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Poloxamer 407 (Pluronic® F127)-based polymeric micelles for amphotericin B: *In vitro* biological activity, toxicity and *in vivo* therapeutic efficacy against murine tegumentary leishmaniasis

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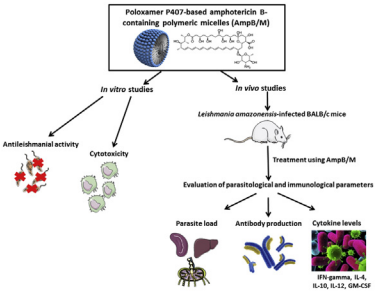
HIGHLIGHTS

- Poloxamer 407-based amphotericin B-containing micelles (AmpB/M) were developed.
- AmpB/M showed satisfactory anti-leishmanial activity and selectivity index.
- AmpB/M was also shown to be effective in treating *Leishmania amazonensis*-infected BALB/c mice.

GRAPHICAL ABSTRACT



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ABSTRACT

In the present study, a Poloxamer 407-based amphotericin B (AmpB)-containing polymeric micelles system (AmpB/M) was employed in the treatment of *Leishmania amazonensis*-infected BALB/c mice. Initially, the *in vitro* antileishmanial activity (IC_{50} value) of AmpB/M and B-AmpB/M (empty micelles) against stationary promastigotes and amastigotes-like forms of the parasites was determined, and results were of 1.83 ± 0.4 and 22.1 ± 0.7 μM, respectively, for the promastigotes, and of 2.27 ± 0.5 and 33.98 ± 2.6 μM, respectively, for the amastigotes-like. The cytotoxic concentration (CC_{50}) values of these products were also evaluated, and we found the results of 119.5 ± 9.6 and 134.7 ± 10.3 μM, respectively. With these values, the selectivity index (SI) was calculated and results were of 65.3 and 5.4, respectively,

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for the promastigotes, and of 59.3 and 3.96, respectively, for the amastigotes-like of the parasites. Free AmpB showed IC_{50} values of 1.2 ± 0.3 and 2.5 ± 0.5 μM for the promastigotes and amastigotes-like, respectively, whereas the CC_{50} value was of 9.5 ± 0.4 μM. The SI values of this drug were of 7.9 and 3.8, respectively, for the promastigote and amastigote-like stages of the parasites. After, animals were infected and received saline or were treated subcutaneously with free AmpB, AmpB/M or B-AmpB/M. In the results, free AmpB-treated and infected mice showed reductions in their body weight, which were associated with hepatic and renal damage; however, no organic alteration was observed in the AmpB/M-treated animals. In addition, these animals showed significant reductions in their lesion average size and in the parasite burden in all evaluated infected tissue and organs, when compared to the other groups; as well as significantly higher levels of antileishmanial IFN-γ, IL-12, GM-CSF and nitrite, which were associated with low production of IL-4, IL-10 and IgG1 isotype antibodies. In conclusion, this AmpB/M system could be considered as an alternative for future studies in the treatment of tegumentary leishmaniasis.

1. Introduction

Leishmaniasis affects about 12 million people in 98 countries worldwide (Alvar et al., 2012). The severity of the disease depends primarily of the parasites infective species and of the immune response generated by the infected mammalian hosts (Kaye and Scott, 2011). Tegumentary leishmaniasis (TL) is the most common form of leishmaniasis, presenting an incidence of about 1.2–1.5 million new cases each year (WHO, 2010). This disease is endemic in more than 70 countries, where 90% of the cases have been registered in Afghanistan, Algeria, Brazil, Pakistan, Peru, Saudi Arabia and Syria (Desjeux, 2004). The disease exhibits distinct clinical manifestations such as cutaneous leishmaniasis (CL), diffuse cutaneous leishmaniasis (DCL) and mucosal leishmaniasis (ML) (Grimaldi and Tesh, 1993; Reithinger et al., 2007). In Brazil, TL is caused mainly by infection with *Leishmania (Viannia) braziliensis, Leishmania (V.) guyanensis* and *Leishmania (Leishmania) amazonensis* species (Marzochi and Marzochi, 1994; Silveira et al., 2004). *Leishmania amazonensis* presents particular importance, since it is able to cause a broad spectrum of clinical manifestations in the infected hosts, ranging from cutaneous to visceral leishmaniasis (Barral et al., 1991; Garcez et al., 2002).

The current treatment for leishmaniasis is still based on the administration of pentavalent antimonials; however, their use presents problems such as the requirement of intramuscular or intravenous injections, as well as the occurrence of side effects such as anorexia, myalgia, arthralgia, pancreatitis, leukopenia, besides of renal, hepatic and cardiac disorders in the patients (Vyas and Gupta, 2006; Minodier and Parola, 2007). Pentamidine, miltefosine and paramomycin have been applied as alternatives drugs, but their use is also limited due to the high toxicity (Chawla and Madhubala, 2010). Since the drug discovery is a long and very costly process, requiring an investment of more than $1.0 billion to identify, characterize and develop new pharmaceuticals (Hughes et al., 2011), the research for new delivery systems to carry out conventional drugs, with the purpose to reduce their toxicity, but without losing their biological activity, could be considered relevant (Neves et al., 2010; Zhang et al., 2007, 2010; Carvalho et al., 2013; Ribeiro et al., 2014).

