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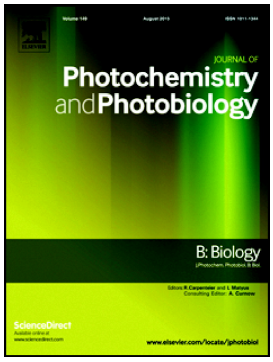

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Photodynamic Therapy treatment of onychomycosis with Aluminium-Phthalocyanine Chloride nanoemulsions: a proof of concept clinical trial

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Abstract

The conventional treatment of onychomycosis, a common fungal infection, consists in the use of local and systemic drugs for 4-6 months. This long protocol is often ineffective due to patient compliance, and usually promotes important collateral effects such as liver and kidney failure. As the alternative, Photodynamic Therapy (PDT) has been used as a noninvasive alternative local treatment for onychomycosis due to the reduction of systemic side effects, fact indicates their use for patients undergoing other systemic treatments. In the present article, we evaluated the effectiveness, as well as the safety of PDT mediated by Aluminium-Phthalocyanine Chloride, entrapped in nanoemulsions, as a drug carrier, to treat onychomycosis in a Proof of Concept clinical trial. To the date, this is the first published clinical trial that uses PDT mediated by nanomedicines to treat onychomycosis. As main results, we can highlight the safety of the clinical protocol and the antifungal effectiveness similar to the conventional treatments. We observed the (1) clinical cure of 60% of treated lesions; (2) the absence of local and systemic adverse effects; (3) from these clinically healed lesions, 40% were negative for fungal infection in laboratorial exams; and (4) nails that presented negative fungal culture were kept without fungal infection for at least four weeks. The innovation of this approach is the absence of collateral effects, due to the local therapeutically treatment, and the possibility to repeat the treatment without inducing fungal resistance, a fact that indicates this approach as a possible alternative protocol for onychomycosis management.

Keywords: photodynamic therapy; nanoemulsion; onychomycosis.

1. Introduction

Onychomycosis is a fungal nail infection usually caused by dermatophytes. It is considered one of the most common mycosis diagnosed worldwide, with a prevalence near to 10-15%, resulting in important aesthetic and functional problems [1]. Besides that, secondary bacterial infections are facilitated, condition that is potentially dangerous in immunocompromised and elderly patients. The standard treatment for onychomycosis consist in a combination of local and systemic treatments, usually with the administration of oral antifungal for at least 4 to 6 months. Important to highlight that protocols with local antimycotic drugs alone are ineffective for onychomycosis control, thus, combination of these two therapeutic procedures are fundamental for treatment success [2, 3].

The two most important drawbacks of these therapeutic protocols are patient adherence, which is reduced, since treatment is too long; and the possible toxic side effects of systemic antifungal administration for long periods, what can lead to the treatment interruption, especially in elderly patients or under others systemic treatments[4]. Considering that, innovative protocols are under investigation to overcome these inconveniences.

Photodynamic Therapy (PDT) emerge as alternative noninvasive local treatment for onychomycosis. PDT involves the photoactivation of photosensitizer drugs (PS) with light in specific wavelengths. This activation increases PS energetic level and this energy can be transfer to molecular oxygen producing a series of reactive oxygen species (ROS), especially singlet oxygen, that are toxic for fungal cells. As main advantages, we can highlight PDT as a rapid harmless local treatment. Moreover, PDT do not induce systemic side effects and can be repeated several times without fungal resistance [5].

Some research groups have already published clinical trials with PDT application against onychomycosis, most of them using first and second generation PS such as 5-aminolevulinic acid and methylene blue respectively [6-8]. In the present report, we use the

Aluminium-Phthalocyanine Chloride (AlClPc), entrapped in oil in water nanoemulsions (NE) as PS, in a *Proof of Concept* clinical trial. This association (AlClPc-NE), PS and NE, is defined as a third-generation PS [9] and has some advantages over the first generations PS such as: photoactivation with longer wavelengths that allow the treatment of deeper nail layers; can be easily dispersed in the nail and skin structures; and do not stain nail structures as methylene blue.

