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Original article

In vivo antileishmanial efficacy of a naphthoquinone derivate incorporated into a Pluronic® F127-based polymeric micelle system against *Leishmania amazonensis* infection

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ABSTRACT

New therapeutic strategies against leishmaniasis are desirable, since the treatment against disease presents problems, such as the toxicity, high cost and/or parasite resistance. As consequence, new antileishmanial compounds are necessary to be identified, as presenting high activity against *Leishmania* parasites, but low toxicity in mammalian hosts. Flau-A is a naphthoquinone derivative recently showed to presents an *in vitro* effective action against *Leishmania amazonensis* and *L. infantum* species. In the present work, the *in vivo* efficacy of Flau-A, which was incorporated into a Poloxamer 407-based micelle system, was evaluated in a murine model against *L. amazonensis* infection. Amphotericin B (AmB) and Ambisome® were used as controls. The animals were infected and later treated with the compounds. Thirty days after the treatment, parasitological and immunological parameters were evaluated. Results showed that AmB, Ambisome®, Flau-A or Flau-A/M-treated animals presented significantly lower average lesion diameter and parasite burden in tissue and organs evaluated, when compared to the control (saline and micelle) groups. Flau-A or Flau-A/M-treated mice were those presenting the most significant reductions in the parasite burden, when compared to the others. These animals developed also a more polarized antileishmanial Th1 immune response, which was based on significantly higher levels of IFN-γ, IL-12, TNF-α, GM-CSF, and parasite-specific IgG2a isotype; associated with low levels of IL-4, IL-10, and IgG1 antibody. The absence of toxicity was found in these animals, although mice receiving AmB have showed high levels of renal and hepatic damage markers. In conclusion, results suggested that the Flau-A/M compound may be considered as a possible therapeutic target to be evaluated against human leishmaniasis.

1. Introduction

Leishmaniasis is a neglected disease with approximately 350 million people at risk of infection by *Leishmania* parasite, and with 2 million new cases being reported annually, occurring mainly in developing countries [1]. The clinical manifestations of the disease range from cutaneous leishmaniasis to the visceral disease [2]. Tegumentary leishmaniasis (TL) is considered endemic in Latin America, and the

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main species able to cause the disease are *Leishmania braziliensis, L. amazonensis, L. guyanensis, L. panamensis,* and *L. mexicana*. The disease can be subclinical or produces self-healing cutaneous lesions, although the uncontrolled parasite replication leading to mutilation and morbidity can be observed in the patients [3,4]. Visceral leishmaniasis (VL), which can be fatal if acute and untreated, is mainly found in India, Bangladesh, Sudan, South Sudan, Ethiopia, and Brazil. The disease is caused by *L. donovani* and *L. infantum* species, and about 20,000 to 40,000 deaths are registered annually [5].

Among the distinct *Leishmania* spp. able to cause human disease, the *L. amazonensis* parasite is an important etiological agent of TL in the South America, being responsible by the cases of diffuse cutaneous leishmaniasis [6]. Regarding to the disease in BALB/c mice, the animals are highly susceptible to this parasite species, showing progressive swelling at the inoculation site followed by metastasis and visceralization [7,8]. This mouse lineage develops a parasite-specific Th2 immune response, which is characterized by production of high levels of IL-4, IL-10, among other anti-inflammatory cytokines; while the resistance is associated with the development of an specific Th1 immune response, primed by the production of IFN-γ, IL-2, GM-CSF, IL-12, among other pro-inflammatory cytokines [9,10].

The treatment against leishmaniasis is based on the use of pentavalent antimonials in Brazil and other developing countries. Other second-line products, such as miltefosine, amphotericin B (AmB), paromomycin, and pentamidine are also used; however, side effects such as hepatic, cardiac, and renal toxicity are also registered [11]. In addition, parasite resistance is increasing, leading to the necessity to use higher doses and longer-term therapy, thus causing higher toxicity in the patients [12]. AmB-containing lipid-based formulations have been employed for the treatment against leishmaniasis. These compounds present higher efficacy and lower toxicity in the patients, when compared to the use of free drug. However, the high cost is an impeditive factor, and limits their use in developing countries [13]. In this context, the search for new, safer, and cheaper drugs to treat against leishmaniasis is urgently needed, and one strategy could be based on the employ of new synthetic and/or natural products.

Quinones are a known class of molecules exhibiting distinct biological activities, such as antitumor [14], molluscicidal [15], bactericidal [16], fungicidal [17], and trypanocidal [18,19] functions. Studies have showed also the use of this molecules´ class against *Leishmania* parasites [20–22]. However, the further development of these compounds has been hampered, due to the absence of *in vivo* experiments or due to their low efficacy, when they are tested as therapeutics in mammalian models [22].