Amphotericin B (AmpB) has been shown to present an effective *in vitro* antileishmanial activity against different *Leishmania* species, such as *Leishmania infantum, Leishmania braziliensis* and *L. amazonensis* (Ordóñez-Gutiérrez et al., 2007; Chávez-Fumagalli et al., 2015; Cunha et al., 2015; Duarte et al., 2016). However, its clinical use is also hampered due to the toxicity in the patients (Croft and Coombs, 2003; Vyas and Gupta, 2006). As a consequence, lipid-based formulations carrying AmpB have been formulated with the purpose of reducing the toxicity of this drug (Bern et al., 2006; Rosenthal et al., 2009; Ribeiro et al., 2014). The development of these formulations made it possible to solve this problem, and the World Health Organization has recommended the use of liposomal AmpB for the treatment of visceral leishmaniasis (VL) (WHO, 2010). However, the main restrictions against the widespread use of these formulations have been based on their intravenous application and high cost (Chávez-Fumagalli et al., 2015).

In this context, the search for obtaining new delivery systems that can be used for the treatment of leishmaniasis continues. In the present study, a Poloxamer P407 (Pluronic® F127)-based AmpB-containing polymeric micelles system (namely AmpB/M) was developed and evaluated *in vitro* and *in vivo* against *L. amazonensis*. Firstly, the *in vitro* antileishmanial activity of the free AmpB (as a drug control), AmpB/M and B-AmpB/M (as a delivery control) was evaluated against stationary-phase promastigote and amastigote-like stages of the parasites, aiming to establish their *Leishmania* minimum inhibitory concentrations (IC_{50}), as well as their cytotoxic effects on murine macrophages (CC_{50}). Secondly, the formed compounds were employed in the treatment of TL, by evaluating their therapeutic efficacy to treat *L. amazonensis*-infected BALB/c mice. Experiments were developed to evaluate the parasite load in the infected tissue and organs of the treated and infected animals, as well as to investigate the immune response generated after the treatments performed, by investigating the cytokine production, the humoral response and the nitrite secretion in these animals.

2. Materials and methods

2.1. Mice

Female BALB/c mice (8 weeks age) were obtained from the breeding facilities of the Department of Biochemistry and Immunology, Institute of Biological Sciences, Federal University of Minas Gerais (UFMG), Belo Horizonte, Minas Gerais, Brazil. Animals were maintained under pathogen-free conditions. The study was performed in compliance with the National Guidelines of the Institutional Animal Care and approved by Committee for the Ethical Handling of Research Animals (CEUA) from UFMG (protocol number 182/2012).

2.2. Parasites

L. amazonensis (IFLA/BR/1967/PH-8) strain was used. Parasites were grown in complete Schneider's medium (Sigma, St. Louis, MO, USA), which was composed by Schneider' s medium supplemented with 20% heat-inactivated fetal bovine serum (FBS, Sigma) and

20 mM L-glutamine, pH 7.4, at 24 °C. The soluble *Leishmania* antigenic (SLA) extract was prepared from stationary-phase promastigotes, as described (Coelho et al., 2003). The protein concentration was estimated by the Bradford method (Bradford, 1976). The amastigote-like forms were obtained following a technical protocol (Doyle et al., 1991), with few modifications. Briefly, 1×10^{10} stationary-phase promastigotes of *L. amazonensis* were washed in sterile phosphate saline buffer (PBS 1×). Then, parasites were incubated in 5 mL of FBS for 48 h at 37 °C. After, they were washed two times in sterile PBS 1× and visualized in an optical light microscopy. The cellular density was estimated by counting in a Newbauer chamber and their morphology was evaluated after staining by Giemsa (Valadares et al., 2011).

2.3. Formulation of polymeric micelles

Poloxamer 407 (Kolliphor® P407, catalog 62,035) composed by 71.5–74.9% of oxyethylene, and AmpB from *Streptomyces* sp. (catalog A2421) presenting 80% of purity, both purchased from Sigma-Aldrich (St. Louis, MO, USA), were used. The AmpB-containing polymeric micelles were prepared as described (Barichello et al., 1999). Briefly, Poloxamer P407 (18% w/w) was added to a phosphate saline buffer pH 7.4, under moderate magnetic agitation and in ice bath. To ensure the complete dissolution of the polymer, the solution was kept at 4 °C in incubation for a period of 18 h. Then, AmpB (1 mg/mL) was added to a microtube containing 500 μL of dichloromethane and solubilized using vortex. The solution was immediately added to the Poloxamer P407 under vigorous magnetic agitation and in an ice bath, until a viscous emulsion had been obtained. The dichloromethane was completely evaporated under vacuum by using a conventional rotary evaporation method (Buchi, Flawil, Switzerland). The final weight of the formulation was set up with purified water to obtain the final concentration of 18% w/w (weight/weight) of Poloxamer P407 and 1 mg of AmpB, per gram of formulation. The final composition was a transparent yellow gel at room temperature, which becomes a transparent yellow liquid at temperatures around 15 °C. The AmpB content present in the micellar solution was evaluated spectrophotometrically by using an ultraviolet method, as described (Filippin and Souza, 2008). Briefly, samples of the AmpB/M solution were collected and diluted in methanol PA. Absorbances were measured in a UV/Vis spectrophotometer (Double beam AJX-6100 PC; Micronal, São Paulo, Brazil), at a wavelength of 380 nm. The concentration of AmpB was calculated using a standard curve (0–18 μM), which was previously prepared in methanol PA. The analyses were carried out using three replicates. Empty micelles (18% w/w) were prepared using the same technical protocol described above, but without adding AmpB. All products were maintained at room temperature, until their use. Free AmpB was resuspended in 2 mL (per 1 mg of drug) of a methanol/DMSO (9:1 v/v) solution, and it was maintained at −80 °C until use. This preparation was named stock solution and it was diluted in saline before subcutaneous injection into the animals.

