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Leishmanicidal activity of amphotericin B encapsulated in PLGA–DMSA nanoparticles to treat cutaneous leishmaniasis in C57BL/6 mice

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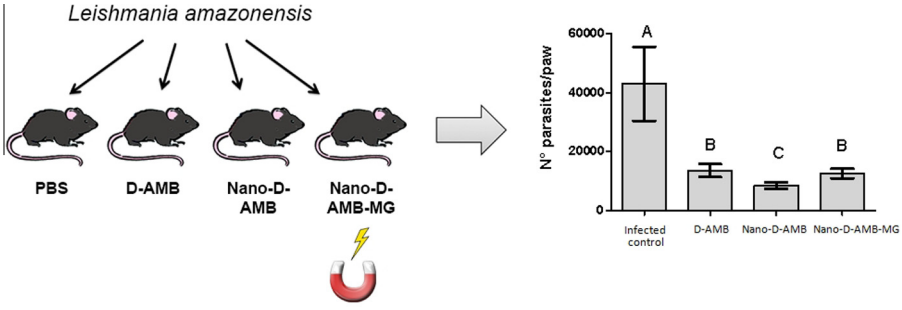
HIGHLIGHTS

- D-AMB and Nano-D-AMB had the same efficacy to reduce paw diameter of infected mice.
- Nano-D-AMB promoted a greater reduction in parasite number and cell viability.
- Nano-D-AMB favored a longer interval between drug administrations.

GRAPHICAL ABSTRACT



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ABSTRACT

The major goal of this work was to design a new nanoparticle drug delivery system for desoxycholate amphotericin B (D-AMB), based on controlled particle size, looking for the most successful release of the active agents in order to achieve the best site-specific action of the drug at the therapeutically optimal rate and dose regimen. For this, AMB nanoencapsulated in poly(lactic-co-glycolic acid) (PLGA) and dimercaptosuccinic acid (DMSA) nanoparticles (Nano-D-AMB) has been developed, and its efficacy was evaluated in the treatment of experimental cutaneous leishmaniasis in C57BL/6 mice, to test if our nano-drug delivery system could favor the reduction of the dose frequency required to achieve the same therapeutic level of free D-AMB, and so, an extended dosing interval. Magnetic citrate-coated maghemite nanoparticles were added to this nanosystem (Nano-D-AMB-MG) aiming to increase controlled release of AMB by magnetohyperthermia. Female mice ($N = 6$/group) were infected intradermally in the right footpad with promastigotes of *Leishmania amazonensis* in the metacyclic phase, receiving the following intraperitoneal treatments: 1% PBS for 10 consecutive days; D-AMB at 2 mg/kg/day for 10 days (totalizing 20 mg/kg/animal); Nano-D-AMB and Nano-D-AMB-MG at 6 mg/kg on the 1st, 4th and 7th days and at 2 mg/kg on the 10th day, also totalizing 20 mg/kg/animal by treatment end. The Nano-D-AMB-MG group was submitted to an AC magnetic field, allowing the induction of magnetohyperthermia. The evaluations were through paw diameter measurements; parasite number and cell viability were investigated by limiting dilution assay. D-AMB-coated PLGA–DMSA nanoparticles showed the same efficacy as free D-AMB to reduce paw diameter; however, the Nano-D-AMB treatment also promoted a significantly greater reduction in parasite number and cell viability compared with free D-AMB. The nano-drug AMB delivery system

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appeared more effective than free D-AMB therapy to reduce the dose frequency required to achieve the same therapeutic level. It thus favors a longer interval between doses, as expected with development of a new nano drug delivery system, and may be useful in the treatment of many different pathologies, from cancer to neurodegenerative diseases.