Since structural modifications in known molecules can be considered a cheaper and faster pathway to develops new antileishmanial agents [23]; in the present study, a quinoline derivate, namely 2-(2,3,4-tri-O-acetyl-6-deoxy-β-L-galactopyranosyloxy)-1,4-naphthoquinone or Flau-A, was investigated against *L. amazonensis* infection in BALB/c mice. This molecule was previously showed to presents an *in vitro* antileishmanial activity against *L. amazonensis* and *L. infantum* species, as well as low toxicity in murine macrophages and human red cells [24]. Here, Flau-A was incorporated into a Poloxamer 407-based micelle system, and the therapeutic efficacy of this product was evaluated in *L. amazonensis*-infected BALB/c mice, comparing with the use of free AmB and its liposomal formulation, Ambisome®. The efficacy of the products was evaluated 30 days after the treatment, when immune response and parasite burden in the infected tissue and organs were evaluated in the treated and infected animals.

2. Materials and methods

2.1. Chemicals

A technical protocol recently developed [24] was used for the preparation of Flau-A. Briefly, 0.30 g (1.72 mmol) lawsone was dissolved in 5 mL dichloromethane PA and transferred to a 100 mL round bottom flask. A solution containing potassium carbonate (10% w/v) was added, and the mixture was stirred for 30 min at room temperature. Next, 2,3,4-tri-*O*-acetyl-6-deoxy-β-L-galactopyranosyl bromide (3.44 mmol) and n-Bu_4NBr (22% mol) were added, and the mixture was again stirred for 18 h at room temperature. Then, 6 mol/L hydrochloric acid were added to reach at pH 3.0, and the mixture was transferred to a separator funnel to obtain the organic phase. Thus, it was washed in 100 mL distilled water, dried by using anhydrous sodium sulfate and concentrated. The product was passed in a column chromatography (hexane/ethyl acetate, 7:3 v/v), and the purified molecule was obtained in a 34% yield. The product was considered pure according to its proton and 13-carbon NMR spectra, besides TLC analysis. For the *in vivo* experiments, Flau-A was diluted in phosphate-buffered saline (PBS 1x) pH 7.4. Poloxamer 407 (Pluronic® F127) was purchased from Sigma-Aldrich (catalog number 16,758; St. Louis, MO, USA). AmB (Cristália, São Paulo, São Paulo, Brazil) was resuspended in methanol/DMSO (9:1 v/v), and maintained at -80° until use. Ambisome® (Gilead Sciences, Inc. San Dimas, USA) was obtained as a lyophilized powder, and resuspended in water to prepare a stock solution.

2.2. Preparation of the Flau-A/M

The polymeric micelles were prepared as described previously [25]. Briefly, Poloxamer 407 (18% w/w) was diluted in PBS 1x, pH 7.4, under magnetic agitation for 18 h at 4 °C. Then, Flau-A (8 mg) was diluted in 500 μL dichloromethane PA and solubilized by using vortex. The mixture was added to the previously prepared Poloxamer solution under vigorous magnetic agitation and in an ice bath, until a viscous emulsion has been obtained. The dichloromethane solution was evaporated by using a rotary evaporate (Buchi, Flawil, Switzerland), and the product was obtained as a transparent yellow gel at room temperature. The Flau-A content in the micellar solution was evaluated spectrophotometrically using an ultraviolet method, as described previously [26]. Briefly, samples of the Flau-A/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 (nm). The concentration of Flau-A 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 by using the same technical protocol described for preparation of the Flau-A/M.

2.3. Parasite and mice

L. *amazonensis* (IFLA/BR/1967/PH-8) was used. The parasites were grown in complete Schneider's medium (Sigma-Aldrich, St. Louis, MO, USA), which was composed by the Schneider's medium plus 20% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich, USA), 20 mM L-glutamine, 200 U/mL penicillin, 100 μg/mL streptomycin, and 50 μg/mL gentamicin pH 7.4, at 24 °C. Female BALB/c mice (8 weeks age) were obtained from the breeding facilities of the Department of Biochemistry and Immunology, Institute of Biological Sciences (ICB), Federal University of Minas Gerais (UFMG), Belo Horizonte, Minas Gerais, Brazil. The animals were maintained under pathogen-free conditions. The study was approved by Committee for the Ethical Handling of Research Animals from UFMG (protocol number 085/2017).

2.4. Infection and therapeutic schedules

Mice (n = 10 per group) were infected with 10^7 L. *amazonensis* stationary promastigotes in their base of the tail through subcutaneous injection. Fifty days after infection (with lesions presenting between 2–3 millimeters), animals were separated in groups according to lesion size, aiming to ensure similar average lesion diameter among the evaluated groups. Then, they were treated with one of the following

regimens:

(i) Saline (control) group: mice received 50 μL of PBS 1x by subcutaneous route, once a day during 15 days;

(b) Empty micelle (micelle) group: mice received 50 μL of non-incorporated micelles (10 mg/kg body weight) by subcutaneous route, every two days during 15 days;

(c) Amphotericin B (AmB) group: mice received 50 μL of AmB (1 mg/kg body weight) by intraperitoneal route, every two days during 15 days;

(d) Ambisome® group: mice received 50 μL of product (2 mg/kg body weight) by intravenous route, every five days during 15 days;

(e) Flau-A group: mice received 50 μL of Flau-A (10 mg/kg body weight) by subcutaneous route, every two days during 15 days;

(f) Flau-A/micelle (Flau-A/M) group: mice received 50 μL of Flau-A-containing micelles (5 mg/kg body weight) by subcutaneous route, every two days during 15 days.