In the present article, we aimed to evaluate the effectiveness, as well as the safety of PDT mediated by AlClPc-NE in a *Proof of Concept* clinical trial. To the date, this is the first published clinical trial that use PDT mediated by nanomedicines to treat patient onychomycosis. In this study, twenty patients diagnosed with onychomycosis were included in the clinical trial protocol. As main results, we can highlight (1) clinical cure of 60% of treated lesions; (2) the absence of important local and systemic adverse effects; (3) from these clinically healed lesions, 40% were negative for fungal infection in laboratorial exams; and (4) nails that presented negative fungal culture were kept without fungal infection for at least four weeks.

2. Methodology

2. 1. Clinical Trial

The clinical protocol included twenty volunteers patients (sample of convenience) diagnosed, by laboratory procedures, with onychomycosis. All the demographic data, as well as the anatomical site of infection are described in Table 1. The Human Ethics Committee from the Medicine Faculty of Brasília approved all the clinical and laboratory procedures described in the present article (Project number 32301214.9.0000.5558 /2014). Investigator number 1, which is a dermatologist with fifteen years of experience, was responsible for patient's selection according to the previous established inclusion and exclusion criteria. Inclusion criteria were: patients more than 18 years of age, and laboratory diagnosis of onychomycosis. Exclusion criteria were: pregnancy; patient with diabetes, hypertension, cardiac disease; use of systemic antifungal therapy. Investigator number 2 that is also a fifteen-year experience dermatologist was responsible for all the clinical treatment procedures.

2. 2. Clinical Procedures

During the first visit, patients read and sign the consent term that explains all the clinical procedure, risks and benefits involved in the study. Then we collected peripheral blood sample from patients for hematological and biochemical exams; and nail samples to confirm onychomycosis clinical diagnosis. Fungal infection was confirmed by direct microscopic examination of subungueal nail samples stained with potassium hydroxide (20%) and by fungal culture in Saboraud glucose agar. Patients positive for any one of the microbiological exams were included in the study. All the laboratorial procedures were conducted in good laboratory practices by an external clinical laboratory (www.sabinonline.com.br).

After inclusion in the clinical protocol, patients were oriented to use a 40% urea topical solution during one week previous to the first PDT session. This step aims to increase the permeation of PS formulation. For PDT, a PS formulation composed by an oil in water

nanoemulsion (NE), with a gelified consistency, containing 65 µM of AlClPc was applied on the dorsal face of infected nail. The NE-AlClPc was kept protected from light during fifteen minutes to allow PS permeation. After that, the NE was removed, and the nail was exposed to red LED (660 nm) irradiation for ten minutes with a power density of 51.5 mW/cm^2 and total energy of 30.9 J/cm^2. After treatment, patients were asked about any kind of local painful, and discomfort using a visual analog scale (VAS) of pain (0-10) to access any possible adverse symptoms of the applied PDT.

2. 3. Photodynamic Therapy Procedures

2. 3. 1. Nanoemulgel containing AlClPc preparation and characterization

The nanoemulgel containing AlClPc (65 µM) was adapted form a self-emulsification protocol previously published by us [10]. The difference was the amount of water used in the protocol to produce a gelified version of the nanoemulsion. For nanoemulgel preparation, 9 grams of Cremophor ELP® (Sigma-Aldrich) and 3 grams of castor oil (Sigma-Aldrich) were weighted, this mixture was kept in mild magnetic stirring (300 RPM for 15 minute). After this initial homogenization process, 5 mL of a AlClPc solution (Ethanoic 260 µM) was added to the system and kept in agitation for 45 minutes until all the ethanol evaporation. After this process, 20 mL of PBS (pH 6.8) were added to the system and kept under agitation for 25 minutes for nanoemulgel formation. The final nanoemulgel concentration was 65 µM of AlClPc.

The nanoscopic and photophysical proprieties of the nanoemulgel containing AlClPc (NE) used in this clinical trial were study to evaluate the nanodroplets size dispersion and the fluorescent emission of the samples. Nanodroplets hydrodynamic diameter (nm) was evaluated by measuring the Dynamic Light Scattering using Zeta Sizer (ZS90, Malvern®, USA). NE fluorescence emission (Excitation 350 nm; Emission 680 nm) was measured using a

spectrofluorometer (SpectraMax). These same characterization protocol was also used in previous publication to evaluate the similar lipid nanoemulsion containing AlClPc [10].