1. Introduction

Leishmaniases comprise a complex group of non-contagious infectious vector-borne diseases caused by protozoa of the *Leishmania* genus, which affect more than 12 million people in 88 countries worldwide. Although its clinical forms are particularly diverse, the disease can be classified in three main forms: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), and visceral leishmaniasis (VL) (de Souza et al., 2010; Desjeux, 2004; Launois et al., 2008; Murray et al., 2005; Wang et al., 2011; Zauli-Nascimento et al., 2010). VL is typically caused by the *Leishmania donovani* complex, which includes the species *L. donovani*, *Leishmania infantum* and *Leishmania chagasi* (Sundar and Rai, 2002; Wang et al., 2011), whereas CL and MCL forms are caused by several *Leishmania* species, including *Leishmania major*, *Leishmania tropica*, *Leishmania amazonensis* (CL) and *Leishmania braziliensis* (CL and MCL) (Minodier and Parola, 2007; Souza et al., 2011; Wang et al., 2011).

The causative species of CL determines the clinical features and courses, and the treatments (Minodier and Parola, 2007). This disease presents a broad spectrum of clinical features: it may be limited to a single part of the skin (localized cutaneous leishmaniasis) or may produce a large number of lesions (diffuse cutaneous leishmaniasis), causing severe skin lesions and, exceptionally, leading to fatal systemic infection (de Souza et al., 2010; Minodier and Parola, 2007; Nogueira and Sampaio, 2001; Silveira et al., 2009). *L. amazonensis* is clinically important in the New World, where it can cause localized CL or diffuse CL, especially in immuno-compromised hosts (de Souza et al., 2010; Zauli-Nascimento et al., 2010).

Systemic antimonials are generally required for the treatment of CL in the New World because of the risk of mucosal involvement (Minodier and Parola, 2007). This current chemotherapy has a series of limitations such as: high cost, intravenous administration and high toxicity, associated with many other undesirable side effects. The antibiotic amphotericin B (AMB), a second-choice drug, has showed good clinical results, although its use in CL treatment requires more extensive studies. However, its effectiveness is also limited and sometimes causes significant hypersensitivity reactions, nephrotoxicity, hepatotoxicity, cardiotoxicity, and other adverse effects (Launois et al., 2008; Minodier and Parola, 2007; Vyas and Gupta, 2006). Thus, the development of alternative therapies is a priority for the treatment of this infection.

The design of nano drug delivery systems for conventional drugs represents one of the most promising antimicrobial therapies due to its higher therapeutic efficacy, low toxicity, higher target delivery effect, and prolonged systemic circulation lifetime, releasing drugs in a sustained and controlled manner (Zhang et al., 2007, 2010). Moreover, because nanoformulations as a drug delivery system improve bioavailability, the protection of drugs incorporated from metabolism is a favorable feature of nanosystems, allowing prolonged drug residence in the human body, and therefore prolonging time between administrations (das Neves et al., 2010). Thus, the major goal of this work was to design a new nanoparticle drug delivery system for desoxycholate amphotericin B (D-AMB), based on controlled particle size, looking for the most successful release of the active agents in order to achieve the best site-specific action of the drug at the therapeutically optimal rate and dose regimen. For this, AMB nanoencapsulated in poly(lactic-co-glycolic acid) (PLGA) and dimercaptosuccinic acid (DMSA) nanoparticles (Nano-D-AMB) has been developed, and its efficacy was evaluated in the treatment of experimental cutaneous leishmaniasis in C57BL/6 mice, to test if our nano-drug delivery system could favor the reduction of the dose frequency required to achieve the same therapeutic level of free D-AMB, and so, an extended dosing interval. Because hyperthermia based on magnetic nanoparticles results in controlled release of the drug (Kumar and Mohammad, 2011), magnetic citrate-coated maghemite nanoparticles were added in this nanosystem (Nano-D-AMB-MG), in an attempt to increase the controlled release of AMB by magnetohyperthermia.

