Efficacy of the Photodynamic Antimicrobial Therapy (PACT) with the use of Methylene Blue Associated with the λ660nm laser in *Leishmania (Leishmania) amazonensis*: In Vitro Study

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ABSTRACT

The present studied evaluated the *in vitro* effects of PDT on *Leishmania (Leishmania) amazonensis* promastigotes. For this examination *L. amazonensis* promastigotes, stain Josefa, were used and maintained in Warren media supplement with fetal bovine serum at 26°C for 96 hours. A viability curve was accomplished using different concentrations of methylene blue photosensitizer associated to red laser light in order to obtain the most effective interaction to inhibit the parasite's growth. Two pre-irradiation periods, 5 and 30 minutes, were evaluated and the promastigotes were counted by colorimetry. On fluorescence microscopy the autophagic processes and reactive oxygen species were detected. Promastigotes treated with Photodynamic Therapy (PDT) by concentrations of 5 and 0,315ug/mL, presented cellular proliferation inhibition when compared to the control. In the first condition, the cells had structural alterations such as truncated cells, cells with two flagella, bleb formation and cells body deformation, while none of these modifications could be visualized in the control group. When analyzed through fluorescence microscopy, the promastigotes treated were positives for free radicals immediately after light application and also 1 hour after treatment presenting signs of autophagia. PDT on *L. (L.) amazonensis* is effective causing alterations that can help elucidate the mechanisms of the parasite's death when treated with methilene blue associated to laser light. Therefore, new studies of PDT intra-parasite's proceedings are still being accomplished.

Keywords: *Leishmania,* Photodynamic Therapy, light, fluorescence microscopy

1. INTRODUCTION

Leishmaniasis is a zoonosis that is considered an important public health problem and is a parasitosis distributed in at least 88 countries of tropical and sub-tropical regions. With a prevelance of 12 million cases and an incidence of around 500.000 cases of Visceral leishmaniasis (VL) and 1.5 million cases of Cutaneous leishmaniasis (CL), this disease presents an annual estimate of 70.000 deaths and 350.000 millions of people within the risk of getting infected [1].

The CL (known as ulcer of Bauru, Oriental button or brave wound) is the most common form of the disease and is characterized by only one or multiple ulcerous, nodules or crusted lesions, usually painless, formed by the infected insect on the hematophagy site [2]. Some species can cause a variant form known as diffuse cutaneous leishmaniasis (DCL). This form is characterized by disseminated cutaneous nodules, nonulcered, that can compromise extended areas of the skin with plaques [3]. The Mucocutaneous Leishmaniasis is characterized by metastatic lesions on mucosa, which can cause disfiguration by progressive ulceration, especially in the nasal, oral and pharynx region.

The pentavalent antimonials [Sb (V)], such as the sodium stibogluconate (Pentostam®) and the meglumine antimoniate (Glucantime®), introduced as quimiotherapics in the 50's decade, are still the main drugs used in the treatment and control of leishmaniasis [4]. These compounds inhibit the synthesis of ATP in the amastigote forms, through the inhibition of glycolysis and fatty acids ß-oxidation and are potent proteins phosphatases inhibitors, especially of PTPs (protein tyrosine phosphatases), which are important components of the leishmaniasis pathogenesis, and are involved in pathways that depend on phosphorylation which are essential in the control of protozoa infection [5,6]. These

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pathways are important targets of the virulence mechanism of *Leishmania* to deactivate the host's cell [7-12]. The therapy consists of daily injections during a months period, causes collateral effects, has been ineffective in many endemic regions such as India and contributes to selection of tolerant parasites to high doses of the drug [13,14].

The pentavalent antimonials [Sb (V)], work as a pro-drug being converted in a trivalent form [Sb (III)], which is highly toxic for both types of *Leishmania* leading to a reduction of ATP availability for the parasite since the compound interferes with the bioenergetics oxidation process of the protozoa. Patahak e Yi (2001) referes to the antimonials in its pentavalent form as active agents of cellular signaling pathway inhibiting PTPs, increasing JAK-STAT phosphorylation, that has a relevant role on leishmania progress and on cellular response induced by cytokines, especially IFN-□ [16,17]. Meanwhile, the vanadium derivative bring a previous concept of its use in diabetics animal models as potent inhibitors of protein phosphatases, especially PTPs, important in *Leishmania* virulence [6,18].

