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ORIGINAL ARTICLE

Leishmanicidal effect of antiparasitic photodynamic therapy—ApPDT on infected macrophages

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Abstract The aim of this study is to evaluate the effects of ApPDT (antiparasitic photodynamic therapy) on the interaction of *Leishmania braziliensis* with J774 macrophages, used as a photosensitizer, methylene blue associated with red laser. The tests are in triplicate and the samples divided into four groups: control, photosensitizer, laser, and ApPDT. The photosensitizer used was the methylene blue at concentration of 12.5 μg/mL. The parameters of the laser were λ = 660 nm, 40 mW, and 8.4 J/cm^2. Samples are analyzed by optical microscopy through the identification and counting of infected and uninfected macrophages, parasite load, infectivity, and infection index. Statistical analysis used ANOVA test with Tukey post-test, being considered statistically significant $p < 0.05$. The analysis of the interaction tests shows that the infection rate in the ApPDT group in relation to the control group presents a statistically significant reduction ($p < 0.0001$) of 71% at both 24 and 48 h ($p < 0.0001$) of 62%. ApPDT reduces the number of macrophages infected by *Leishmania braziliensis*, as well as the number of intracellular parasites, being a possible alternative therapy in the treatment of cutaneous leishmaniosis.

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Introduction

The earliest forms of treatment for cutaneous leishmaniosis were based on oral, topical, or systemic administration of antimonial drugs. For decades, sodium stibogluconate was considered the gold standard for treatment of cutaneous leishmaniosis. However, this drug was reported to be quite toxic to humans, expensive and inefficient to the various species of *Leishmania* [1]. Therapy with meglumine and allopurinol antimoniate promotes not only a clinical improvement but also a marked decrease in parasitic load on blood, skin, and lymph nodes [2]. The treatment usually lasts for more than 6 months in cases of large lesions and lesions located in the joints or face [3].

Among the problems reported in the treatment of cutaneous leishmaniosis are difficulty in determining clinical diagnosis due to lack of access to microscopy on many basic health services [3]; the serious side effects of pentavalent antimonial drugs, for example: bone and muscle pain, renal failure, hepatotoxicity, and cardiotoxicity [4]; and the variability of the efficacy against the different forms of *Leishmania* [5], making drugs and medical attention an expensive treatment because of the side effects [6]; besides, there have also been reports of patients not responding to drugs due to drug resistance or increased immunosuppression [7].

In this context, ApPDT appears to be a promising technique in the treatment of cutaneous leishmaniosis due to its low toxicity, lower costs and adherence of patients to the treatment. Unlike conventional drugs that act only on a target, the photosensitizers act via production of singlet oxygen or reactive oxygen species [8], inducing damage to biomolecules that will lead to loss of appropriate biological functionality, leading to inactivation of the parasite cell [9]. The evident advantages of ApPDT over conventional treatments such as chemotherapy are target

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selectivity and reduction of toxicity [10]; few adverse effects; high cure rates and excellent esthetic results in affected areas [11]. ApPDT is non-invasive and can be repeated when necessary without damage to the patient's health and without causing resistance in the parasite [12].

It was hypothesized that ApPDT could be used as a reliable procedure on the treatment of cutaneous leishmaniosis. The aim of this study was to assess, in vitro, the effect of ApPDT in the interaction between *Leishmania braziliensis* and J774 cells, using methylene blue and red laser.

Materials and methods

Sampling

For the development of this study, four experimental groups were used: negative control (where the macrophage/*Leishmania* interaction was exempt from any treatment), photosensitizer (treated only with methylene blue), laser (treated only with light), and ApPDT (where antiparasitic photodynamic therapy was performed) in triplicate as shown in Table 1.

Parasite strain

The strain of *Leishmania braziliensis* (MHOM / BR / 01BA788) was obtained from the Fiocruz-Bahia Immunoparasitology laboratory was cultured in Warren medium supplemented with 10% inactivated fetal bovine serum (Cripion, Brazil), 2 mM L-glutamine, penicillin 100 U/mL, and 100 I/ml streptomycin (Sigma, USA) at 23 °C.