2.4. Antileishmanial activity and cytotoxicity

The inhibition of 50% of the *Leishmania* growth was assessed *in vitro* by cultivating stationary-phase promastigotes or amastigotes-like of *L. amazonensis* (5×10^6 cells per well) in the presence of individual concentrations of AmpB/M, B-AmpB/M or free AmpB (108.2, 54.1, 27.1, 13.5, 6.8, 3.4, 1.7 and 0.8 μM) in 96-well culture plates (Nunc, Nunclon®, Roskilde, Denmark), for 48 h at 24 °C. Cell viability was assessed by measuring the cleavage of 2 mg per mL of MTT [3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide] (Sigma). Absorbances were measured by using a multi-well scanning spectrophotometer (Molecular Devices, Spectra Max Plus, Canada), at 570 nm. The concentration needed to inhibit 50% of *Leishmania* viability (IC_{50}) was determined by applying the sigmoidal regression of a concentration-response curve using the tested concentrations. As a cytotoxicity parameter, the inhibition of 50% of the macrophages viability (CC_{50}) was evaluated. For this, murine macrophages were collected from the peritoneal cavities of BALB/c mice elicited with 3 mL of thioglycolate, which was administered five days before the experiments. Then, cells (5×10^5 per well) were incubated with AmpB/M, B-AmpB/M or free AmpB (108.2, 54.1, 27.1, 13.5, 6.8, 3.4, 1.7 and 0.8 μM) in 96-well plates, for 48 h at 37 °C in 5% CO_2. Finally, the cell viability was assessed also by the MTT assay and the CC_{50} values were calculated by applying the sigmoidal regression of a concentration-response curve using the tested concentrations. In addition, the selectivity index (SI) of the compounds was calculated by the ratio between the CC_{50} and IC_{50} values.

2.5. Infection and therapeutic regimen

Mice (n = 12 per group) were infected with 1×10^7 stationary-phase promastigotes of *L. amazonensis* through subcutaneous injection in the base of the tail, after trichotomy, and lesion development was monitored using an electronic caliper (799-6/150 model, Starrett®, Brazil). After the development of ulcerated lesions (2–3 mm, approximately 60–70 days post-infection), animals were divided into groups, aiming to ensure a similar lesion average size among them. Then, animals were treated during 15 days, once a day, receiving subcutaneous injections in their left hind footpad by using one of the following regimens:

i) Saline group: mice received 100 μL of saline.
ii) Free AmpB group: mice received 100 μL of AmpB (25 μg) derived from a stock solution.
iii) B-AmpB/M group: mice received 100 μL of a solution composed by 75 μL of saline and 25 μL of B-AmpB/M.
iv) AmpB/M group: mice received 100 μL of a solution composed by 75 μL of saline and 25 μL of AmpB/M (corresponding to a final concentration of 25 μg of AmpB).

Every day during the treatment, the lesion average size was measured to evaluate the development of the lesions. Further observations including the appearance of relapses and/or nodules, as well as metastasis or other clinical symptoms in the animals were also performed. The treatments efficacy was evaluated by measuring the lesion average diameter and by the estimation of the parasite load in the infected tissue (base of the tail), as well as in the spleen, liver and draining lymph nodes (dLN) of the animals, which was performed one-day after the end of treatments.

2.6. Parasite burden

A limiting-dilution technique developed by Titus et al. (1985) and modified by Martins et al. (2013) was used to evaluate the parasite load in the treated and infected animals. For this, infected tissue fragments, spleen, liver and dLN of the animals were collected, weighed and homogenized using a glass tissue grinder in sterile PBS 1×. Tissue debris were removed by centrifugation at 150 × *g* and cells were concentrated by centrifugation at 2000 × *g*. The pellet was resuspended in 1 mL of complete Schneider's medium. Two hundred 20 μL of the resuspension was plated onto 96-well flat-bottom microtiter plates (Nunc), and diluted in log-fold serial dilutions in complete Schneider's medium with a 10^{-1}–10^{-12} dilution. Each sample was plated in triplicate and read 7 days after the beginning of the culture at 24 °C. Pipette tips were

discarded after each dilution to avoid carrying adhered parasites from one well to another. Results were expressed as the negative log of the titer (*i.e.*, the dilution corresponding to the last positive well) adjusted per milligram of infected tissue or organ.

2.7. Immune response

2.7.1. Cytokine and nitrite production

To evaluate the cellular response developed in the end of the treatments, spleen cells culture and cytokine assays were performed as described (Martins et al., 2015). For this, single-cell suspensions (5×10^6 cells per well) were plated in duplicate in 24-well plates (Nunc) and incubated in DMEM (Dulbecco's Modified Eagle Medium, Sigma-Aldrich; background control), which was supplemented with 20% FBS and 20 mM L-glutamine, pH 7.4, or stimulated with *L. amazonensis* SLA (25 μg/mL), for 48 h at 37 °C in 5% CO_2. IFN-γ, IL-4, IL-10, IL-12p70 and GM-CSF levels were assessed in the supernatants by a sandwich ELISA provided in commercial kits (BD OptEIA TM set mouse IFN-γ, IL-4, IL-10, and IL-12 and GM-CSF, Pharmingen®, San Diego, CA, USA), following the manufacturer's instructions. The involvement of $CD4^+$ and $CD8^+$ T cells in the IFN-γ production by spleen cells of the AmpB/M-treated and infected mice was evaluated after the *in vitro* stimulation with SLA (25 μg/mL), in the presence or absence of monoclonal antibodies against mouse IL-12 (C17.8), CD4 (GK 1.5) or CD8 (53–6.7), at a 5 μg/mL concentration. Appropriate isotype-matched controls [rat IgG2a (R35-95) and rat IgG2b (95-1)] were used in the assays. All antibodies (no azide/low endotoxin™) were purchased from BD (Pharmingen). The nitrite production was also evaluated in the culture supernatants by the Griess method (Green et al., 1982). Data were expressed as μM (per 5×10^6 cells).