Then, the lesion average diameter was measured weekly by using an electronic caliper (799-6/150 model, Starrett®, Brazil), and 30 days after the treatment, the animals were euthanized, when parasitological and immunological parameters were evaluated.

2.5. Cellular response evaluated by capture ELISA and flow cytometry

Aiming to evaluate the antileishmanial cellular response induced after the treatment, the soluble L. *amazonensis* antigenic (SLA) extract was prepared as described previously [27]. Briefly, 10^9 stationary promastigotes were washed three times in cold sterile PBS 1x, pH 7.4. After six cycles of freezing and thawing, followed by ultrasonication (Ultrasonic processor, GEX600), with six cycles of 30 s at 38 MHz, the suspension was centrifuged at 9000 x *g* for 30 min at 4 °C, and the supernatant containing L. *amazonensis* SLA was collected. The protein concentration was estimated by the Bradford method [28], and aliquots were stored at −80 °C until use. Murine splenocytes (n = 10 per group) were collected 30 days after the treatment, and cells (5×10^6) were plated in 24-well plates (Nunc) and incubated in DMEM plus 20% FBS and 20 mM L-glutamine, at pH 7.4. Then, cells were unstimulated (medium, background control) or stimulated with SLA (25.0 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 supernatants using commercial kits (BD Pharmingen®, San Diego, CA, USA), according to the manufacturer's instructions. The nitrite production was evaluated in the cell supernatant by Griess reaction [29].

To investigate the origin of the IFN-γ production in the treated and infected animals, splenocytes were stimulated with SLA (25.0 μg/mL) and incubated in the presence of 5.0 μg/mL of monoclonal antibodies (mAb) against mouse CD4 (GK 1.5) or CD8 (53-6.7). Appropriate isotype-matched controls – rat IgG2a (R35-95) and rat IgG2b (95-1) – were used. Antibodies (no azide/low endotoxin™) were purchased from BD (Pharmingen®). A flow cytometry assay was also performed to evaluate the IFN-γ, TNF-α and IL-10-producing $CD4^+$ and $CD8^+$ T cell frequency as described previously [30]. Results were expressed as indexes, which were calculated by the ratio between the cytokine-producing $CD4^+$ and $CD8^+$ T cell percentages *versus* the values obtained in the unstimulated cultures (SLA/CC ratio).

2.6. Humoral response

The antileishmanial IgG1 and IgG2a isotype production was evaluated in serum samples of the treated and infected animals. For this, *L. amazonensis* SLA was used as an antigen (1.0 μg per well), and samples were 1:100 diluted in PBS-T (PBS 1x plus 0.05% Tween 20), with incubation for 1 h at 37 °C. After washing plates five times, the anti-mouse IgG1 and IgG2a horseradish-peroxidase conjugated antibodies were added (in 1:5000 and 1:10,000 dilutions, respectively, in PBS-T, Sigma-Aldrich), and reactions were developed by incubation with 2 μL H_2O_2, 2 mg ortho-phenylenediamine and 10 mL citrate-phosphate buffer pH 5.0, for 30 min and in the dark, and stopped by the addition of 20 μL H_2SO_4 2 N. The optical density was determined by an ELISA microplate spectrophotometer (Molecular Devices, Spectra Max Plus, Canada), at 492 nm.

2.7. Parasite burden

The parasite load was evaluated in the infected tissue, draining lymph node (dLN), spleen, and liver of the animals by a limiting dilution technique [31]. For this, tissue and organs were weighed and homogenized using a glass tissue grinder in sterile PBS. Tissue debris were removed by centrifugation at 150 × *g* and cells were concentrated by centrifugation at 2000 x *g*. The pellet was resuspended in 1 mL of complete Schneider's medium and 220 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} to 10^{-12} dilution. Each sample was plated in triplicate and read 7 days after the beginning of the culture at 24 °C. Results were expressed as the negative log of the titer (*i.e.*, the dilution corresponding to the last positive well), which was adjusted per milligram of tissue or organ.

The parasite load was also evaluated in the infected tissue by a qPCR technique as described previously [30]. Briefly, infected tissue DNA was extracted by using a phenol-chloroform method. Standard curves were obtained from DNA extracted from 10^8 parasites for kDNA and of 10^8 peritoneal macrophages for β-actin, under the same conditions used to extract the other samples. PCR was performed on Step One™Instrument (48 wells-plate; Life Technologies) using 2x SYBR® Green PCR Master Mix (5 μL, Applied Biosystems), with 2 mM of each primer (1 μL) and 4 μL of DNA (5 ng/μL). Samples were incubated at 95 °C for 10 min, and submitted to 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Results were converted into number of parasites per nucleated cell (multiplied by one thousand to facilitate visualization).

2.8. Toxicology study

To evaluate the toxicity of the treatment, the nephrotoxicity was investigated by the dosage of urea nitrogen and creatinine, while the hepatic function was analyzed by dosage of the alanine transaminase (ALT) and aspartate transaminase (AST), in the serum samples of the treated and infected animals, by means of commercial kits (Labtest Diagnostica®, Belo Horizonte). As control, serum samples from naive (non-treated and non-infected) mice (n = 8) were used.

