

Photodynamic therapy for American cutaneous leishmaniasis: The efficacy of methylene blue in hamsters experimentally infected with *Leishmania (Leishmania) amazonensis*

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ABSTRACT

The aim of this study was to investigate the effectiveness of Photodynamic Therapy (PDT) using Methylene Blue (MB) as the photosensitizing compound and a Light-Emitting Diode (LED) in American cutaneous leishmaniasis (ACL). Hamsters were experimentally infected with *Leishmania (Leishmania) amazonensis*. After the development of the lesions in the footpad, the animals were treated with MB three times a week for 3 months. Ten minutes after each application of MB, the lesions were irradiated with LED for 1 h. The lesions were evaluated weekly by the measurement of the hamster footpad thickness. At the end of the treatment the parasitic load was quantified in the regional lymph node of the hamsters. The treatment promoted a decrease in the thickness of infected footpad ($P = 0.0001$) and reduction in the parasitic load in the regional lymph node ($P = 0.0007$) of the animals from group treated with MB + LED. PDT using MB + LED in ACL caused by *L. amazonensis* shows a strong photodynamic effect. This therapy is very promising, once it is an inexpensive system and the own patient can apply it in their wound and in their house without the need of technical assistance.

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1. Introduction

Leishmaniasis, is one of commonest parasitic diseases in the world and according to the World Health Organization more than 12 million people in 88 countries are infected and around 350 million people are at risk (WHO, 2009). The annual incidence is estimated as 1–1.5 million cases of cutaneous leishmaniasis and 500,000 cases of the visceral form (WHO, 2009).

Cutaneous leishmaniasis (CL) is characterized by single or multiple lesions in the skin or in the mucosa tissues which develop into oral, nasal and pharyngeal destruction (Neves et al., 2000). American cutaneous leishmaniasis (ACL) is caused by *Leishmania* of the subgenus *Leishmania* and *Viannia*, the most common being *Leishmania braziliensis*, *Leishmania guyanensis*, *Leishmania panamensis*, and *Leishmania amazonensis* (Neves et al., 2000).

The treatment of leishmaniasis is based, mainly, on injection of pentavalent antimony derivatives, as first choice, and also amphotericin B, paromomycin and pentamidine isothionate (Brazil, 2007). Because the high incidence of collateral effects (arthralgia, myalgia, anorexia, nausea, vomiting, swelling and local pain) and the discomfort of daily injections, patients tend to interrupt treatment and the disease may evolve with serious complications and may develop drug resistance (Brazil, 2007). Therefore, application of a topical formulation is seen as more desirable than local injections or systemic therapy, resulting in better treatment compliance.

Photodynamic Therapy (PDT), using a photosensitizing compound (PS) and visible light producing reactive oxygen species (Macdonald and Dougherty, 2001; Mang, 2004), has been successfully applied for treatment of local and topical diseases such as macular degeneration, skin lesions and cancer (Levy, 1995; Machado, 2000; Sternberg and Dolphin, 1996; Sternberg et al., 1998; Gollnick et al., 2003) and *Leishmania* eradication with low scar formation (Akilov et al., 2007a; van der Snoek et al., 2008).

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Photosensitizers such as tetracationic porphyrins (Bristow et al., 2006), cationic and anionic PS (Akilov et al., 2006), aminolevulinic acid (ALA) and protoporphyrin IX (Akilov et al., 2007b), and two cationic PS (Akilov et al., 2007a) have produced encouraging results in *Leishmania* treatment. All were tested against *Leishmania major* and to a lesser extent *Leishmania donovani* and *Leishmania tropica* (Gardlo et al., 2003; Enk et al., 2003; Asilian and Davami, 2006; Ghaffarifar et al., 2006; Sohl et al., 2007) which are more common in Europe.

Few PDT studies have been carried out against *Leishmania* species causing ACL and we could only find reports of phthalocyanine derivatives used against *L. amazonensis* (Dutta et al., 2005), *L. panamensis* and *Leishmania chagasi* (Escobar et al., 2006). As ACL are endemic diseases in forest areas and, usually, the native population from these areas cannot afford expensive treatments, low cost treatments are desirable and may help to control this disease. Additionally, it is well known that the combination of Methylene Blue (MB) and different light sources such as Light-Emitting-Diodes (LED) or low potency lasers are effective against prokaryote and eukaryote cells (Tardivo et al., 2004; Tardivo et al., 2005; Wainwright et al., 1997; Zeina et al., 2001; Floyd et al., 2004; Huang et al., 2004; ÓRiordan et al., 2005; Peloi et al., 2008). However there are no reports of effectiveness of this combination against *Leishmania* species. Therefore, the aim of our study was to investigate the photodynamic effectiveness of MB and Light-Emitting Diode (LED) against ACL in hamsters experimentally infected with *Leishmania (L.) amazonensis*.