2.7.2. Humoral response

The antibody production was evaluated one-day after the end of the treatments. For this, the parasite-specific IgG1 or IgG2a isotype antibodies levels were measured by an ELISA technique, as described (Coelho et al., 2003). Briefly, *L. amazonensis* SLA was used as an antigen (at a concentration of 1.0 μg per well), whereas serum samples were diluted at 1:100 in a PBS-T (PBS 1× plus 0.05% Tween 20) buffer and anti-mouse IgG1 and IgG2a horseradish-peroxidase conjugated antibodies (Sigma-Aldrich) were both used at a 1:10,000 dilution (also diluted in PBS-T). Reactions were developed by incubation with a solution composed by 2 μL H_2O_2, 2 mg ortophenylenediamine and 10 mL citrate-phosphate buffer at pH 5.0, for 30 min in the dark and stopped by adding 20 μL H_2SO_4 2 N. The optical density was determined in an ELISA microplate spectrophotometer (Molecular Devices, Spectra Max Plus, Canada), at 492 nm.

2.8. In vivo toxicity evaluation

Serum samples were collected of the infected and treated animals to perform the biochemical analysis. Samples from naive (non-treated and non-infected) mice were used as a control. The hepatic function was analyzed by dosage of the aspartate aminotransferase (AST) and alanine aminotransferase (ALT), whereas the nephrotoxicity was evaluated by examining the levels of blood urea nitrogen (BUN) and serum creatinine (CRTN). The biochemical assays were developed using commercial kits (Labtest Diagnostica®, Belo Horizonte, Minas Gerais, Brazil) and an auto-analyzer apparatus (Thermo Plate TP analyzer), according to manufacturer's instructions.

2.9. Statistical analysis

The results of *in vitro* experiments were entered into Microsoft Excel (version 10.0) spreadsheets and analyzed by GraphPad Prism™ (version 6.0 for Windows; GraphPad Software, Fay Avenue, La Jolla, CA, USA). The one-way analysis of variance (ANOVA) followed by Tukey's post-test were used for comparisons between the experimental groups. Differences were considered significant when $P < 0.05$. Data of the *in vivo* experiments shown in this study represent the mean ± standard deviation of two different experiments, which presented similar results.

3. Results

3.1. In vitro antileishmanial activity and cytotoxicity

Initially, the *in vitro* antileishmanial activity of the AmpB/M and B-AmpB/M against stationary-phase promastigote and amastigote-like stages of *L. amazonensis* was evaluated. In the results, the IC_{50} values of these products were of 1.83 ± 0.4 and 22.1 ± 0.7 μM, respectively, in the promastigotes, and of 2.27 ± 0.5 and 33.98 ± 2.6 μM, respectively, in amastigotes-like. Their CC_{50} values were also determined and the results were of 119.5 ± 9.6 and 134.7 ± 10.3 μM, respectively. With these values, the SI was calculated and values were of 65.3 and 5.4 for the AmpB/M and B-AmpB/M, respectively, in promastigotes, and of 59.3 and 3.96, respectively, in the amastigotes-like. Free AmpB showed IC_{50} values of 1.2 ± 0.3 and 2.5 ± 0.5 μM for the promastigotes and amastigotes-like of *L. amazonensis*, respectively, whereas its CC_{50} value was of 9.5 ± 0.4 μM. The SI values for this drug were of 7.9 and 3.8, respectively, for the promastigotes and amastigotes-like of the parasites.

3.2. Treatment efficacy using AmpB-containing polymeric micelles against L. amazonensis

In our study, BALB/c mice were infected with a high inoculum of stationary-phase promastigotes of *L. amazonensis* and, during the development of the chronic disease, they were treated with AmpB, which was administered in a free format or incorporated into a delivery system composed by Poloxamer 407-based polymeric micelles. The treatment was performed during 15 days and the products were administered by a subcutaneous route in the hind footpad of the animals, once a day. During this time, the lesion average size was evaluated in the treated and infected animals, and the results were expressed in mm^2 (area). In the results, reductions were observed in the animals that were treated with free AmpB, AmpB/M and B-AmpB/M, when compared to the saline group (Fig. 1A). However, when the three treated groups were compared between them, the AmpB/M-treated mice showed a reduction of 37% and 45% in their lesion average size in relation to the free AmpB and B-AmpB/M groups, respectively. The parasite load was evaluated in infected tissue fragments (Fig. 1B), dLN (Fig. 1C), liver (Fig. 1D) and spleen (Fig. 1E) of the animals. In the results, it was observed that all treated animals presented reductions in the parasite log, when compared to the saline group. However, when the treated groups were compared between them, the AmpB/M-treated mice showed an improved therapeutic response against infection, when compared to the free AmpB and B-AmpB/M groups, since their parasite load was lower in the infected tissue fragment (3.3- and 4.9-log reductions, respectively), dLN (2.3- and 4.2-log reductions, respectively), liver (1.0- and 2.8-log reductions, respectively) and spleen (1.0- and 3.5-log reductions, respectively) in relation to the others.