2.9. Statistical analysis

Results 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) and Tukey´s post-test were used for comparisons between the groups. Differences were considered significant when $P < 0.05$. The experiments were repeated and results were similar. Data shown in this study are representative of the first experiment.

3. Results

3.1. Efficacy of the therapeutics against L. amazonensis infection

The *in vivo* therapeutic efficacy employing AmB, Ambisome®, Flau-A or Flau-A/M was evaluated in BALB/c mice against *L. amazonensis* infection. For this, the average lesion diameter and parasite burden in the infected tissue, liver, spleen and draining lymph node of the animals were investigated, 30 days after the treatment. Results showed that treated and infected animals presented significant reductions in the edema in the infected tissue, when compared with the controls (saline and micelle). The reduction percentage in the lesion diameter in the

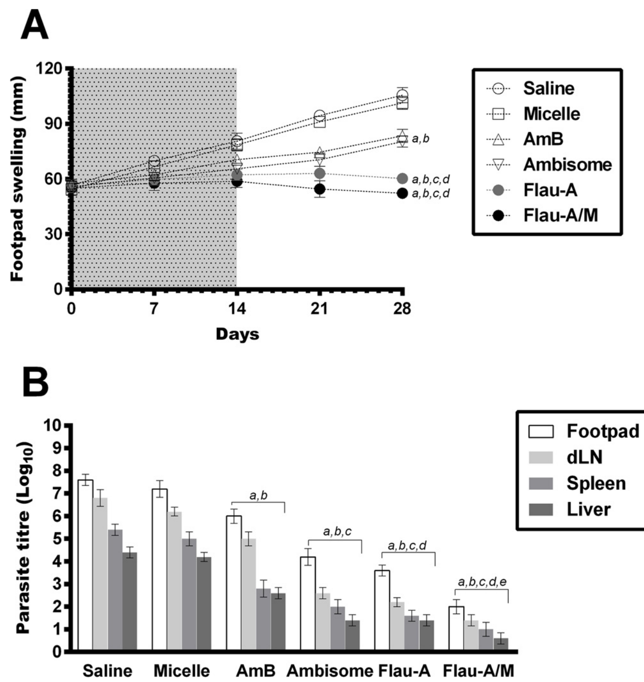

Fig. 1. Average lesion diameter and parasite burden in the treated and infected animals. BALB/c mice were infected with 10^7 *L. amazonensis* stationary promastigotes and, 50 days post-infection, they were separated into groups (n = 10 per group), and received saline or were treated with empty micelles, AmB, Ambisome®, Flau-A or Flau-A/M. The evolution of lesion size was monitored weekly, and during four weeks, in the treated and infected mice. Lines represent the lesion average diameter (area) expressed as the mean ± standard deviation of the groups (A). The parasite load was evaluated 30 days after the treatment in the infected tissue, draining lymph node, spleen, and liver by a limiting dilution technique (B). Bars represent the mean ± standard deviation of the groups. The letters *a*, *b*, *c*, and *d* indicate statistically significant differences in relation to the saline, micelle, AmB, and Ambisome® groups, respectively ($P < 0.001$).

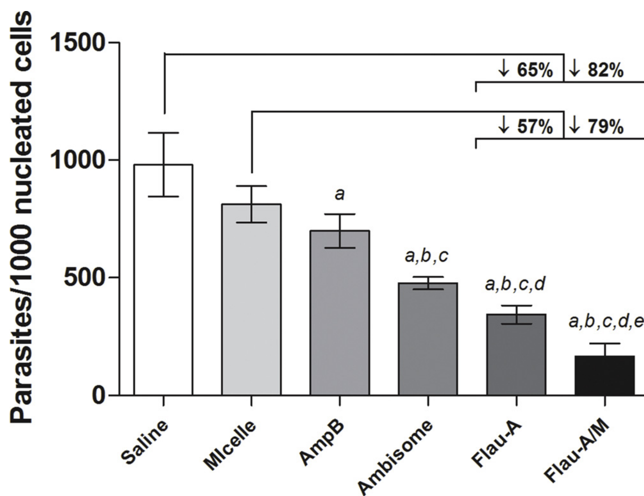

Fig. 2. Parasite burden evaluated by qPCR technique. The parasite load was also evaluated 30 days after the treatment in the infected tissue, by using a qPCR technique. The detection of parasites was expressed as number of parasites per 1000 nucleated cells in the treated and infected animals. Data are showed in linear scale, and bars represent the mean plus standard deviation of the groups. The letters *a*–*e* indicate statistically significant differences in relation to the saline, micelle, AmB, Ambisome®, and Flau-A groups, respectively ($P < 0.005$).