2. Material and methods

2.1. Syntheses of nanostructured D-AMB samples

The polylactic acid (PLA), polyglycolic acid (PGA), DMSA and the D-AMB used to prepare Nano-D-AMB were purchased from Sigma (St Louis, MO, USA). The sample of Nano-D-AMB was prepared according to Amaral et al. (2009), with slight modification. The polymers (50 mg of PLA and 50 mg of PGA) were first dissolved in 10 ml of dichloromethane. This organic solution received the addition of 120 mg of D-AMB and 0.05 M DMSA as an additive. To another solution of 40 mL of a phosphate buffer saline solution (PBS) containing polyvinyl alcohol (PVA) 1%, was added the initial organic solution of PLA-PGA with vigorous agitation in a blender operating ultra turrax system (10,000 rpm) to obtain the initial water–in-oil emulsification. The organic solvent was removed from the solution by stirring at room temperature and evaporation under reduced pressure. The nanoparticles were centrifuged (25 °C, 5000 rpm) in intervals of 10 min. The preparation was washed three times in distilled water, suspended in 1.0 mL physiological PBS solution, and stored at 4 °C. All procedures were developed in a sterile room with all the manipulation in sterile wood. The stability of the suspension was analyzed over time and maintained for 3 weeks. The process was protected by a patent deposited in the INPI (National Institute of Intellectual Property, Brazil), PI # 0700446-0.

Magnetic D-AMB polymer sample (Nano-D-AMB-MG) was developed following the same protocol described above. The magnetic fluid sample based on citrate-coated maghemite nanoparticles was synthesized and characterized as previously described (da Silva et al., 2003), with maghemite nanoparticles being obtained via oxidation of magnetite nanoparticles by co-precipitation of Fe (II) and Fe (III) ions in alkaline medium (Soler et al., 2004). The precise volume of the stock solution of citrate-coated maghemite nanoparticles at 2.3×10^{16} particles/mL was added to the initial phosphate buffer saline solution (PBS) containing polyvinyl alcohol (PVA 1%). To this solution was finally added the organic phase containing D-AMB and the DMSA additive with vigorous agitation in a blender operating ultra turrax system (10,000 rpm) to obtain the initial water–in-oil emulsification. The final isolation of the Magnetic D-AMB sample was identical to the procedure described above for the non-magnetic one, rendering a stabilized fluid with 7.8 ± 2.6 nm average-diameter maghemite nanoparticles coated with citrate, at the concentration of 3.4×10^{13} particles/mL, used in the experiments.

2.2. Characterization of nanostructured D-AMB samples by electron microscopy and Zetasizer

To determine the diameter of nanoparticles, samples were diluted in distilled water at a ratio of 1:500 and placed on two screens (2 μL) for transmission electron microscopy (TEM) previously coated with Formvar. After drying for 2 h at room temperature (25 ± 2 °C) screens were analyzed in a Jeol JEM-1010 electron microscope and photomicrographed by an UltraScan® with Digital Micrograph 3.6.5 software (Gatan/USA). Nanoparticle diameters ($N = 300$) were measured using the Image Pro Plus 6.0 software. For the JEOL 840A scanning electron microscope (SEM) analysis, samples (5 μL) were dripped in spots previously cleaned with a double-sided adhesive tape carbon containing a thin layer of fixed mica, and allowed to dry at room temperature (25 ± 2 °C) without pretreatment for 24 h, and coated in gold.

The hydrodynamic average diameter of the samples was determined by dynamic light scattering (DLS), while the surface charge of the nanoparticles was evaluated by measuring the zeta potential on the equipment Zetasizer (Zetasizer Nano-ZS90 Malvern Instruments Limited, Malvern, UK). For this, approximately 1 mL of the diluted solution in the ratio 1:20 in distilled water was used; analyses were performed in triplicate at 25 °C, with a fixed detection angle of 173°. The pH was measured using a pH indicator strip.

2.3. Leishmania Strain

Leishmania (*Leishmania*) *amazonensis* promastigotes forms (MHOM/BR/PH8) obtained from the World Health Organization (WHO) and identified by the techniques of isozymes and monoclonal antibodies were maintained in cultures and cryopreserved in liquid nitrogen (−196 °C) in the Laboratory of Dermatology, Faculty of Medicine, University of Brasilia, Brazil. These cultures were regularly inoculated in hamsters to ensure and guarantee the infectivity of virulence of the inocula. Following the appearance of lesions, parasites were collected with a 1 mL syringe containing 0.2–0.5 mL of saline, and the aspirate was transferred to the NNN medium for one week. Subsequent to this period, the culture was transferred to bottles for cultivation of 60 mL (growth area of 25 cm^2) containing RPMI 1640 medium (Gibco®) added to 50 mg/mL gentamicin and 20% inactivated fetal bovine serum (FBS), at final pH of 7.2.