Photodynamic therapy (PDT) is a method of a light-sensitive drug, in combination with light of a visible wavelength, to eliminate target cells. Photosensitisers can be administered by the vein or topically, which targeted cells then preferentially absorb. These intermediates irreversibly oxidize, a vital cellular component, producing tissue injury and necrosis.[19]

2. MATERIALS AND METHODS

In this study *L. amazonensis* promastigotes, stain Josefa, were used and maintained in medium Warren supplement with fetal bovine serum at 26°C for 96 hours.

The susceptibility of the parasite to the association of the photosensitizer methylene blue in different concentrations to the laser light was initially evaluated (*Tab. 1*). To do so, a viability curve was performed, in the presence and absence of the aforementioned to find the most effective interaction to inhibit the parasite's growth.

For the bioassays, $4x10^5$ parasites were applied in culture plates with 24 wells containing 2mL of Warren media, final volume, complying the growth premise. Two pre-irradiation periods, 5 and 30 minutes, were evaluated and the promastigotes were counted by colorimetry with methylene blue.

Table 1: Laser Parameters used in the studied

PARAMETERS	LASER
Wavelength (nm)	660
SAEF (J/cm^2)	4
Power Output (W)	0.040
Illuminated Area (cm^2)	1
Mode	CW
Spot (cm^2)	0.04
Intensity (mW/cm^2)	100.000
Exposure Time (per session)	1 min

The autophagic process was detected by the use of a fluorescence monodansylcadaverine (MDC) probe, which is a marker of autophagic vacuoles. The parasites, in different periods, were applied on a lamina, and 1 □L/mL of MDC probe was added in the culture media containing the cells in the presence or absence of the substances. After 10 minutes of incubation with the marker, the culture media was removed and the cover slips were withdrawn to observe the trophozoites. This observation was full field in an Olympus BX51 fluorescence microscope, were micrographs with 0.15ms of exposition were obtained.

To detect the Reactive Oxygen Species (ROS) a probe DHE was used. This probe reacts with free radicals and emits red fluorescence. For assays, aliquots of a stash solution were applied in the wells, till the final concentration of 0,4

□M and incubated for 10 minutes at 26ºC in the absence of light. Later, the promastigotes were observed and micrographs were taken in Olympus BX51 fluorescence microscope at a wavelength of 460-495 nm.

3. RESULTS AND DISCUSSION

In the bioassays triage of the association, two of them, 5ug e 0.315ug, presented inhibition in the growth of promastigotes within the pre-irradiation period of 5 minutes (*Fig. 1*), however the effect of PDT was potenciallized when increasing the pre-irradiation period to 30 minutes.

In the presence of the association it was possible to observe that the parasites formed structures where cells in the process of division were found, beyond that, cellular morphology also presented alterations with bleb formation (*Fig. 2*), truncated cells and cellular volume alterations (*Fig. 3*) and loosening of flagellum membrane. These alterations were not found in the control.

In 96 hours after treatment with the association, no unharmed cells were found, however previous experiments observed that with the use of only the photosensitizer in 96 hours, the parasite would be inhibited, nevertheless unharmed cells could be seen in this condition.

The aforementioned alterations are tied to actions initiated by free radicals as the ones produced by the photosensitizer after photoactivation. However, it is possible to associate the bleb formation of the membrane to a lipid peroxidation triggered by the release of free radicals, and due to the location of the majority of these membrane formations be related also to a loss of the mitochondrial function, located in the flagellum base of the parasite, that occurs when molecules responsible of detoxification are saturated. Another effect of the free radicals is on the cytoskeleton, in which some cases may lose its physiological shape turning the affected cell pleomorphic and also cause truncated cells.