2. 3. 2. LED Device Prototype

The LED device used in the present clinical trial is a prototype developed at University of Brasília. We used a LED model GP-100Wr6-G42M-Z3GL, 100W electric power, 660nm wavelength and direct operating voltage of 20-24V from Green Powertech Solutions Limited. The power was controlled using pulse width modulation, PWM, applied to a 480Hz square wave signal emitted by an ATMEGA328P microcontroller embedded in the Arduino Open Source Hardware (OSH) platform. The duty cycle ranges from 31% up to 71% duty. The irradiance calibration was performed, as recommended [11], using a FIELD MAX II Energy and Power meter, item # 1098580, serial # 0099L11R from Coherent getting individual data from each point in the duty cycle range. The temperature was controlled via a commercial watercooler Corsair H95 actuating as a heat sink attached to the high-power LED. The LED emission was recorded with a room spectrophotometer Ocean Optics (USB 2000+). The measurement interval was less than 1 nm of resolution (Figure 1).

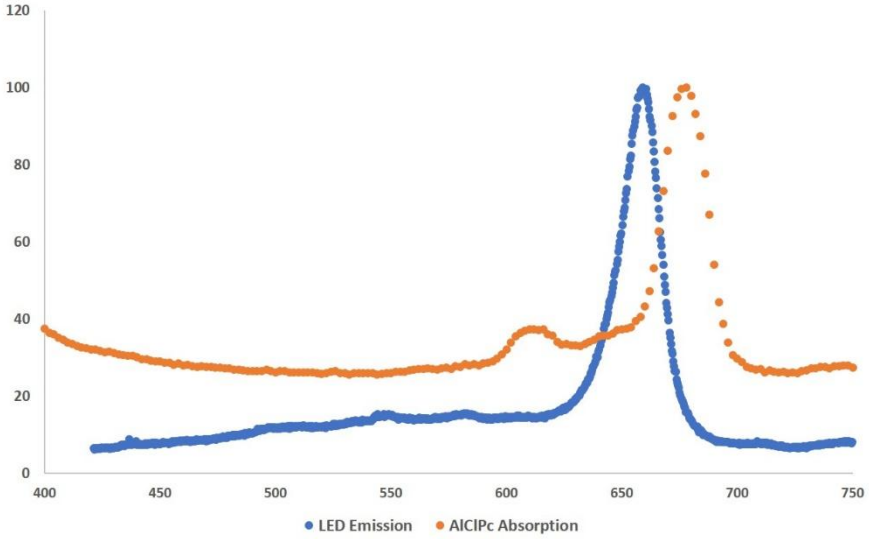

Figure 01: LED prototype spectrum emission (blue dots) and the AlClPc absorption spectrum (red dots).

2. 3. 3. Photodynamic therapy protocol, mycological and clinical evaluation

PDT sessions were repeated every fifteen days until the complete fungal infection remission, diagnosed by clinical observation. Clinical investigators were responsible for evaluate individual treatment clinical responsiveness and define the number of PDT sessions for each patient. The clinical diagnosis of onychomycosis was defined after observing some altered signals detected by the investigator 1. For the present study, the presence of subungual hyperkeratosis and onycholysis, presence of color changes, such as dark, green or yellowish colored nails, or a combination of these signals were considered as a nail with onychomycosis. The absence of these signs was also considered to define the clinical lesion cure. These diagnosis, as well as clinical cure, parameters were based in a previous literature report [1].

After this preliminary clinical diagnosis, samples from the treated nails were collected to evaluate the presence or absence of fungal infections under microbiological laboratory culture exams. These exams were repeated after at least one month after the last PDT procedure to confirm the treatment protocol efficacy. Some of the patients were examined for more time and the exams were repeated in longer intervals.

3. Results and Discussion

Conventional onychomycosis treatment is long, usually 4-6 months, and often-ineffective due to patient compliance. Data from the literature indicates that systemic drugs such as itraconazole and terbinafine can promote complete healing of 25-35% of severe cases of onychomycosis [12]. Thus, alternative of more effective and faster treatments, such as PDT, are under development to overcome these drawbacks and provide a better alternative therapeutic protocol for this common infection.