As in in vitro culture media, metacyclogenesis occurs at the end of the logarithmic phase – early stationary phase – so that this culture is enriched with metacyclic forms (end of replicative cycle) (Ramos et al., 2011), from inocula of 10^7 promastigotes/mL growth curves were performed in triplicate at 24-h intervals for seven consecutive days in a Neubauer chamber, to quantify the number of parasites at the end of the logarithmic phase/early stationary phase (enriched with metacyclic forms), and to calculate the correspondent volume of the culture medium to be inoculated in the animals.

2.4. Animals and experimental design

Female C57BL/6 mice, 10 weeks old, weighing 30.6 ± 2.9 g and purchased from the animal facility of the University of Brasilia (Brazil), were housed in polypropylene cages (6/cage) at room temperature (20 ± 2 °C) under a 12 h light/dark cycle with lights on at 6 a.m. and free access to food and water. Animals were infected intradermally in the right footpad with a volume of 100 μL of RPMI 1640 medium containing 3.57×10^6 promastigotes of *Leishmania* (*Leishmania*) *amazonensis* in the metacyclic phase. The infection was confirmed by cultures and smears of lymph taken from the inoculated paws sixty days after inoculation. The infected animals were divided into four groups to be treated intraperitoneally, as follows: 1% PBS for ten consecutive days; D-AMB at 2 mg/kg/day for 10 days (totalizing 20 mg/kg/animal); Nano-D-AMB and Nano-D-AMB-MG (3.4×10^{13} particles/mL) at 6 mg/kg on the 1st, 4th and 7th days and at 2 mg/kg on the 10th day, also totalizing 20 mg/kg/animal at the end of the treatment. The Nano-D-AMB-MG group was submitted to an alternating current (AC) magnetic field (40 Oe amplitude AC magnetic field oscillating at 1 MHz) to produce magnetohyperthermia. The lesions were exposed for ten minutes, according to the protocol described by Portilho et al. (2011). A fifth group of uninfected and non-treated animals (group without infection) was kept as negative control. The choice of the doses used in the treatments was based on Amaral et al. (2009). The animals were previously anesthetized by an intraperitoneal administration of ketamine (Cetamin®, Syntec, 80 mg/kg) and xylazine (Calmiun®, Agener União, 10 mg/kg) solution.

All procedures were reviewed and approved by the institutional Ethics Committee for Animal Research (UnBDOC number 62393/2010).

2.5. Treatment evaluations

The diameters of the frontal and sagittal sections of the infected right planar footpad of each mouse were measured, using a precision digital pachymeter, as follows: before the infection, before starting treatment, 24 h and 10 days after the treatment. The evaluation criteria were based on the measurement of infiltration of the inoculated paw and counting parasites in cultures. After this period, animals were euthanized in a carbon dioxide chamber. Paws underwent aseptic dissection, which consists of crushing them in 1.5 ml RPMI supplemented with 20% FBS and 0.2% gentamicin sulfate, and the limiting dilution test was carried out in duplicate to estimate the parasitological growth and the cytotoxicity of the treatments, following the protocol proposed by Costa Filho et al. (2008).

2.6. Statistical Analysis

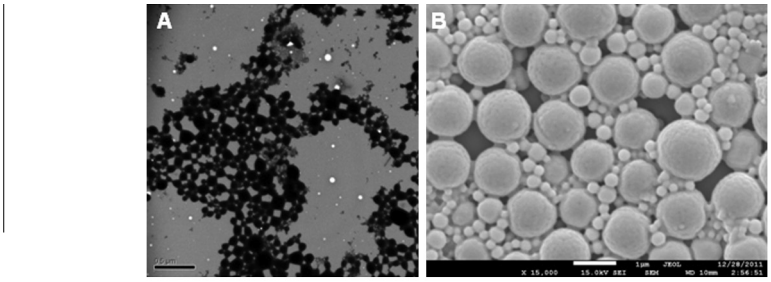
Statistical analysis was carried out using SPSS (Statistical Package for the Social Sciences) version 17.0. The continuous variables were tested for normal distribution with Shapiro–Wilk. For the paw diameters, differences between the treatment groups were checked by one-way ANOVA followed by the Tukey post hoc test, while the repeated measures analysis of variance (ANOVA) followed by the Tukey test were used to investigate changes in mean scores over the four used time points (before the infection, before starting treatment, 24 h and 10 days after the treatment). Values of $p < 0.05$ were considered statistically significant.