The ultrastructure of treated parasites can be completely altered due to action of free radicals that are produced after PDT and can induce different death cell processes such as autophagic death, necrosis and apoptosis. This first death process should be evaluated more precisely, for the treated parasites presented positive diffuse labeling for autophagy within the period of 1 hour. This analysis is being achieved through techniques such as transmission electron microscopy and flow cytometry to help in the death process description.

The first drug choice in Leishmania is the antimonial substance, existing under the form of the antimoniate of N-metilglucamine. The second choice of treatment is the Amphotericin B, that is used when occur a reply to the treatment with antimoniate substance. As disadvantage of the traditional treatments are the collateral effects, as arthralgia, myalgia, chronic headache, fever, vomits, giddiness and swell in the place of the application. The cardio and kidney damage made by antimonial substance constitute an important limitation to its security. New modalities of treatment are need, for better results in the treatment of the disease [20].

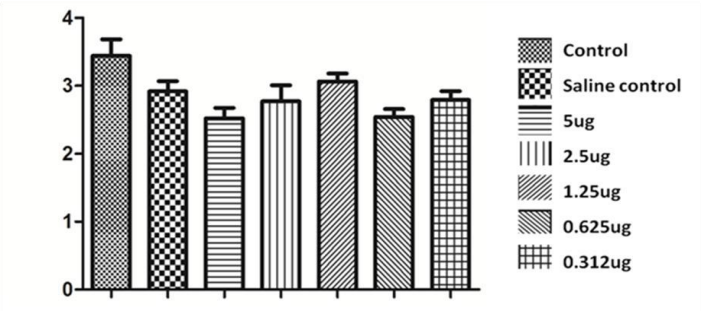

Figure 1: Graphic of the proliferation of *L. amazonensis* in different conditions. Control) parasites without any type of treatment. PDT) parasites treated with different (0.312 – 5ug/mL) concentrations of methylene blue associated to red LASER, 660 nm, 2,4J/cm², for 1 minute, with pre-irradiation time of 5 minutes.

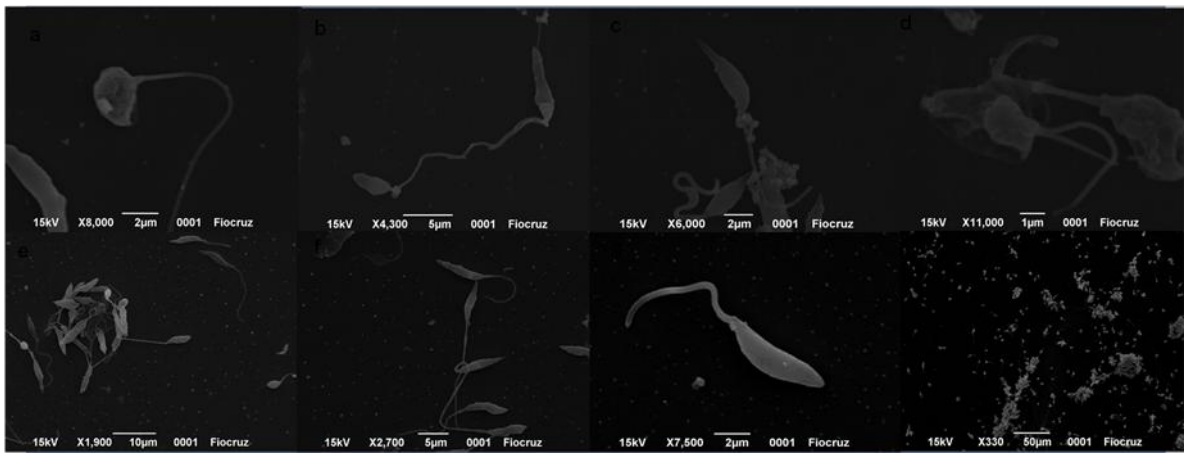

Figure 2: Scanning electron micrograph of *Leishmania amazonensis* promastigotes. a-d) parasites treated with methylene blue associated with red LASER. e-h) control parasites.