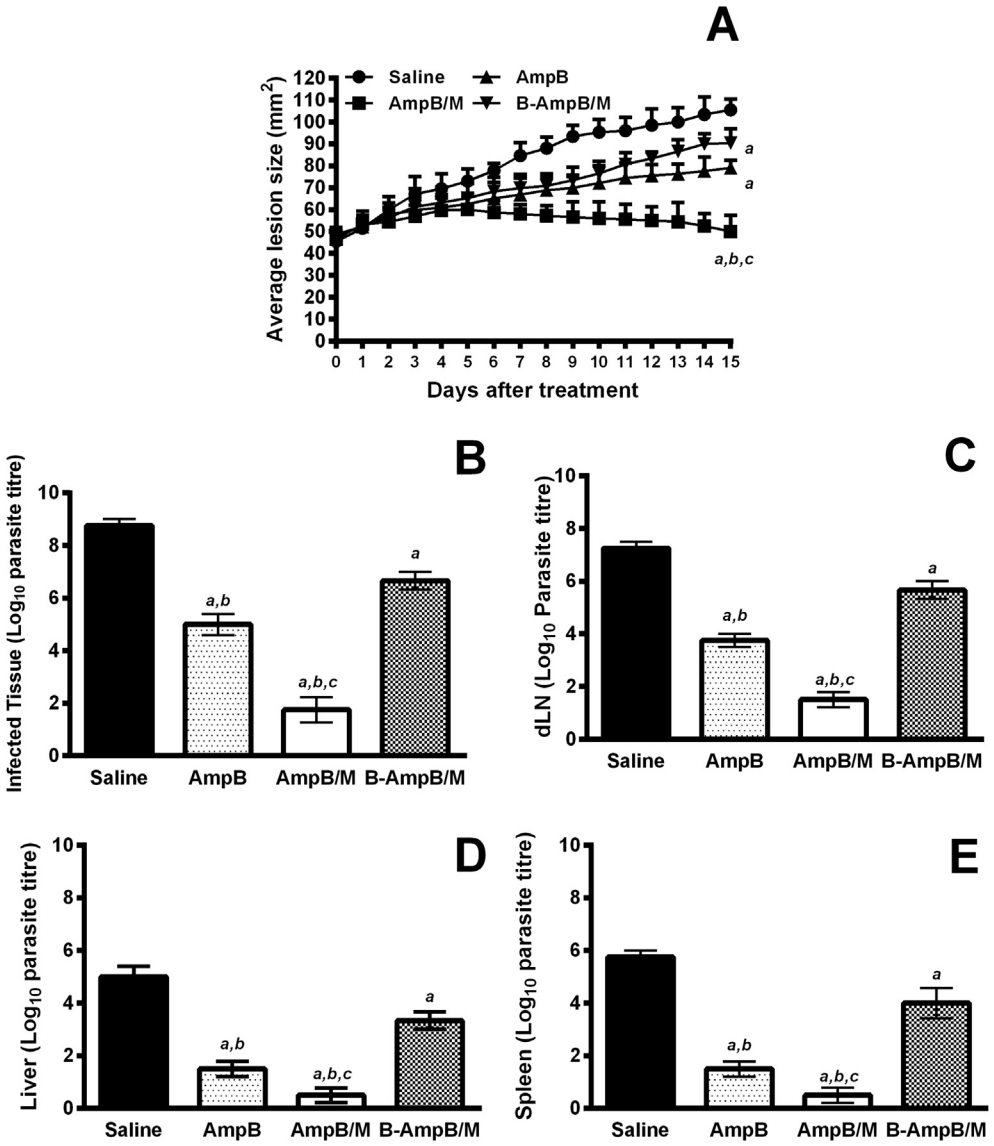

Fig. 1. Treatment efficacy using amphotericin B-containing polymeric micelles in *L. amazonensis*-infected mice. Animals (n = 12 per group) were subcutaneously infected in the base of the tail, after trichotomy, with 1×10^7 stationary-phase promastigotes of *L. amazonensis*. When they developed ulcerated lesions presenting an average diameter of 2–3 mm (between 60 and 70 days after infection), animals were divided into groups and received saline or were treated with free amphotericin B (AmpB), non-incorporated micelles (B-AmpB/M, as a micelles control) or with AmpB-containing polymeric micelles (AmpB/M) during 15 days, once a day, by a subcutaneous route in their left hind footpad. In **A**, lines represent the lesion average diameter (area) expressed as the mean plus standard deviation of the groups. The parasite burden was evaluated in infected tissue fragments (**B**), draining lymph nodes (dLN) (**C**), liver (**D**) and spleen (**E**) of the treated and infected animals, by a limiting dilution technique. Bars represent the mean ± standard deviation of the groups. The letters *a*, *b* and *c* indicate statistically significant differences in relation to the saline, B-AmpB/M and AmpB groups, respectively ($P < 0.05$).

3.3. *Immune response generated in the treated and infected BALB/c mice*

In the present study, the antileishmanial immune response was evaluated in the treated and infected animals. For this, the *Leishmania*-specific cytokine levels, as well as the antibody production and nitrite secretion were evaluated. In the results, spleen cells of all treated mice showed significantly higher levels ($P < 0.05$) of parasite-specific IFN-γ, IL-12 and GM-CSF than those produced by spleen cells of the saline group. Comparatively, AmpB/M-treated animals presented higher levels of IFN-γ, IL-12 and GM-CSF, when compared to those found in the free AmpB and B-Amp/B groups (Fig. 2A). On the other hand, animals from the saline and B-AmpB/M groups showed higher levels of IL-4 and IL-10, demonstrating that these animals developed a non-protective immune response against *L. amazonensis* infection.

The involvement of $CD4^+$ and $CD8^+$ T cells in the antileishmanial IFN-γ production in the AmpB/M group was evaluated in their spleen cells stimulated with SLA, in the absence or presence of anti-CD4 or anti-CD8 monoclonal antibodies. In the results, the use of an anti-CD4 antibody induced a lower IFN-γ production, possibly reflecting that this T cell subtype was the main responsible for the production of this cytokine in the treated animals (Fig. 2B). Serum samples were collected and antileishmanial antibodies were also evaluated. In the results, a predominant IgG2a response was found in the AmpB/M-treated mice, whereas animals from the saline and B-AmpB/M groups showed a predominance of the IgG1 isotype (Fig. 2C). The nitrite secretion showed that spleen cells from