Macrophages

J774 macrophages were cultured in DMEM culture medium (Sigma, USA), supplemented with 10% fetal bovine serum (Sigma, USA), in a CO_2 oven with 5% CO_2 at 37 °C. Passages were performed at every 72 h.

Antiparasitic photodynamic therapy

For the realization of the ApPDT, a laser device (TwinFlex©, MMOptics, São Carlos, São Paulo, Brazil) was used as shown in Table 2, with a pre-irradiation time of 5 min. The laser probe was standardized fixed by a holder to maintain the same irradiation conditions to all samples. The compound used was methylene blue at a concentration of 12.5 μg/mL (Laboratory Fórmula, Salvador, Bahia, Brazil), being stored at 4 °C and protected from light.

Table 1 Experimental groups tested on the study

	Control	Laser	Photosensitizer	ApPDT
Compound	−	−	+	+
Laser	−	+	−	+

Table 2 Parameters of the laser

Parameters	Laser
Wavelength (nm)	660
Mode	CW
Spot of the probe (mm^2)	4
Power output (W)	0.04
Exposure time (s, per session)	300
Energy density (J/cm^2)	8.4

Assessment of the parasite-macrophage interaction

For the assessment of the parasite-macrophage interaction, the concentration of macrophages used in the experiments was 3×10^5 cells/well cultured in 24-well plate with complete DMEM medium. The samples were distributed in alternated wells, the wells without samples were added a black ink to block the light. After 2 h of incubation, *Leishmania braziliensis* promastigotes were added at 3×10^6 concentration, so the parasite/macrophage ratio was 10:1. The time of infection of the macrophages was 24 h. The treatment was performed and evaluated at 24 and 48 h. The evaluation was performed by counting the infected and uninfected macrophages of *Leishmania braziliensis* in five randomly determined fields.

Parasitic load calculation

The parasite load was determined by averaging the intracellular amastigote count in 100 macrophages. As described in the equation:

$$parasitic\ load = \frac{(AM\text{Ø}1 + AM\text{Ø}\ n)}{n}. \quad (1)$$

Determination of infectivity

Infectivity was determined by the ratio of infected macrophages to total count of macrophages counted. As described in the equation:

$$Infectivity = \frac{M\text{Ø}infected}{100M\text{Ø}} \quad (2)$$

Determination of the infection rate

The infection rate was determined by multiplying the infectivity of an experimental group by their respective parasitic load. As described in the equation:

$$infection\ index = infectivity \times parasitic\ load. \quad (3)$$

Statistical analysis

Statistical analysis was performed using GraphPad Prism® software version 5.0 using ANOVA with Tukey's post-test, and values of $p < 0.05$ were considered statistically significant.

Results

In the evaluation of the parasite load after 24 h after the treatment, it was noticed that there was a statistically significant difference ($p < 0.001$) between the control and the photosensitizer groups, with a 17.4% reduction in the parasitic load in the photosensitizer group. Significant statistic reduction ($p < 0.0001$) was also seen when comparing the control and the ApPDT group in which a 33.1% reduction in the parasite load was observed. However, there was no difference between the control and the laser groups in that same time (Fig. 1a). In the evaluation of the parasitic load after 48 h after treatment, it was noticed that there was no statistically significant difference between the control and the photosensitizer and laser groups. However, there was a statistically significant difference ($p < 0.0001$) between the control and ApPDT groups, with a 38.4% reduction in parasite load in the ApPDT group (Fig. 1b).

Data from this study show that the percentages of infected and uninfected macrophages are different between the experimental groups of this assay in the 24- and 48-h periods as shown in Table 3.

The evaluation of infectivity in 24 h showed that, although there was a reduction on the number when comparing control and laser groups, this difference was not significant. However, there was a statistically significant difference ($p < 0.05$) between the control and the photosensitizer groups, with a 33.4% reduction in the infectivity of the photosensitizer group. Significant differences ($p < 0.0007$) were also observed when comparing control and the ApPDT groups, with a 57.9% reduction in infectivity in the ApPDT group (Fig. 2a). The evaluation of infectivity in 48 h showed a difference between the control and the other experimental groups. The difference between the control and the ApPDT group was statistically significant ($p < 0.0032$), with a reduction in infectivity (41.6%) in the ApPDT group (Fig. 2b).

