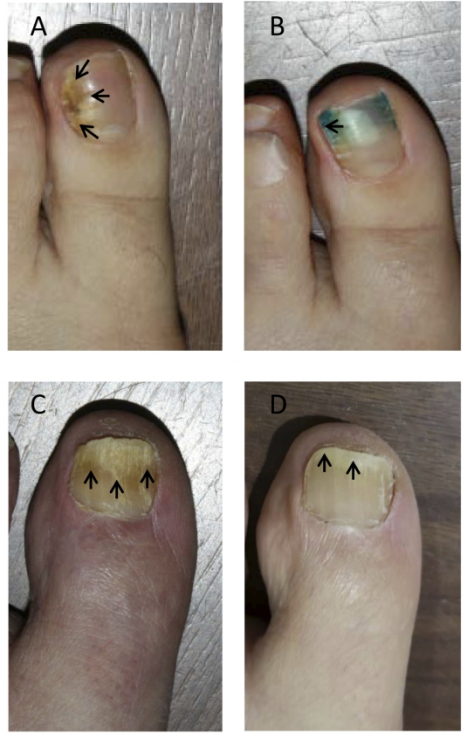


Figure 2

Figure 3.- Clinical evolution of two toenail treated for onychomycosis with MB/PDT (A,B) and MAL/PDT (C,D), respectively: (A) before treatment (30% of nail involvement), (B) at 24 week after MB/PDT (40-week follow-up) (2% of nail involvement) (C) before treatment (40% of nail involvement) and (D) at 24 week after MAL/PDT (40-week follow-up) (5% of nail involvement).