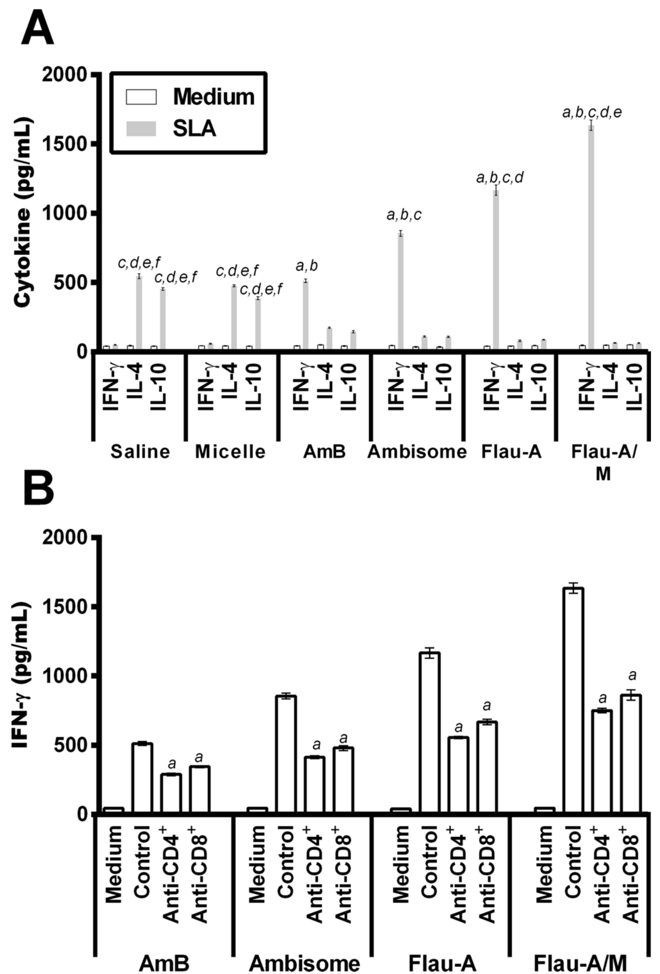

Fig. 3. Cellular response generated after the treatment. Spleen cells of the treated and infected mice (n = 10 per group) were obtained 30 days after the treatment, and cells (5×10^6) were unstimulated (medium, background control) or stimulated with *L. amazonensis* SLA (25 µg/mL) for 48 h at 37 °C in 5% CO_2. IFN-γ, IL-4, and IL-10 levels were measured in the supernatant by capture ELISA (A). The letters *a*, *b*, *c*, *d*, and *e* indicate statistically significant differences in relation to the saline, micelle, AmB, Ambisome®, and Flau-A groups, respectively ($P < 0.0001$). The involvement of $CD4^+$ and $CD8^+$ T cells in the IFN-γ production was evaluated by means of the incubation of the splenocytes (5×10^6 cells) with SLA (25 µg/mL), for 48 h at 37 °C in 5% CO_2, in the absence (control) or presence of monoclonal antibodies (mAb) against mouse $CD4^+$ or $CD8^+$. The supernatant was collected and the IFN-γ production was measured by capture ELISA. Bars represent the mean ± standard deviation of the groups. The letter *a* indicates the existence of statistically significant difference in relation to the control group ($P < 0.0001$).

AmB, Ambisome®, Flau-A and Flau-A/M groups was of 21.0%, 24.0%, 43.0%, and 51.0%, respectively, when compared to the saline group; and of 18.0%, 21.0%, 41.0%, and 49.0%, respectively, when compared to the micelle group (Fig. 1A). Flau-A and Flau-A/M-treated mice presented the most significant reductions in the average lesion diameter, in the order of 28.0% and 37.0%, respectively, when compared to the AmB group; and of 25.0% and 35.0%, respectively, when compared with the Ambisome® group. In the evaluation of the parasite load, the AmB, Ambisome®, Flau-A and Flau-A/M-treated mice´ groups showed also significant reductions in the tissue and organic parasitism, when compared to the controls, being the most significant reductions also found in the Flau-A and Flau-A/M groups (Fig. 1B). A qPCR assay was performed in the infected tissue, and results showed also that Flau-A and Flau-A/M groups were those presenting the most significant reductions in the parasitism, when compared to the others (Fig. 2).