For the limiting dilution test (parasitological test), data were obtained by counting the number of wells in which promastigotes of *Leishmania* presented growth, when observed under an inverted light microscope. The average values obtained for the treatment groups in the cell viability test were calculated using the Elida® program.

3. Results

3.1. Nanostructured D-AMB samples

The Zeta potential of both Nano-D-AMB samples showed a negative superficial charge, in the range that allowed the stability of the formulation in the nanometric size scale to be confirmed, with a reduced aggregation process that could compromise the biodistribution of the active material, and its interaction with the biological target. The observed distribution in size while associated with low polydispersity index represents normal behavior for this kind



Sample	TEM diameter (nm)	Hydrodynamic diameter (nm)	PDI	Zeta Potential (mV)	pH
Nano-D-AMB	365.34 ± 70.86	456.60 ± 133.60	0.431 ± 0.000	-32.60 ± 4.64	7.2 ± 2.0
Nano-D-AMB-MG	608.20 ± 87.72	675.20 ± 40.28	0.584 ± 0.000	-28.00 ± 4.86	7.2 ± 2.0

Fig. 1. Physicochemical characterization of the Nano-D-AMB suspension by transmission electron micrography (TEM), Scanning electron photomicrography (SEM) and Zetasizer. (A) Transmission electron micrography (TEM); (B) Scanning electron photomicrography (SEM); PDI = polydispersity index; hydrodynamic diameter = diameter of the samples determined by dynamic light scattering (DLS). Table data are expressed as mean ± SD (standard deviation) and summarize results of nanostructured D-AMB samples analyses.