In this study, we present results showing the safety and effectiveness of PDT mediated by AlClPc-NE to treat onychomycosis in a clinical trial, involving twenty volunteer patients. As presented in Table 1, sixteen (80%) of the selected patient (n=20) finished the protocol and the other four (20%) did not. Patients who drop out of the study showed no justification, nor provided information about their decision. Review articles state that less than fifty percent of patients undergoing onychomycosis oral and local therapy complete the therapeutic protocol [4, 13]. Important to note that PDT treatment duration, compared to conventional treatment (16-24 weeks), is shorter, fact that may increase patient compliance and adherence to the therapy.

The number of PDT sessions is a key point for onychomycosis treatment success. It is a consensus that is impossible to treat onychomycosis with just a single PDT application, thus, authors are investigating which is the best clinical protocol to optimize the therapy. The number of PDT sessions in the present clinical trial (4.45 ± 1.76) was defined considering clinical cure by clinical investigators (1 and 2) and respected the individual clinical responsiveness to the treatment.

Revising the PDT clinical trials in the literature [8, 14-16], the number of sessions vary from 3-12. The limitation of PDT sessions includes patient compliance [4] and the occurrence of adverse local signs and symptom, especially pain [6], which impairs PDT application. In the

present study, no important local adverse signs, nor nail temperature changes (unpublished data) during PDT application, were observed during the clinical visits. For the local symptom, we measured local pain discomfort using a visual analogue score (VAS; 0-10 score) to measure pain. This data (2.76±1.87) varies among patients and is in accordance with individual painful sensitivity. Most of the PDT applications (n=50/69; 72%) had score of pain less than 3, data considered acceptable. Figure 2A presents the visual pain score of each PDT session applied in this clinical trial. In this chart we can note that most of the PDT sessions presented scores at the bottom part of the graph. As comparison, some reports in literature attest that PDT protocol needs to be interrupted due to the presence of pain after PDT sessions [6].

Table 1: Baseline patient demography; clinical trial characteristics; and patient outcome.	
Patients *n*=20	
Gender *n* (%)	
Male	8 (40%)
Female	12 (60%)
Age (mean ± SD)	
53.3 ± 14.7 years	
Number of PDT sessions (n=total; mean ± SD)	
Total sessions, n=69	4.45 ± 1.76
Patient Outcome *n* (%)	
Lack of follow up	4 (20%)
Clinical diagnosis of Cure	12 (60%)
Mycological examination negative	8 (40%)
Lack of efficacy	8 (40%)

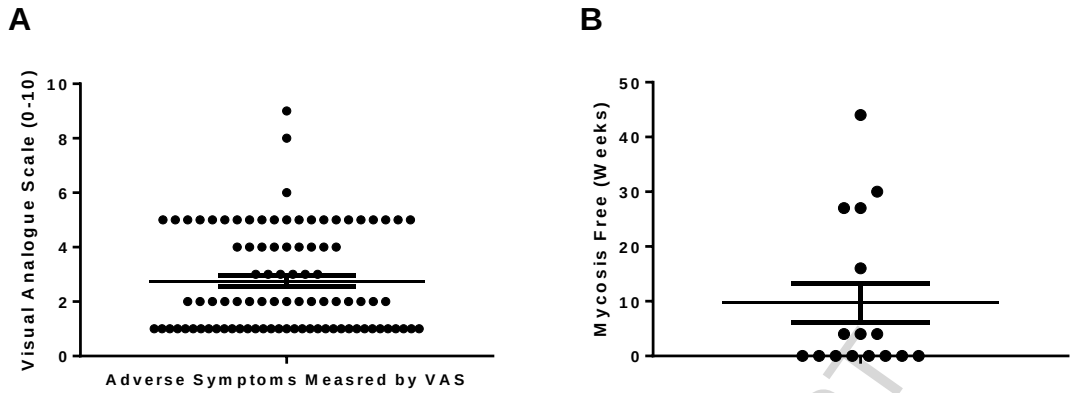

Figure 02: In section A, the Visual Analogue Scale (0-10) adverse symptom measured in each individual PDT session. In section B the number of mycosis free patient measured with laboratory procedures. Each dot presented in the graphs represents the data collected from an individual patient.