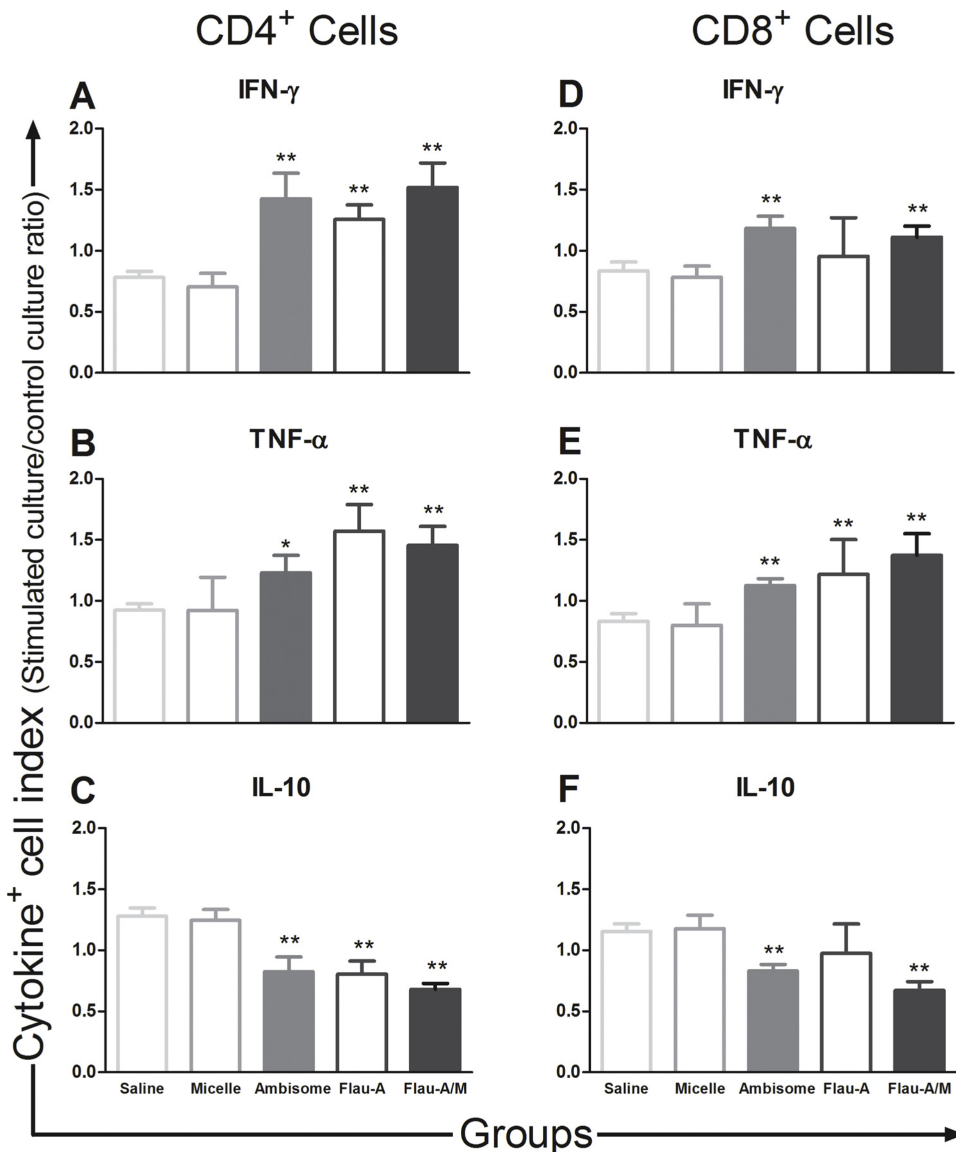

Fig. 4. Frequency of intracytoplasmic cytokine-producing CD4$^+$ and CD8$^+$ T cells. BALB/c mice were infected with 10^7 *L. amazonensis* stationary promastigotes and, 50 days post-infection, they received saline or were treated with empty micelles, Ambisome®, Flau-A or Flau-A/M. The IFN-γ, TNF-α, and IL-10-producing CD4$^+$ and CD8$^+$ T cell frequency was evaluated in the spleen cells (n = 10 per group), 30 days after the treatment. Results were reported as cytokine indexes, which were calculated by the ratio of cytokine$^+$-T cell percentage in the stimulated cultures *versus* the values obtained in the unstimulated cultures (SLA/CC ratio). Bars represent the mean plus standard deviation of the groups, for CD4$^+$ (A, B and C) and CD8$^+$ (D, E and F) T cells. (*) and (**) indicate the existence of statistically significant difference in relation to the saline and micelle groups, respectively ($P < 0.05$).

3.2. Immunogenicity generated by the therapeutics against L. amazonensis infection

The profile of Th1 and Th2 cytokines was evaluated in the cellular supernatant of the SLA-stimulated cultures, 30 days after the treatment. Results showed that spleen cells collected from the AmB, Ambisome®, Flau-A, and Flau-A/M groups produced higher IFN-γ and lower IL-4 and IL-10 levels, when compared with the values found in the controls (Fig. 3A). The involvement of CD4$^+$ and CD8$^+$ T cells in the IFN-γ production was also evaluated, and results showed a significantly lower IFN-γ production when anti-CD4 or anti-CD8 antibody was added in the cell cultures in the treated groups, thus demonstrating the importance of both T cell subtypes in the production of this cytokine (Fig. 3B). A flow cytometry assay was developed to investigate the cytokine-producing T cell frequency, and results showed that Ambisome®, Flau-A and Flau-A/M-treated mice were those presenting higher levels of IFN-γ and TNF-α-producing CD4$^+$ and CD8$^+$ T cells, when compared to the others, as well as lower levels of IL-10-producing T cell (Fig. 4); thus corroborating with the immune profile found in the ELISA experiments.

It is known that macrophages stimulated by IFN-γ are able to activate NO synthesis to destroy intracellular amastigotes. In an attempt to evaluate the parasite-specific activation of macrophages in the treated and infected animals; the nitrite, IL-12 and GM-CSF production was investigated in the cell supernatant. Results showed higher levels of these markers in the AmB, Ambisome®, Flau-A, and Flau-A/M groups; although higher production has been observed in the Flau-A and Flau-A/M groups (Fig. 5). The humoral response was evaluated, and results showed that Flau-A and Flau-A/M-treated mice also presented a predominance of antileishmanial IgG2a isotype, when compared to the IgG1 levels (Fig. 6).