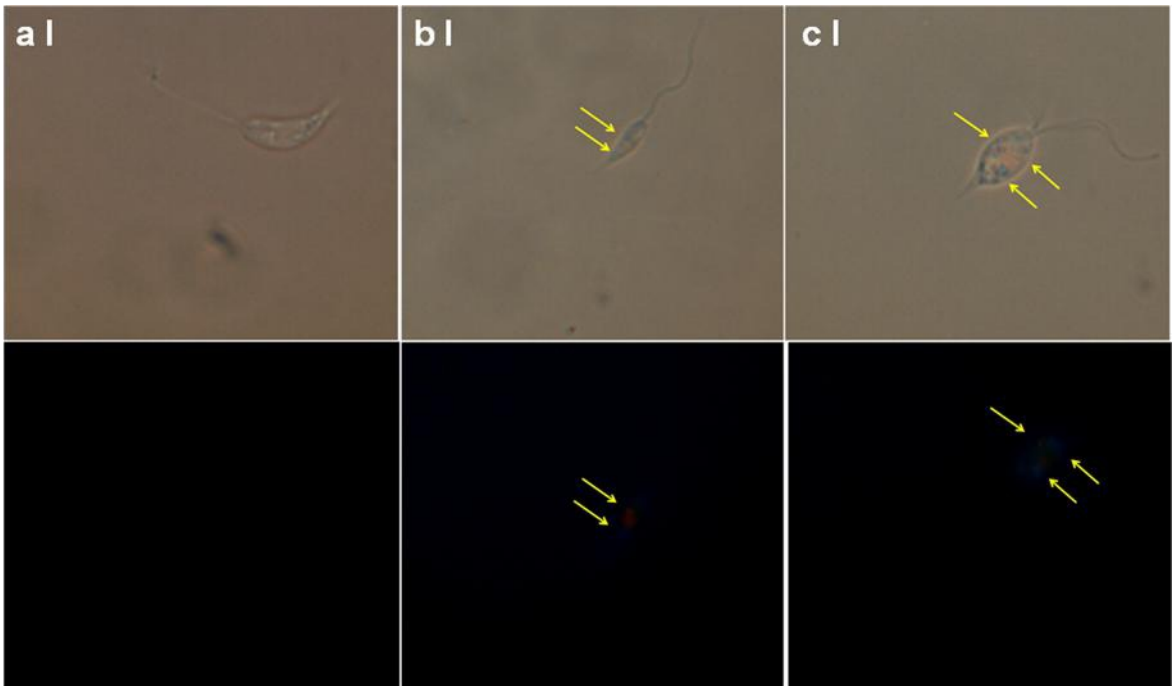

Figure 3: Micrograph presenting promastigotes, incubated with autophagic probes (MDC – in blue) and free radicals (DHE - red), control (aI, aII) and treated (bI, bII, cI e cII). In the control the absence of labeling suggests that the autophagic processes and production of free radicals of these parasites are basal, however in the treated species there is a labeling of free radicals near the nucleus and a diffuse labeling for autophagy**.**

4. CONCLUSION

The PDT protocol here presented is efficient for it increases the production of intracellular free radicals, as well as initiates an autophagic process and promotes inhibition of the parasites proliferation. However, more studies are

necessary for the death pathways and effects are still not completely elucidated. A more thorough study of the protocol will be accomplished in order to potentize the PDT effect, since in the pilot test it was observed that with a pre-irradiation period it was possible to increase the *in vitro* treatment efficacy.

REFERENCES

[1] http://www.who.int/leishmaniasis/en/

[2] Hepburn, N. C., "Cutaneous leishmaniasis," Clin. Exp. Dermatol. 25, 363-370 (2000).

[3] Grevelink, S. A. and Lerner, E., "Leishmaniasis," J. Am. Acad. Dermatol. 34(2 Part 1), 257-272 (1996).

[4] Croft, S. L. and Yardley, V., "Chemotherapy of leishmaniasis," Curr. Pharm. Des. 8, 319-342 (2002).

[5] Balaña-Fouce, R., Reguera, R., Cubria, J. C. and Ordonez, D., "The pharmacology of leishmaniasis," Gen. Pharmacol. 30, 435-444, (1998).