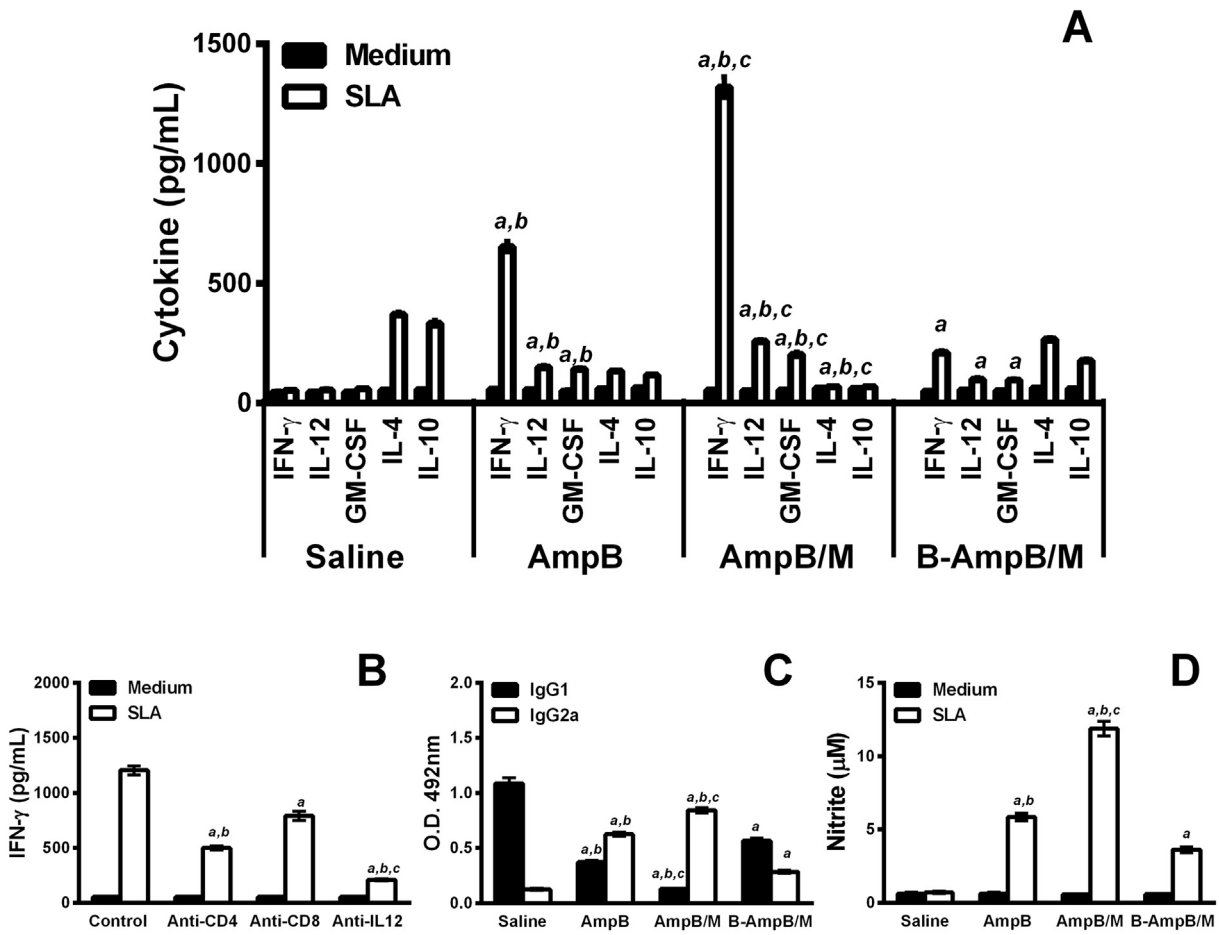

Fig. 2. Immune response developed in the treated and infected animals. Single cells suspensions were obtained from the spleen of mice (n = 12 per group), one-day after the end of the treatments, when cells were non-stimulated (medium; background control) or stimulated with *L. amazonensis* SLA (25 μg/mL), for 48 h at 37 °C in 5% CO_2. IFN-γ, IL-4, IL-10, IL-12p70 and GM-CSF levels were measured in the culture supernatants, by using a capture ELISA (**A**). Mean ± standard deviation of the cytokine levels of the groups are shown. In addition, spleen cells of the AmpB/M-treated mice were *in vitro* stimulated with SLA (25 μg/mL) in the absence (control) or presence of monoclonal antibodies (mAb) against mouse IL-12, $CD4^+$ or $CD8^+$, and the IFN-γ production was evaluated by capture ELISA (**B**). The levels of antileishmanial IgG1 and IgG2a isotypes antibodies were investigated by an ELISA technique, and the results are also shown (**C**). The nitrite secretion, as an indicator of nitric oxide production, was analyzed in the supernatants of the parasite-stimulated cultures by the Griess method, and results are shown (**D**). In all cases, bars represent the mean ± standard deviation of the experimental groups. The letters *a*, *b* and *c* indicate statistically significant differences in relation to the saline, B-AmpB/M and AmpB groups, respectively ($P < 0.05$).

animals of the free AmpB, AmpB/M and B-AmpB/M groups presented a high production of this molecule, which was more pronounced in the AmpB/M-treated group (Fig. 2D).

3.4. *In vivo* toxicity evaluation

Clinical symptoms such as ataxia, loss of body weight and weakness were observed in free AmpB-treated mice (all animals). These mice showed a negative variation in their body weight in the order of 14%, possibly reflecting the toxicity of drug. On the other hand, animals treated with AmpB/M showed a positive variation in their body weight reaching a maximum of 3%, when compared to the beginning of the treatment, whereas mice that received saline or that were treated with B-AmpB/M presented negative variations in their body weight in the order of 7% and 1%, respectively, possibly reflecting the chronicity of the infection. In addition, enzymatic markers of hepatic and renal damage showed that free AmpB-treated mice presented higher levels of AST, ALT, BUN and CRTN, when compared to the values found in the AmpB/M and B-Amp/M groups, as well as in relation to the values obtained evaluating the non-treated and non-infected mice (Fig. 3).