2. Materials and methods

2.1. Chemical and LED device

Methylene Blue (MB, Vetec, $C_{16}H_{18}ClN_3S \cdot 3H_2O$, MW 373.90 g mol⁻¹) and all other chemicals were analytical grade and were used without purification. The LED (EverLight Co.) light system was constructed using 6 units (in series) that emit red light, and their individual output was determined using a Handheld Laser Power Meter (Edmund Optics Inc.). The LED emission (maximum at 663 nm) and MB absorption (maximum at 665 nm) spectra were recorded in a SPEX Fluorolog2 model 1680 and in a Varian Cary 50 spectrophotometer, respectively.

2.2. Parasite

Leishmania (L.) amazonensis (MHOM/BR/73/M2269) was isolated from experimentally infected hamsters and cultured in medium 199 supplemented with 10% of fetal calf serum, 20 mM L-glutamine, 1% human urine and antibiotics (100 UI/mL penicillin G and 100 µg/mL streptomycin) at 26 °C, by weekly passages.

2.3. Experimental infection

Thirty-eight male golden hamsters (90 days old) from the Animal Laboratory of Universidade Estadual Paulista (UNESP, Assis – SP, Brazil) were intradermally inoculated with 100 µL of physiological saline (PS) containing 5×10^6 promastigote forms of *L. amazonensis* in the posterior right footpad. The posterior left footpad, inoculated with 100 µL of uninfected PS was used as a vehicle control. The development of the lesions in the footpads were observed and measured for up to 12 weeks.

2.4. Photodynamic Therapy of MB/LED-PDT

The animals were separated in three groups: Control group ($n = 8$) treated with MB half in lotion and half in water without

LED, group A ($n = 14$) treated with oil/water (O/W) lotion with 10 nM MB and group B ($n = 15$) treated with 10 nM MB aqueous solution (15 animals), and treatment started 90 days after infection. Before each treatment, the animals were anaesthetized with a combination of xylazine (10 mg/kg – Rompum®) and ketamine chlorohydrate of (50 mg/kg – Ketamina®). The footpad of the infected animals were treated with MB three times a week for 3 months. 10 min after the each application of MB, LED (665 nm, 5 mW cm⁻²) was used for 1 h (12 J cm⁻²). The control group received only MB without any irradiation.

2.5. The evaluation of lesions

The lesions were evaluated weekly by measurement of footpad thickness using a dial thickness gauge (Mitutoyo®, Japan). The lesion thickness was expressed as the difference between the infected footpad and the contralateral uninfected footpad.

2.6. Quantification of parasites in lymph node and spleen

Parasites were quantified in the lymph node and spleen of the hamsters after the end of the treatment, following the method described by Buffet et al. (1995), with slight modifications. The animals were euthanized under deep anesthesia. From each animal the popliteal lymph node and the spleen were aseptically removed and weighed. Each organ was macerated and the suspension was passed through a needle (insulin type) several times to disrupt the cells. The suspensions were diluted 1:4 and transferred to 96-well plates containing medium 199 (Gibco, Invitrogen Corporation) and incubated at 26 °C for 30 days. Next, each well was examined in an inverted light microscope searching for promastigote forms and the titer was considered as the highest dilution where least one parasite could be found. The incubation was extended for a further 15 days and re-examined. The parasitic load in each organ was calculated as geometric mean of the titers multiplied by 16.7 and divided by the total mass of the organs (in grams), where 16.7 represents the fraction of the suspension of each organ inoculated in the first well of the culture plate.

2.7. Statistical analysis

All results were analyzed by Shapiro Wilk's normality tests followed by Student's *t* test using the StatSoft® software; significant differences were considered at the 5% level.

2.8. Ethical aspects

All procedures were performed according to protocols approved by the Committee on Research Animal Care of the State University of Maringá (report 028/2006 on 11/Jul/2006).

3. Results

The development of the lesions in experimentally infected hamster were measured weekly for 12 weeks. After this, the hamster footpads were treated with 10 nM MB dissolved in lotion (group A) or water (group B) and irradiated with LED for 1 h three times a week for 12 more weeks (Fig. 1).