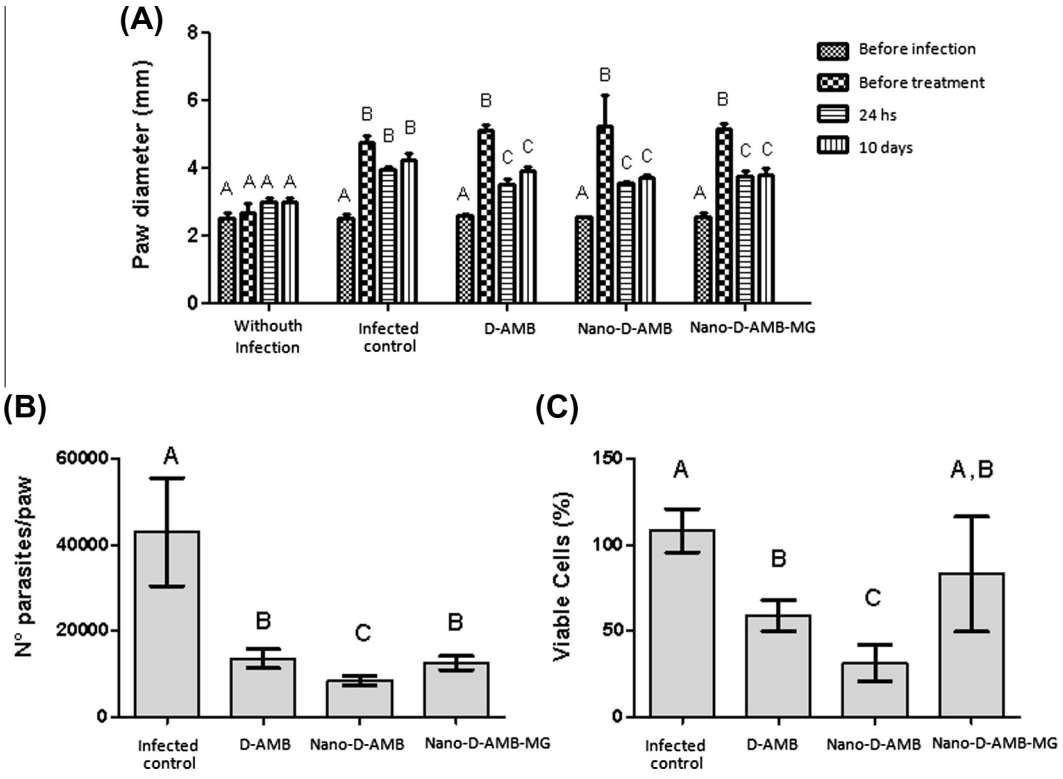


Fig. 2. Distribution of the paw diameter (mm) of the mice according to duration of treatment (A), number of parasites per paw (B), and percentage of viable cells (C). Without infection = negative control group; Leishmania = infected animals treated with% PBS; D-AMB = infected animals treated with free D-AMB; Nano-D-AMB = infected animals treated with Nano-D-AMB; Nano-D-AMB-MG = infected animals treated with Nano-D-AMB-MG and AC magnetic field to produce magnetohyperthermia. Different letters indicate significant differences ($p < 0.05$) detected by the Tukey post hoc test, where comparisons were made between the treatment groups (A and B) and also within the groups: before infection, before treatment, 24 h and 10 days (A). Bar graphs were expressed as standard deviation.

of complex drug delivery system. Results of TEM and SEM of Nano-D-AMB showed adequate size and diameter distributions for the nanomaterial, in agreement with the parameters measured by the Zetasizer. For Nano-D-AMB-MG the presence of magnetic nanoparticles led to an increase in size in respect to Nano-D-AMB, due to particle aggregation by the effect of the magnetic fluid (particle grows) (Fig. 1).

3.2. Treatment evaluations

Untreated animals (“infected control” group) showed the same paw diameter throughout the experimental time after infection. All the D-AMB treatments significantly reduced the paw diameter, but on average this reduction did not allow paw diameter to return to what it was before infection. The reduction in the paw lesions

remained stable throughout the treatment period; i.e., independently of treatment group, there were no significant differences in the paw diameters among the groups at each one of the four used time points (before infection, before treatment, 24 h and 10 days after the treatment) (Fig. 2A). There were no nodules or metastases to other areas of the skin.

All the D-AMB treatments significantly reduced the number of parasites on the paw, and this reduction was higher for the treatment with Nano-D-AMB (Fig. 2B). AMB-related treatments also decreased about 50% cell viability when compared to the Leishmania group, and the reduction was significantly higher for the Nano-D-AMB treatment in particular (Fig. 2C).

4. Discussion

Leishmania amastigotes live inside resident macrophages in different anatomic sites, and their concealed location is responsible for impairing the access of conventional therapeutic drugs, which are poorly selective, or must be administered in repeated and high doses by parenteral routes (Chang et al., 2003; Romero and Morilla, 2008). Thus, current drugs for the treatment of leishmaniasis are far from satisfactory and no effective vaccine against the disease in humans is available (Launois et al., 2008; Okwor and Uzonna, 2009).

Desoxycholate amphotericin B (Fungizome®), a polyene antibiotic obtained from a strain of *Streptomyces nodosus*, was the pioneered intravenous AMB formulation (Patel and Patravale, 2011; Vyas and Gupta, 2006). Despite its wide spectrum of activity, encompassing a large number of fungi, *Leishmania* and *Naegleria* (amoeba) species (Barratt and Bretagne, 2007), its usefulness is limited due to its poor solubility in aqueous solution and its severe nephrotoxicity, which may lead to kidney failure (Barratt and Bretagne, 2007; Jung et al., 2009; Patel and Patravale, 2011; Vyas and Gupta, 2006).