4. Discussion

AmpB has been used as a second-line drug for the treatment of VL and its mechanism of action is based on the binding with parasites membrane sterols (Seifert et al., 2011). This product has demonstrated an effective antileishmanial activity against different *Leishmania* species (Lage et al., 2013; Morais-Teixeira et al., 2014); however, its clinical use has been hampered due to its high toxicity (Annaloro et al., 2009). Liposomal delivery of AmpB has allowed the increase of drug concentration in target organs, while decreasing the systemic side effects of the drug. Similarly to other conventional liposomes, Ambisome® is the most efficient formulation in terms of action spectrum against VL, and it is the least toxic among the lipid-AmpB formulations, but presents problems of stability and a high price due to the cost of phospholipids, thus putting limitations. In addition, Ambisome® and other lipid-AmpB formulations are usually administered by an intravenous route for the treatment of disease (Larabi et al., 2003; Musa et al., 2005; Gershkovich et al., 2010; Gupta et al., 2014).

The parenteral and intravenous routes are used for the majority of antileishmanial drugs, as they ensure the drug distribution to the

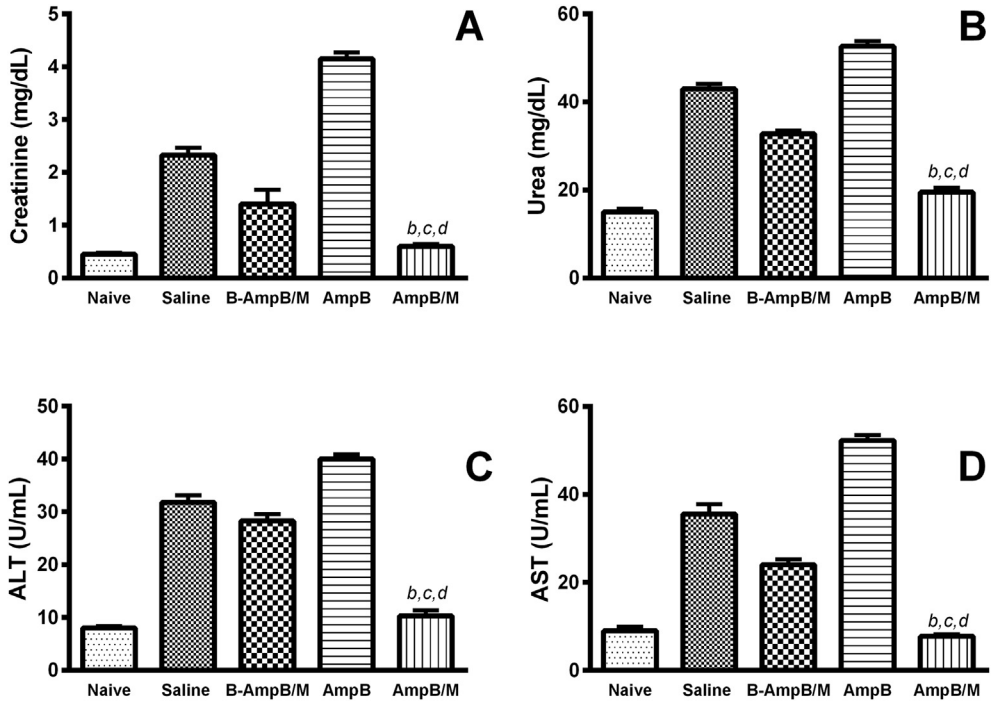

Fig. 3. Evaluation of the *in vivo* toxicity. The levels of blood urea nitrogen (BUN), serum creatinine (CRTN), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) enzymes in the infected mice and that received saline or were treated with free AmpB, B-AmpB/M or AmpB/M were evaluated. Non-treated and non-infected (naive) mice were used as a control. Bars represent the mean ± standard deviation of the groups (n = 12 per group). The letters *b, c* and *d* indicate statistically significant differences in relation to the saline, B-AmpB/M and AmpB groups, respectively ($P < 0.05$).

systemic sites of infection. Studies have shown that the treatment using the intravenous route is effective in reducing parasites in liver and spleen, making it possible to reduce the time necessary to perform the treatment. However, the inhibition of cutaneous parasitism has been more problematic when drugs are administered by these routes, since phagocytic cells within the liver and spleen will remove particles from the systemic circulation and, in this way, less drug-loaded particles remain for uptake in the cutaneous infection sites, where parasites were inoculated and persist. Therefore, the disease is developed and the treatment usually presents poor results (Shaw and Carter, 2014).

Poloxamer P407-based AmpB-containing polymeric micelles administered by subcutaneous injections in murine models will turn into a semi-rigid gel, when in contact with the local tissue, creating a reservoir system and keeping the drug in the extracellular space, then allowing its action against the parasites in the local infection site. In course of time, as the gel matrix is diluted by body fluids and phagocytosis, the drug will be gradually released into the systemic circulation, enabling its systemic action in a controlled manner (Barichello et al., 1999). This fact can be considered relevant, when compared to the use of the lipid-based formulations such as Ambisome®, since that when the AmpB/M system was applied in the present study, an effective antileishmanial activity was reached in BALB/c mice that were chronically infected with *L. amazonensis*. This fact can be corroborated with the low parasitism found in the systemic organs of these animals, such as spleen and liver, as well as in the local infection site, represented by the infected tissue fragments and dLN of the AmpB/M-treated animals.