Reactive oxygen species produced during PDT are the main responsible for pain induction [17]. The amount of ROS produced depends upon different factors such as accumulation in the ungueal bed, PS concentration, and nail exposition to PS. As stated before, in comparison with other reports in the literature [6, 18], our VAS measurements were significantly lower. One possible explanation for that is the time of PS exposition during PDT. For the conventional first and second generation PS, such as ALA and methylene blue, exposition time ranges from 30 to 180 minutes. For ALA the time is usually longer because the molecule needs to be enzymatically converted to a protoporphyrin to be active for PDT [15]. NE-AlClPc, a third-generation PS, was exposed to infected nails for 15 minutes. Reports describe that drugs incorporated in nano-carriers, such as nanoemulsion, permeate nail structures faster in comparison with free drug, fact that reduce significantly the PDT session duration [19]. As an aqueous dispersion, we hypothesize that lipid droplets carrying AlClPc present in NE-AlClPc solution can permeate nail structures by passing through aqueous tunnels created after nail pretreatment with urea solution.

For the clinical trial, we developed a nanoemulgel consisting of an oil in water nanoemulsion, containing AlClPc. As a topical formulation, during study concept design, we plan to produce a gelified consistency product (Figure 3A) with the objective to increase the contact time between PS formulation and nail surface. Important to highlight that even in that consistence, after aqueous dilution the lipid droplet size carrying AlClPc was kept in the nano-size range, with a hydrodynamic diameter of 30.49 nm and a polydispersity index (PDI) of 0.166 (Figure 3B). The maintenance of these nanoscopic properties reinforce our hypothesis of NE nail permeation presented previously, where the lipid nanodroplets carrying the AlClPc permeated the nail structure via the aqueous pores created by the pretreatment with urea.

In addition to the nanoscopic characterization, we also studied the maintenance of the NE photophysical proprieties. In Figure 3C is possible to observe the fluorescence emission pattern of AlClPc in different solutions. As expected, as a strongly hydrophobic compound the AlClPC aqueous solution has the lowest fluorescent emission, fact that indicates the PS aggregation and the formation of dimers and trimer forms of the compound, that are ineffective for PDT applications [20].

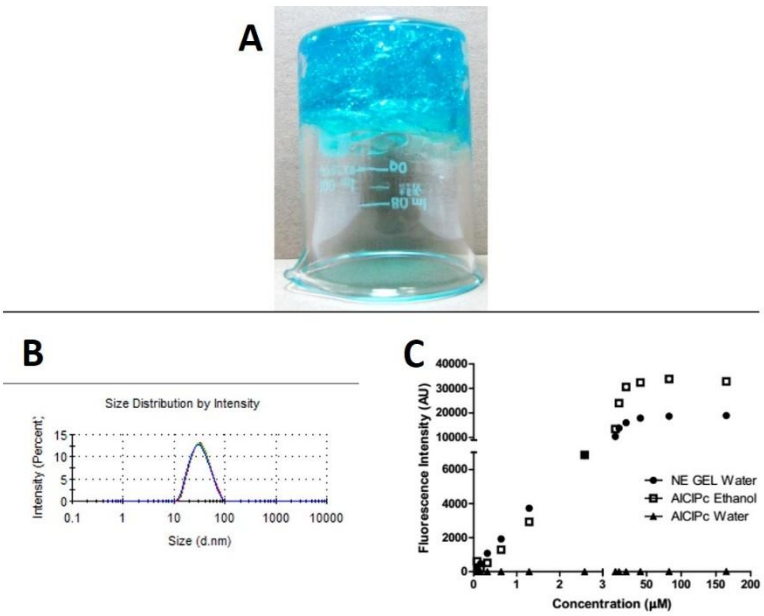

Figure 3: In section A, the macroscopic appearance of the gelified aspect of the nanoemulgel containing AlClPc. In section B the hydrodynamic size distribution, measured by dynamic light scattering. And the fluorescence emission of AlClPc dispersed in the nanoemulsion, in water and in ethanol (Section C).

Phthalocyanines, are a group of porphyrin-related macrocyclic molecules with a planar structure, that are responsible by the generation of the molecules photophysical proprieties, and makes them prone to aggregation by stacking. This aggregation behavior impairs it use in aqueous solutions, fact that justified the use a lipid nanocarrier such as the nanoemulgel used in our clinical trial [10]. In Figure 3C, we can also note that in alcoholic solution, AlClPc has the best fluorescent emission in higher concentrations. However, after serial dilutions (<2.58 µM) this alcoholic AlClPc solution has a similar fluorescent emission patter to the NE-AlClPc. This result indicates that AlClPc, when incorporated in NE, is useful and functional in lower concentrations. If we agree that the NE is diluted during nail permeation, this might be a useful characteristic of the NE-AlClPc and may contribute for the effectiveness of the clinical antifungal activity.