Table 3 Percentage distribution of infected and uninfected macrophages in the different experimental groups in the evaluation periods of 24 and 48 h

Time/percent	24 h		48 h	
	Infected	Not infected	Infected	Not infected
Control	76.8	23.2	54.7	45.3
Laser	68.8	31.2	45.7	54.3
Photosensitizer	51.0	49.0	43.0	57.0
ApPDT	32.2	67.8	32.0	68.0

The infection rate measures the number of parasites that infected macrophages. In this context, the results of this study show that, the control group presented the highest concentration of parasites among all the experimental groups. In addition, there was a significant difference ($p < 0.001$) between the control and the photosensitizer groups, with a 45.1% lower infection rate in the photosensitizer group. The control and the ApPDT group were also statistically significant different ($p < 0.0001$), being the infection rate 71% lower in the ApPDT group. Statistical difference between the photosensitizer and the ApPDT groups was also significant ($p < 0.01$), being the infection rate 48.7% lower in the ApPDT group (Fig. 3a). When evaluating the infection rate after 48 h of treatment, the data point to the existence of a significant difference in relation to the control in the same groups that presented differences in 24 h, the statistical difference between the control and the photosensitizer groups was significant ($p < 0.01$), being the infection rate 26.9% lower in the photosensitizer group. Comparing the control and the ApPDT group, statistically significant difference ($p < 0.0001$) seen and a 62.0% reduction in the infection rate in the ApPDT group was observed. Comparing the photosensitizer and the ApPDT groups, there was also statistically significant difference ($p < 0.001$), with a reduction of 47.9% in the ApPDT group (Fig. 3b).

Figure 4 shows cultures of infected macrophages in the control group and in the ApPDT group. In the control group, the

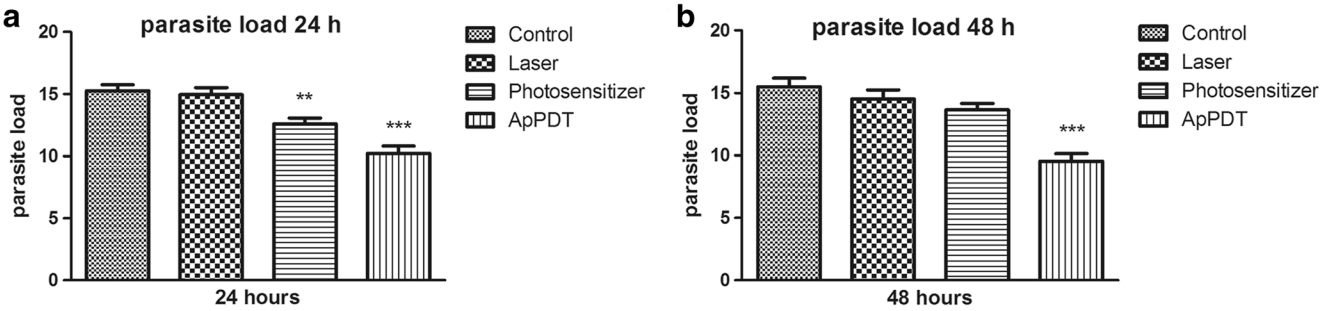

Fig. 1 Parasitic load of the experimental groups 24 h (**a**) and 48 h (**b**) after the protocol. **($p > 0.001$); ***($p > 0.0001$)