Other relevant aspect denoting the importance of the use of AmpB/M in our study was related to the treatment of TL, since most studies using AmpB-containing formulations have been developed against VL. Nevertheless, in a previous work, Ambisome® was shown to present a poor *in vitro* antileishmanial activity against *Leishmania major*, since free AmpB was 3–6 times more active than the liposomal product against both promastigotes and intramacrophage amastigotes of parasites (Vyas and Gupta, 2006). In a murine model of cutaneous infection, Ambisome® administered once a day during 6 alternate days by intravenous route produced a dose-response effect only when applied in concentrations ranging from 6.25 to 50 mg per kg of body weight (Yardley and Croft, 1997). Because the usual treatment of VL has been performed with pentavalent antimonials being administered with 20 mg per kg per day by intravenous or intramuscular injections, our study using AmpB/M shows characteristics that could be considered attractive, since the dosage of formulation employed will be considered low (1 mg per kg per day). This dose was also used by others, when AmpB was administered in a chitosan-chondroitin sulphate nanoparticles system in *Leishmania*-infected BALB/c mice. In this study, nanoparticles were not toxic to animals, although this system was highly effective in treating infected mice (Ribeiro et al., 2014). However, future studies employing different therapeutic regimens, as well as the association with different drugs will be performed, aiming to reduce the period of treatment and evaluate the possibility of the complete elimination of the parasites in the cutaneous infection site.

Poloxamer 407 (Pluronic® F127) has been applied in a very broad range in biomedical and pharmaceutical industries (Torchilin et al., 2001). The ease of production, the stability at 4 °C or at room temperature and the low cost of the formulation, when compared to the phospholipids used in the liposomal formulations, are attractive conditions to their use (Jain et al., 2003). These facts made it possible to develop and test the biological activity of Pluronic®-based polymeric micelles as anti-cancer agents (Wang et al., 2007; Zhang et al., 2011; Lamch et al., 2014) and antileishmanial compounds (Chiellini et al., 2008). However, for the best of our knowledge, this is the first time that a Poloxamer 407-

based system for AmpB delivery has been used to treat *L. amazonensis*-infected BALB/c mice. In the results, animals that were infected with a highly inoculum of stationary promastigotes and received saline showed an exponential parasite burden in the infection cutaneous (infected tissue and dLN) and systemic (spleen and liver) sites, which suggests the occurrence of a chronic disease caused by the parasites. On the other hand, when animals were treated with free AmpB, AmpB/M or B-AmpB/M, all therapeutic regimens were able to reduce the parasite load in the infected animals, although the use of AmpB/M had been able to promote highly significant reductions both in the lesion average diameter and in the parasite load in all evaluated tissue and organs, demonstrating to be a candidate to an effective therapeutic regimen against *L. amazonensis* infection.

As a parameter to evaluate the *in vivo* toxicity in the different treatments performed, the AST, ALT, blood urea nitrogen and serum creatinine levels were evaluated in all mice groups. In the results, high levels of the enzymatic markers were observed in the free AmpB-treated animals. In addition, these mice showed a 14%-reduction in their body weight, possibly reflecting the toxicity of the drug. Mice from the saline and B-AmpB/M groups showed also negative variations in their body weight in the order of 7% and 1%, respectively, maybe reflecting the chronicity of the infection. Our results are in concordance with others, where the use of saline or free AmpB induced a loss of body weight in BALB/c mice that were infected with *L. amazonensis* and later received the therapeutics (Ribeiro et al., 2014). On the other hand, no hepatic or renal damage was observed in the AmpB/M-treated animals, as well as they presented a slight increase in their body weight as compared to the others, possibly reflecting the synergistic action of the treatment in promoting the reduction of infection in the treated and infected animals.

The protective immune response against *Leishmania* infection is dependent on Th1-type $CD4^+$ T cells, which produce macrophage-activating cytokines such as IFN-γ, IL-12, GM-CSF and others, contributing to limit the parasites expansion and to control the infection (Gollob et al., 2014). In our study, spleen cells from the AmpB/M-treated mice were those that presented higher levels of IFN-γ, IL-12 and GM-CSF, which were associated with low levels of IL-4, IL-10 and anti-*Leishmania* IgG1 isotype antibodies. The immune profile found in our study is in concordance with others (Ribeiro et al., 2014), where *L. amazonensis*-infected BALB/c mice and later treated with chitosan-chondroitin sulphate-AmpB nanoparticles also showed higher levels of IFN-γ and IL-12 and lower production of IL-4 and IL-10, whereas controls displayed high levels of IL-4 and IL-10 in their splenic cultures. Our data are also in concordance with Asthana et al. (2013), who showed that low levels of IL-4 and IL-10, associated with a high production of IFN-γ by the splenic cultures of infected hamsters, were related to the efficacy of the treatment using an AmpB-loaded template-based nanoemulsion. Altogether, our results indicate that the treatment using AmpB/M was able to induce an effective antileishmanial Th1 immune response in the treated and infected animals, which was possibly responsible for the control of the infection in the treated animals.

As a limiting factor of the present study, the immunological and parasitological parameters were not evaluated in a longer time after the end of the treatments, since the evaluation was developed one-day after that, due to the ethical aspects involving the severity of infection in the control (saline) group. In this context, evaluations performed in different periods of time could suggest that our therapeutics will be effective in eliminating the parasites, instead of considering that the own immune system of the animals could be reverting the infection. Nevertheless, we believe that our model could be considered adequate, since this formulation showed a good stability and safe to be administered in the mammalian hosts by a subcutaneous route. Thus, our data could be taken as a proof-of-concept of the capacity of this AmpB-containing polymeric micelles system in treating the disease caused by the *L. amazonensis* species, and would serve as a reference for further assays.

5. Conclusion

Taken together, the results showed that the AmpB/M system can be considered as an alternative for the treatment of TL, although additional studies are necessary to be performed in other infected mammalian hosts.

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