Because current therapy is costly, often poorly tolerated and not always efficacious, the development of alternative therapies is a priority in combating *Leishmania* infection (Launois et al., 2008). To this end, AMB was one of the first therapeutic agents to be marketed commercially as nanosized formulations in which the drug is associated with lipids as liposomes or lipid complexes (e.g. AmBisome®, Amphocil®, Abelcet®) (Barratt and Bretagne, 2007; Minodier and Parola, 2007; Patel and Patravale, 2011). Although these liposome formulations were found to be more effective and less toxic than Fungizome®, contributing to shorten the course of therapy, the costs of these formulations were found to be exorbitant, restricting their widespread use (Patel and Patravale, 2011). Thus, AMB has also been conjugated to a number of macromolecules in some newer formulations, with the aim of improving its solubility; many of these have been derived from polysaccharides. However, there have been a few reports of nanoparticulated forms of AMB (Barratt and Bretagne, 2007; Jung et al., 2009) and, although PLGA is one of the most widely employed biocompatible and biodegradable polymers utilized in the preparation of nanoparticles (Gaudana et al., 2011), its use in nanosphere formulation of AMB has been less effective in reducing the parasite load than albumin microspheres, which also induced a significant antibody response to *Leishmania* antigens (Barratt and Bretagne, 2007). In this respect, results demonstrated that our new formulation using AMB nanoencapsulated in PLGA–DMSA nanoparticles appears to have improved the efficacy of PLGA nanoparticulate AMB. This is because, besides Nano-D-AMB having shown itself to be effective in reducing *Leishmania* parasites at the lesion site and inducing a greater decrease in cell viability, in a previous study it also showed antifungal efficacy and a favorably extended dosing interval, with fewer undesirable effects (Amaral et al., 2009). This latter finding allowed us to compare, in this preliminary study, only the leishmanicidal activities of free D-AMB with our nanoencapsulated formulation.

It is already known that many infectious agents are sensitive to heat, and so fever acts as a defense mechanism against a number of processes, mostly related to other infections (Loustaunau, 2011; The Lancet, 2013). Based on these facts, we proposed to associate magnetic nanoparticles with these new nano-AMBs; therefore, a better therapeutic response to the Nano-D-AMB-MG treatment could be expected, since hyperthermia based on magnetic nanoparticles could also result in controlled release of the drug (Kumar and Mohammad, 2011; Shao et al., 2011), besides its additive effect in eliminating the infectious agent by raising the temperature in the affected region (Castro et al., 2010). However, this did not take place as expected, possibly due to the aggregation effect associated with Nano-D-AMB-MG, which showed a large increase in the size distribution. Although it still remained on the nanosized scale, the smaller formulations are more effective, just because of their small size (Barratt and Bretagne, 2007). The observed aggregation effect could be associated with lower target delivery, and thus may be responsible for the cancellation of the effect of the AMB. Additionally, mice were exposed for only 10 min to AC magnetic field to produce magnetohyperthermia, and perhaps this was not enough for the intraperitoneally administered magnetic nanoparticles to reach the lesion in the right conditions. These aspects should be further investigated.

It is believed that nanostructured drugs are more effective than traditional therapies in directly eliminating pathogenic microorganisms without triggering the release of chemical mediators (Suri et al., 2007). In our study, Nano-D-AMB did not improve the performance of the free drug (D-AMB) in reducing the diameter of the infected mouse paw, but its use appeared to influence the viability and the number of infective cells, even applied at longer time intervals. Moreover, the same final concentration as used for free D-AMB was applied at greater time intervals and there were no significant differences between findings at 24 h and 10 days after treatments, supporting the suggestion that this treatment favors a longer interval between drug administrations. Also, still considering the equal final total concentrations of free D-AMB and Nano-D-AMB, results suggest that Nano-D-AMB nanoparticles were more effective than free D-AMB therapy in reducing the dose frequency required to achieve the same therapeutic level. They also promoted a significantly higher reduction in parasites and decreased cell viability than free AD-MB.

In conclusion, D-AMB-coated PLGA–DMSA nanoparticles showed the same efficacy as free D-AMB in reducing the paw diameter. Furthermore, the Nano-D-AMB treatment promoted significantly greater reductions in the number of parasites and in cell viability than free D-AMB. This suggests that these nanoparticles were more effective than free D-AMB therapy, allowing the dose frequency required to achieve the same therapeutic level to be reduced, and thus favoring an extended dosing interval.

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