3.3. Evaluation of the in vivo toxicity

The toxicity was analyzed in the treated and infected animals, and results showed significant increase in serum enzymes associated with renal and hepatic damage in free AmB-treated mice, with significantly higher levels of ALT, AST, urea, and creatinine, when compared with the values obtained in the Ambisome®, Flau-A, and Flau-A/M groups (Fig. 7). In addition, clinical signals, such as ataxia and weakness, were found in AmB-treated mice, which presented also a reduction in their body weight in the order of 9.0%, possibly reflecting the toxicity of the drug. On the other hand, Flau-A and Flau-A/M-treated mice presented positive variation in their body weight, in the order of 5.0% and 8.0%, respectively (data not shown).

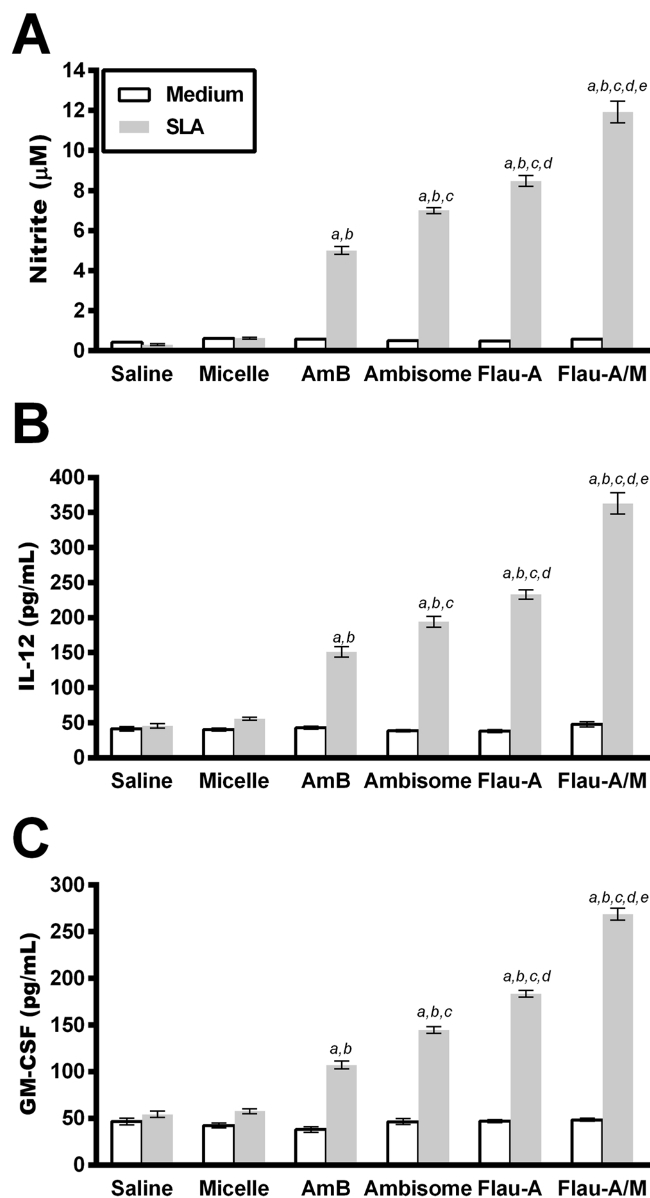

Fig. 5. Nitrite, IL-12 and GM-CSF production. Spleen cells (5×10^6) of the treated and infected mice (n = 10 per group) were collected 30 days after the treatment, and they were unstimulated (medium, background control) or stimulated with SLA (25 μg/mL), for 48 h at 37 °C in 5% CO_2. The nitrite, IL-12 and GM-CSF production was evaluated in the cell supernatant. Bars represent the mean ± standard deviation of the groups. The letters *a, b, c, d,* and *e* indicate statistically significant differences in relation to the saline, micelle, AmB, Ambisome®, and Flau-A groups, respectively ($P < 0.001$).