[6] Posner, B. I., Faure, R., Burgess, J. W., Bevan, A. P., Lachance, D., Zhang-Sun, G., Fantus, I. G., NG, J. B., Hall, D. A., Lum, B. S. and Shaver, A., "Peroxovanadium compounds. A new class of potent phosphotyrosine phosphatase inhibitors which are insulin mimetics," J. Biol. Chem. 269, 4596-4604 (1994).

[7] Martiny, A., Vannier-Santos, M. A., Borges, V. M., Meyer-Fernades, J. R., Cunha-e-Silva, N. L. and de Souza, W., "*Leishmania*-induced tyrosine phosphorylation in the host macrophage and its implication to infection," Eur. J. Cell Biol. 71, 206-215 (1996).

[8] Martiny, A., Meyer-Fernandes, J. R., de Souza, W. and Vannier-Santos, M. A., "Altered tyrosine phosphorylation of ERK1 MAP kinase and other macrophage molecules caused by *Leishmania* amastigotes." Mol. Biochem. Parasitol.102, 1-112 (1999).

[9] Nandan, D. and Reiner, S. L., "Attenuation of gamma interferon-induced tyrosine phosphorylation in mononuclear phagocytes infected with *Leishmania donovani*: selective inhibition of signaling through Janus kinases and Stat 1," Infect. Immun. 63, 4495-4500 (1995).

[10] Olivier, M., Romero-Gallo, B. J., Matte, C., Blanchette, J., Posner, B. I., Tremblay, M. J. and Faure R., "Modulation of interferon-gamma-induced macrophage activation by phosphotyrosine phosphatases inhibition. Effect on murine Leishmaniasis progression," J. Biol. Chem. 273, 13944-13949 (1998).

[11] Matte, C., Marquis J. F., Blanchette, J., Gros, P., Faure, R., Posner, B. I. and Olivier, M., "Peroxovanadium-mediated protection against murine leishmaniasis: role of the modulation of nitric oxide," Eur. J. Immunol. 30, 2555-2564 (2000).

[12] Forget, G., Siminovitch, K. A., Brochu, S., Rivest, S., Radzioch, D. and Olivier, M., "Role of host phosphotyrosine phosphatase SHP-1 in the development of murine leishmaniasis," Eur. J. Immunol. 31, 3185-3196 (2001).

[13] Croft, S. L., "Monitoring drug resistance in leishmaniasis," Trop. Med. Int. Health. 6, 899-905 (2001).

[14] Olliaro, P. L., Guerin, P. J., Gerti, S., Haaskjold, A. A., Rottingen, J. A. and Sundar, S., "Treatment options for visceral lieshmaniasis: systematic review of clinical studies done in India, 1980-2004," Lancet Infect. Dis. 5, 763-774 (2005).

[15] Pathak, M. K. and YI, T., "Sodium stibogluconate is a potent inhibitor of protein tyrosine phosphatases and augments cytokine responses in hemopoietic cell lines," J. Immunol. 167, 3391–3397 (2001).

[16] Murray, H. W. and Delph-Etienne, S., "Roles of endogenous γ-interferon and macrophage microbicidal mechanisms in host response to chemotherapy in experimental visceral leishmaniasis," Infect. Immun. 68, 288-293 (2000).

[17] Olivier, M., Gregory, D. J. and Forget, G., "Subversion Mechanisms by Which *Leishmania* Parasites Can Escape the Host Immune Response: a Signaling Point of View," Clin. Microbiol. Rev. 18, 293- 305 (2005).

[18] Rath, S., Trivelin, L. A., Imbrunito, T. R., Tomazela, D. M., de Jesus, M. N., Marzal, P. C., de Andrade Jr. H. F. And Tempone, A. G., "Antimoniais empregados no tratamento da leishmaniose:estado da arte," Quim. Nova, 26, 550-555 (2003).

[19] Fisher, A. M. R., Murphree, A. L. and Gomer, C. J., "Clinical and preclinical photodynamic therapy," Lasers Surg. Med. 17, 2-31(1995).

[20] Gontijo, B. and Carvalho, M. L. R., "American cutaneous leishmaniasis," Revista da Sociedade Brasileira de Medicina Tropical, 36(1) 71-80 (2003).