The control group treated with MB in lotion or water without LED irradiation, presented a continuous increase in thickness throughout the treatment, showing that MB alone was not able to prevent the development of lesions. In contrast, the thickness of the footpad in the groups treated with MB + LED was significantly decreased ($P = 0.0001$; $P = 0.0001$), indicating a strong

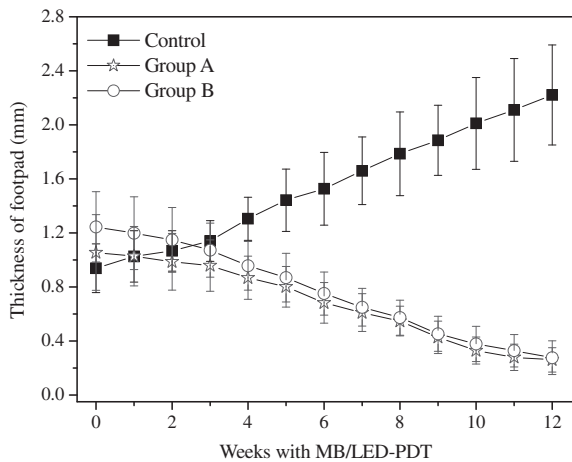


Fig. 1. Effect of MB/LED-PDT on lesions of hamsters infected with *Leishmania (L.) amazonensis*. The thickness of the footpad of hamsters infected with 5×10^6 promastigotes of *Leishmania (L.) amazonensis* was measured weekly for 12 weeks until lesion development and the treatment with MB or MB + LED started and followed weekly for 12 more weeks. The lesion was evaluated by the difference between the thickness of footpad and its contralateral footpad. Half of the control group was treated with MB in lotion and half MB in water ($n = 8$) and group A ($n = 14$) were treated with MB in lotion and group B ($n = 15$) received MB in water. The values represent mean \pm SD of the total number of animals of the each group. $P < 0.001$ compared to control group.

photodynamic effect. No significant difference was found between groups A or B ($P = 0.470$).

The morphology of the lesions was also evaluated. Before MB + LED treatment, all animals had lesions in the infected footpad and more than 40% of them had ulcerated lesions. After 6 weeks of treatment with MB + LED, only 13.3% of the animals in group B and none in group A, had ulcerative lesions. At the end of treatment (12 weeks) 40% and 50% of the lesions in animals in groups A and B, respectively, were completely healed, while all animals in the control group, treated with MB only, had ulcerative lesions.

Fig. 2 shows a sequence of photographs of the infected footpad treated with the combination of MB + LED during and after 12 weeks of treatment, where re-epithelization, regression and healing can be seen.

The parasitic load in the spleen and in the popliteal lymph node of the infected hamsters was also investigated (Fig 3). No parasites were found in the spleens of any treated animal showing that the parasites did not migrate into spleen in the animals treated with MB + LED. In the popliteal lymph node, parasites were found in 87.5% of the control animals, while in the groups treated with MB + LED the percentage was smaller (28.6% for group A and

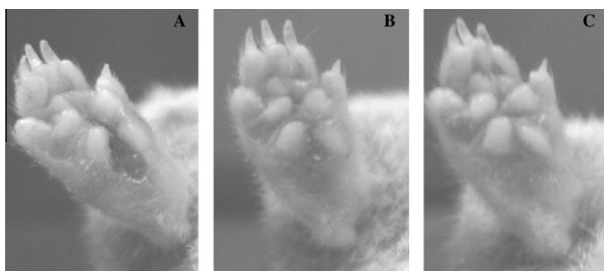


Fig. 2. Development of the lesion in hamster infected with *Leishmania (L.) amazonensis* and treated with MB/LED-PDT. Hamsters were infected with 5×10^6 promastigotes forms of *Leishmania (L.) amazonensis* and after the development of the lesion, the animals were treated with MB/LED-PDT. (A) Before treatment (90 days of infection); (B) After 6th weeks of treatment; (C) After 12 weeks of treatment.

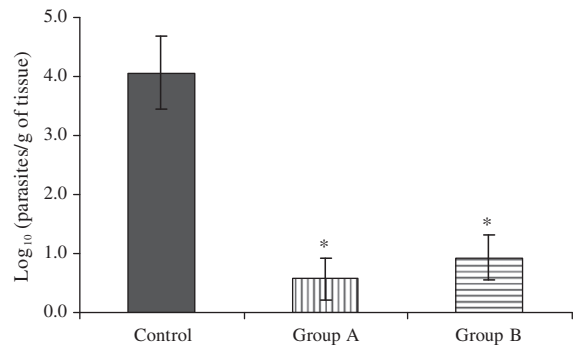


Fig. 3. Effect of MB/LED-PDT on parasite load in hamsters infected with *Leishmania (L.) amazonensis*. Hamsters were infected with 5×10^6 promastigotes forms of *Leishmania (L.) amazonensis* and treated with MB/LED-PDT. Control group ($n = 8$) was treated with MB but was not irradiated with LED. After the treatment for 12 weeks, the parasite load in the popliteal lymph node of infected footpad was evaluated. * $P < 0.05$ compared to control group.