The number of patients free of fungal (Figure 4) after PDT protocol observed in our clinical trial was quite similar to other clinical reports. In general, 20-50% of treated patients are free of fungal infection after PDT sessions. In a recent publication, Gilaberte et al. (2017) [15], using PDT mediated by methyl aminolevulinate to treat onychomycosis, observed a complete response, measured by microbiological investigation, in ~18% of the treated patients. In another study, Figueiredo et (2014) [8] using methylene blue as photosensitizer to treat onychomycosis with PDT observed ~34% of mycological cure. In our study, we observe a complete mycological response in 8 (40%) of the treated patients, showing that this PDT protocol could be useful, and need to be investigated in a larger clinical trial. This result is not so far ideal, however comparing to conventional local and oral antifungal therapy, which is

longer and less effective (25-30% of cure)[12], we consider as an important alternative therapeutic modality to treat onychomycosis.

According to our criteria, 60% of patients included in the clinical trial were considered free of onychomycosis after PDT sessions using clinical parameters. However, after mycological examination, this number reduced to 40%. This result is common in the literature [6], and is relate to the presence of remaining live fungal cells placed in the nail bed that can regrowth and start a new infection. As a complex anatomical structure, the nail bed has low vascularization that difficult the access of immune cells as well as the delivery of systemic antifungal drugs [21]. This condition impairs the process of cure and onychomycosis recurrence is frequent [22]. This data confirms the complexity of onychomycosis diagnosis, and how is difficult to eliminate this kind of infection.