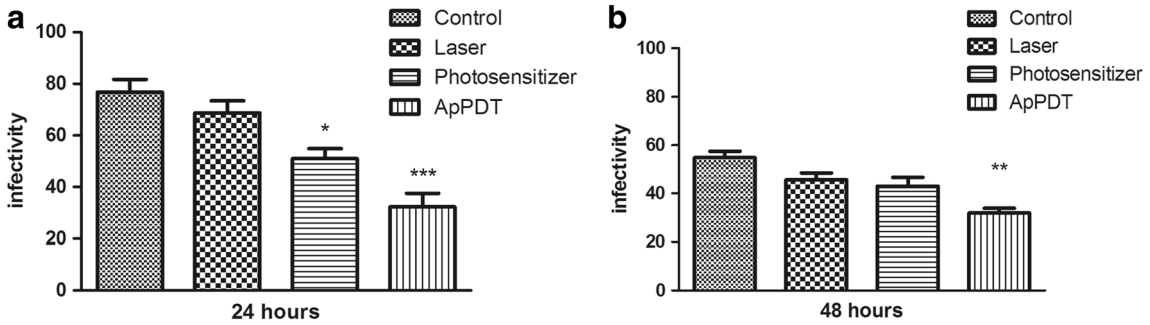

Fig. 2 Infectivity in different experimental groups 24 h. *($p < 0.05$), ***($p < 0.0007$) (**a**) and 48 h (**b**) after treatment **($p > 0.0032$)

macrophages present a greater number of intracellular parasites compared to the group treated with ApPDT.

Discussion

The present investigation is relevant as it reports the efficacy of using a new technique to treat cutaneous leishmaniosis. Besides being effective, its usage prevents the problems reported with the use of pentavalent antimonial drugs [4–7]. Our results are indicative that, ApPDT is a promising technique to be used in the treatment of cutaneous leishmaniosis as it possesses low toxicity, lower costs and is of easy adherence of patients.

Our study indicates advantages of using ApPDT over conventional treatments such as the use of pentavalent antimonial drugs such as selectivity and reduced of toxicity [10]; few adverse effects; high cure rates, and excellent esthetic results in affected areas [11]. It has also to be considered that ApPDT is non-invasive and can be repeated when necessary without damage to the patient's health and without causing resistance in the parasite [12].

The evaluation of the leishmanicidal effect of the protocol adopted on the present study for intracellular parasites was carried out at 24 and 48 h after infection. In this study, it was demonstrated that the number of intracellular parasite, in the ApPDT group, progressively decreased up to 48 h (38.4%) in a significant manner when compared to control group. Our results showed a significant decrease on the infectivity when using ApPDT in comparison to the control group. A single use of the protocol resulted in 57.9% reduction of infected macrophage within 24 h. After 48 h, the difference between ApPDT and control groups was statistically significant ($p < 0.0032$), with a reduction in infectivity of 41.6% when ApPDT was used. Therefore, this is indicative of the potentiating role of ApPDT using phenothiazine compounds. Consequently, optimizing the therapeutic selectivity in a way like the antiparasitic chemotherapy investigations carried out by other researchers [13].

In studies evaluating the treatment of infected macrophages, the reduction of their numbers may be associated with increased photosensitizer concentration. Thus, higher concentrations tend to increase the leishmanicidal effect [13]. Accordingly, since ApPDT potentiates the toxic effect of a compound on its target, it has been shown that the use of this therapy can be as effective as increasing the concentration of a given leishmanicidal compound. However, when ApPDT is used, there is no need to increase the concentration of the chemotherapeutic; therefore, adverse effects can be minimized.

The treatments with the phenothiazinic compound showed a lower infection rate in relation to the control. In the photosensitizer group, the infection rate in 24 h was that of 45.1% and at 48 h presented lower index of infection (26.9%), demonstrating that as time advances, there is a loss in the effectiveness of the photosensitizer. The ApPDT group was able to significantly reduce the number of intracellular amastigotes of the infected macrophages when compared to the control group, in both