13.3% for group B). The parasite load in the groups treated with MB + LED was smaller compared to the control group ($P = 0.0007$ for group A and $P = 0.0001$ for group B). There was no significant difference in the parasitic load between groups A and B ($P = 0.53$) showing that MB in either water or O/W did not interfere in the permeability of MB into parasite membrane.

4. Discussion

Little effort has been made to improve the treatment of leishmaniasis around the world and the medicines recommended for treatment have been used for more than 90 years (Rath et al., 2003). Despite the high incidence of side effects and discomfort for the patient from daily subcutaneous injection, the pentavalent antimonials remain the first choice for treatment (Brazil, 2007; Rath et al., 2003). New therapies which ally lower costs, lower side effects and higher efficiency are urgently needed.

PDT has produced some success in treating superficial diseases (Levy, 1995; Machado, 2000; Sternberg and Dolphin, 1996; van der Snoek et al., 2008) and the approval of protoporphyrin has fueled the search for new photosensitizers (Machado, 2000; Sternberg et al., 1998; van der Snoek et al., 2008).

Several reports have demonstrated the efficacy of PDT for Old World CL using different photosensitizers. Among them, Gardlo et al. (2003) had treated five cases of CL with PDT using Metvix[®] (Photocure, Oslo, Norway), 75 J cm^{-2} of red light, applied twice a week, during 12 weeks followed by application once a week for a further 4 weeks. All patients involved in the study were healed, with excellent cosmetic results and the lesions were clinically and histologically free of parasites with no recurrence throughout the observation time (10 months). Enk et al. (2003) treated 32 lesions in 11 patients, of whom 10 had lesions healed after two PDT applications. Another study (Asilian and Davami, 2006), involving 20 patients with 31 lesions, was carried out with aminolevulinic acid (ALA-PDT), 100 J cm^{-2} , once a week, for 4 weeks. The results were satisfactory with 29 lesions completely healed and the other two being partially cure.

The main limitation to widespread use of PDT is the price of the PS and the light source, since leishmaniasis is mainly found in undeveloped countries where expensive treatments are unaffordable. MB is an inexpensive dye and has been shown to be a good PS for PDT (Peloi et al., 2008) and the combination of MB + LED was successful in our study in treating lesions caused by *L. amazonensis* in experimentally infected hamsters. After 12 weeks, half of the treated animals had their lesion healed

and, from visual analysis, the appearance of the lesions was better and the tissue around the lesions was undamaged. Furthermore, no parasites were found in the popliteal lymph node in more than 79.3% of the treated animals and the mean parasitic load was smaller in treated animals than in control animals.

Studies carried out with animals infected with *L. major* and treated topically with ALA-PDT or PPA904-PDT also had a significant reduction in the parasitic load, although local tissue damage was still observed (Akilov et al., 2007b).

Clinical cure of human patients is considered when the lesions are completely healed (Brazil, 2007). Despite results that showed clinical cure, this was not associated with the clearance of parasites, this being a hallmark of leishmaniasis. It is known that a low number of viable organisms still persist within lymphoid tissue and/or in the site of the former skin lesion after self-cure or successful chemotherapy (Ramírez and Guevara, 1997) and the clinical cure does not always coincide with histopathologic cure (Mendonça et al., 2004). It is possible that healing/cure depends on both the immune response of the host and on genotypic and phenotypic features of the parasite and play a role in the persistence of parasites (Mendonça et al., 2004).

Special and expensive equipment and time-consuming procedures have been emphasised as disadvantages of PDT treatment (van der Snoek et al., 2008), but the system used for this study is simple and cheap. The LED device is inexpensive, easy to handle and to transport, a MB is available in any local market and treatment can be carried out in the patient's house without any special technical training.

5. Conclusions

PDT with MB + LED, a very inexpensive system, promoted significant reduction in the size of the lesion, in the parasitic load in the draining lymph node and healed the lesions in hamsters experimentally infected with *L. amazonensis*. Although the MB/LED-PDT provided promising results, further studies are necessary to better understand its mechanism of action and assure the safety and efficacy for use in humans.

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