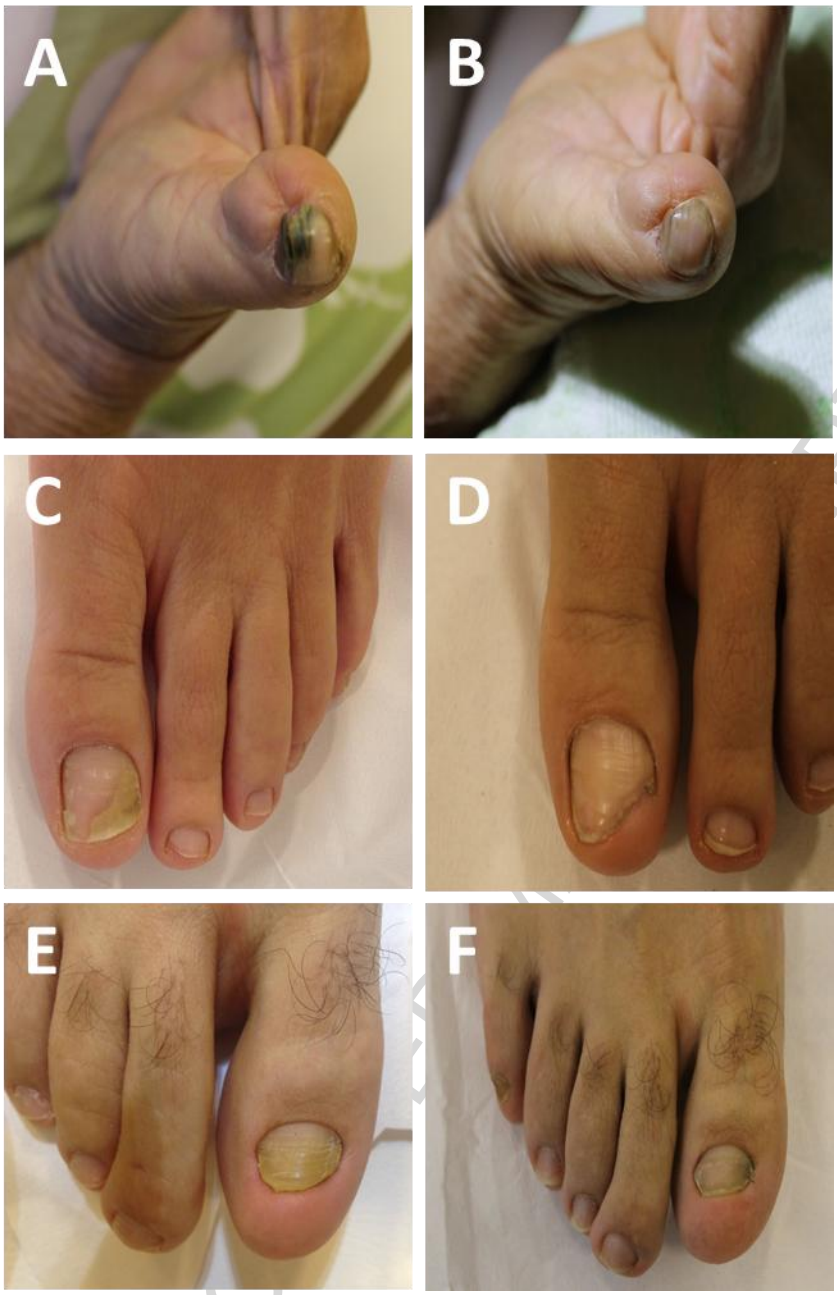

Figure 4: Clinical aspect of onychomycosis previously (A, C, E) and after (B, D, F) PDT treatment.

As commented previously, some factors such as number of sessions, time of interval between sessions, and use of local antifungals in intervals may contribute for the future applications. We plan to reduce the interval between PDT sessions from two to one week, and apply local antifungal products during these sessions' intervals. These approaches may reduce the possibility of fungal cell regrowth, thus improving treatment efficacy and reducing infection recurrence.

Some authors define onychomycosis recurrence as a relapse of the first infection or a completely new infection. This process is frequent and can be observe in 10-53% of patients undergoing onychomycosis conventional treatment [22]. Based in our results, is impossible to ensure if patient with negative clinical diagnosis that was positive for mycological examination had a new infection or an infection relapse. However, patients that were mycological free after at least four weeks of observation demonstrates the feasibility of this therapeutic protocol for the treatment of onychomycosis. Figure 1B presents all patients mycological examinations after different endpoints. In the graph is possible to observe that some patients were free of fungal infections for even longer periods, such as 30-45 weeks.

Based on the data presented in the present report, it is possible to conclude that this therapeutic nanomedicine protocol might be useful as an alternative method to treat onychomycosis. The comparison with conventional oral and local therapies show us that this is a medical field that needs a lot of effort to increase the effectiveness of onychomycosis treatments. The safety, as well as the effectiveness, of this NE-AlClPC mediated PDT protocol are important features that can be successfully incorporate to clinical practice as useful therapeutic options to treat onychomycosis. As comment previously, as an alternative for oral medicines, the protocol presented in this clinical trial might be even more interesting for patient undergoing systemic treatments.