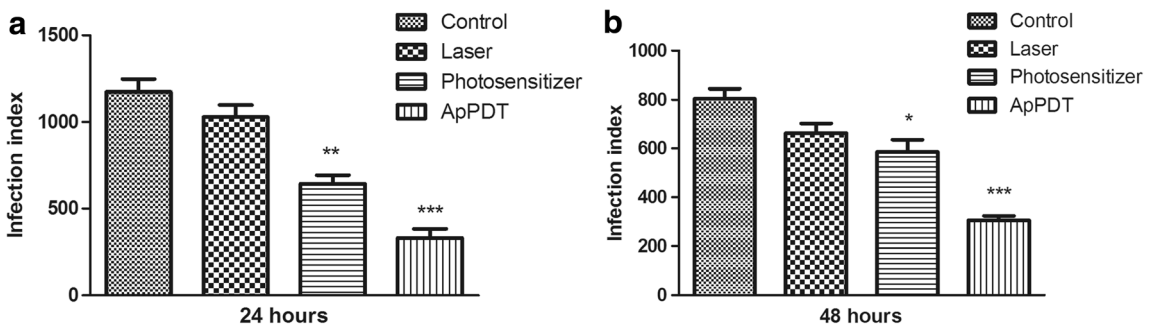

Fig. 3 Index of infection in the different experimental groups 24 h (**a**) and 48 h (**b**) after treatment. **($p < 0.001$); ***($p < 0.0001$)

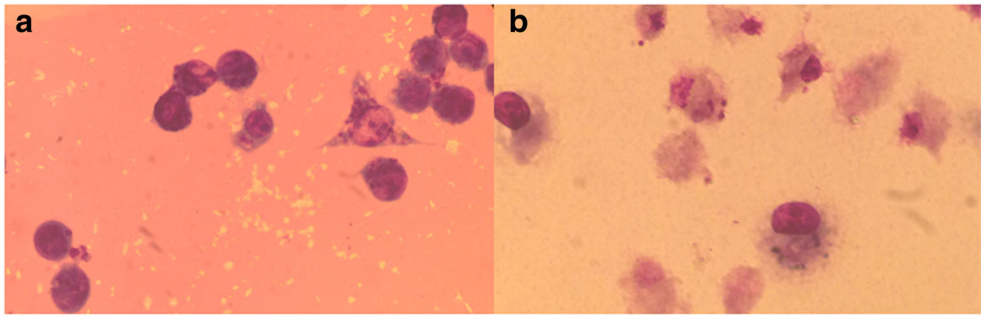

Fig. 4 Photomicrography of cultures of macrophages infected by *Leishmania* amastigotes. **a** Control. **b** Macrophages after ApPDT

24 h (71%) and 48 h (62.0%) periods. These results demonstrate that time does not interfere with the proposed therapy. Although the photosensitizer group demonstrated a reduction in the infection rate, time interfered with its effectiveness. The ApPDT group has also demonstrated a greater reduction in the infection rate when compared to the photosensitizer group, with 48.7% in 24 h and 47.9% in 48 h; however, it effectiveness was not influenced by the time. Therefore, the most effective protocol was of the ApPDT. Recent research has shown concern in new treatments for leishmaniosis pointing to promising results in those who are able to reduce the rates of infection such as the observed in present study [14].

The data from the present study demonstrate that, phototherapy alone does not significantly interfere with the infection rate when compared to the control group; therefore, it is not a therapeutic indication for the antiparasitic treatment of cutaneous leishmaniosis, although phototherapy can be used in tissue repair in cases of ulcers caused by infection with *Leishmania* [15]. On the other hand, light when associated with a photosensitizer compound exhibits antiparasitic photodynamic effect as reported in the literature and in the present investigation [16].

Phagocytic cells such as macrophages use reactive species such as NO and ROS to eliminate intracellular parasites, so one of the leishmanicidal pathways would be the increase of oxidative stress [17]. In this sense, the use of pro-oxidant antiparasitics could be an adopted pathway [18]. In this study, ApPDT was used as it is based on the production of superoxide in a determined target.

There are reports that ApPDT increases 'oxidative burst' in macrophages infected with microorganisms. Thus, ApPDT has an antimicrobial effect due to the production of ROS besides a possible stimulatory effect on infected macrophages [19, 20]. During the evaluation time on this study, a reduction in the number of intracellular parasites was observed in the ApPDT group, as well as the number of infected macrophages in relation to the other groups.

Conclusion

The results of the present investigation indicated that ApPDT reduced the number of macrophages infected by *Leishmania braziliensis*, as well as the number of intracellular parasites. The experimental protocol may be considered an alternative therapeutic in the treatment of leishmaniosis.

Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This in vitro study neither involves humans nor animals; Brazilian regulation do not require approval for this kind of study.

Informed consent As an in vitro study, there is no need for informed consent.

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