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4. References

[1] R.K. Scher, A. Tavakkol, B. Sigurgeirsson, R.J. Hay, W.S. Joseph, A. Tosti, P. Fleckman, M. Ghannoum, D.G. Armstrong, B.C. Markinson, Onychomycosis: diagnosis and definition of cure, Journal of the American Academy of Dermatology 56(6) (2007) 939-944.
[2] R.K. Scher, Onychomycosis: a significant medical disorder, Journal of the American Academy of Dermatology 35(3) (1996) S2-S5.
[3] M. Ghannoum, R. Hajjeh, R. Scher, N. Konnikov, A. Gupta, R. Summerbell, S. Sullivan, R. Daniel, P. Krusinski, P. Fleckman, A large-scale North American study of fungal isolates from nails: the frequency of onychomycosis, fungal distribution, and antifungal susceptibility patterns, Journal of the American Academy of Dermatology 43(4) (2000) 641-648.
[4] R. Hay, The future of onychomycosis therapy may involve a combination of approaches, British Journal of Dermatology 145(S60) (2001) 3-8.
[5] A.P. da Silva, D.J. Chiandrone, J.W.R. Tinta, C. Kurachi, N.M. Inada, V.S. Bagnato, Development and comparison of two devices for treatment of onychomycosis by photodynamic therapy, Journal of biomedical optics 20(6) (2015) 061109-061109.
[6] E. Sotiriou, T. Koussidou-Ermonti, G. Chaidemenos, Z. Apalla, D. Ioannides, Photodynamic therapy for distal and lateral subungual toenail onychomycosis caused by Trichophyton rubrum: Preliminary results of a single-centre open trial, Acta dermato-venereologica 90(2) (2010) 216-217.
[7] A.P. da Silva, C. Kurachi, V.S. Bagnato, N.M. Inada, Fast elimination of onychomycosis by hematoporphyrin derivative-photodynamic therapy, Photodiagnosis and photodynamic therapy 10(3) (2013) 328-330.
[8] L. Figueiredo Souza, S. Souza, A. Botelho, Randomized controlled trial comparing photodynamic therapy based on methylene blue dye and fluconazole for toenail onychomycosis, Dermatologic therapy 27(1) (2014) 43-47.
[9] M.O. Senge, mTHPC–A drug on its way from second to third generation photosensitizer?, Photodiagnosis and photodynamic therapy 9(2) (2012) 170-179.
[10] L.A. Muehlmann, M.C. Rodrigues, J.P.F. Longo, M.P. Garcia, K.R. Py-Daniel, A.B. Veloso, P.E.N. de Souza, S.W. da Silva, R.B. Azevedo, Aluminium-phthalocyanine chloride nanoemulsions for anticancer photodynamic therapy: Development and in vitro activity against monolayers and spheroids of human mammary adenocarcinoma MCF-7 cells, Journal of nanobiotechnology 13(1) (2015) 36.
[11] T.S. Mang, Dosimetric concepts for PDT, Photodiagnosis and photodynamic therapy 5(3) (2008) 217-223.
[12] L.W.F. Souza, S.V.T. Souza, A.C.d.C. Botelho, Distal and lateral toenail onychomycosis caused by Trichophyton rubrum: treatment with photodynamic therapy based on methylene blue dye, Anais brasileiros de dermatologia 89(1) (2014) 184-186.
[13] J.E. Kim, H.J. Park, J.Y. Lee, B.K. Cho, The compliance and long-term follow up of onychomycosis treatment, Korean Journal of Medical Mycology 8(3) (2003) 110-117.
[14] A.P. da Silva, F.M. Carbinatto, V.S. Bagnato, N.M. Inada, A Promising Strategy for the Treatment of Onychomycosis with Curcumin and Photodynamic Therapy, Journal of Pharmacy and Pharmacology 3 (2015) 434-437.
[15] Y. Gilaberte, M. Robres, M. Frías, I. García-Doval, A. Rezusta, C. Aspiroz, Methyl aminolevulinate photodynamic therapy for onychomycosis: a multicentre, randomized, controlled clinical trial, Journal of the European Academy of Dermatology and Venereology (2016).
[16] B. Simmons, R. Griffith, L. Falto-Aizpurua, K. Nouri, An update on photodynamic therapies in the treatment of onychomycosis, Journal of the European Academy of Dermatology and Venereology 29(7) (2015) 1275-1279.

[17] L.A. Muehlmann, G.A. Joanitti, J.R. Silva, J.P.F. Longo, R.B. Azevedo, Liposomal photosensitizers: potential platforms for anticancer photodynamic therapy, Brazilian Journal of Medical and Biological Research 44(8) (2011) 729-737.
[18] J. Qiao, R. Li, Y. Ding, H. Fang, Photodynamic therapy in the treatment of superficial mycoses: an evidence-based evaluation, Mycopathologia 170(5) (2010) 339-343.
[19] A. Mahtab, M. Anwar, N. Mallick, Z. Naz, G.K. Jain, F.J. Ahmad, Transungual Delivery of Ketoconazole Nanoemulgel for the Effective Management of Onychomycosis, AAPS PharmSciTech (2016) 1-14.
[20] J.P.F. Longo, S.C. Leal, A.R. Simioni, M.d.F. Menezes Almeida-Santos, A.C. Tedesco, R.B. Azevedo, Photodynamic therapy disinfection of carious tissue mediated by aluminum-chloride-phthalocyanine entrapped in cationic liposomes: an in vitro and clinical study, Lasers in Medical Science 27(3) (2012) 575-584.
[21] B. Elewski, Onychomycosis. Treatment, quality of life, and economic issues, American journal of clinical dermatology 1(1) (1999) 19-26.
[22] B.M. Piraccini, A. Sisti, A. Tosti, Long-term follow-up of toenail onychomycosis caused by dermatophytes after successful treatment with systemic antifungal agents, Journal of the American Academy of Dermatology 62(3) (2010) 411-414.