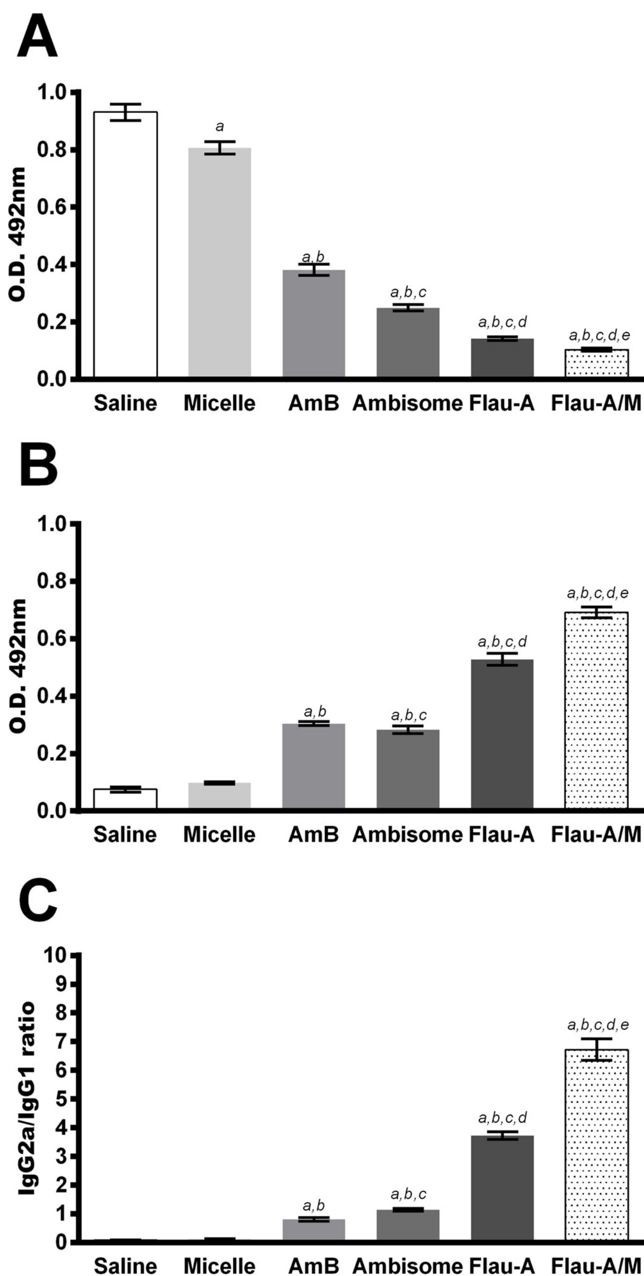

Fig. 6. Antibody production in the treated and infected animals. Sera samples of the treated and infected animals (n = 10 per group) were collected 30 days after the treatment, and they were used to evaluate the parasite-specific IgG1 (A) and IgG2a (B) isotype production. Also, ratios between the IgG2a and IgG1 levels were calculated and are showed (C). Bars represent the mean ± standard deviation of the groups. The letters *a–e* indicate statistically significant differences in relation to the saline, micelle, AmB, Ambisome®, and Flau-A groups, respectively ($P < 0.0001$).

4. Discussion

Leishmaniasis is a tropical disease affecting populations in developing countries in the world. The treatment against disease can causes toxicity, presents high cost and the parasite resistance is increasing [32]. In this scenario, we antileishmanial compounds, such as those plant-derivates, could be identified and applied for a more effective an non-toxic treatment against the disease [33,34]. A previous study demonstrated for the first time an *in vitro* effective antileishmanial activity of a quinoline derivate, Flau-A, against *L. amazonensis* and *L. infantum* species. Results pointed out also for the parasite mitochondria as being the target organelle of this antileishmanial molecule. As a consequence, in the present study, Flau-A was incorporated into a Poloxamer 407-based polymeric micelle system and tested against *L. amazonensis* infection. Results showed that this product (Flau-A/M) was highly effective against *in vivo* infection, as well as was it was not toxic after application in the mice, thus representing a new candidate to be tested against human disease.

Here, the efficacy of the therapeutics was evaluated by means of immunological and parasitological parameters, which were investigated 30 days after the treatment. Higher periods of time were not possible to be analyzed, due to the severity of the infection in control mice, as well as by the Ethical aspects in our University. Regarding to the cytokine profile, the Flau-A or Flau-A/M-treated mice developed a more polarized Th1 immune profile, which was based on higher levels

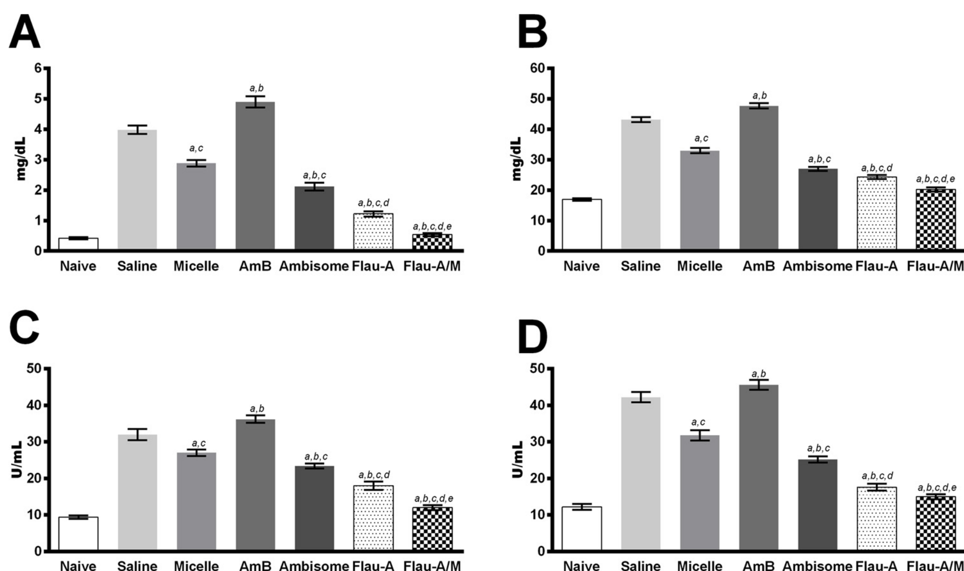

Fig. 7. Toxicity generated in the treated and infected animals. The levels of creatinine (A), urea (B), alanine aminotransferase (C), and aspartate aminotransferase (D) were evaluated in the sera samples of infected and treated mice (n = 10 per group), which were collected 30 days after the treatment. Samples of naive (non-infected and non-treated) mice (n = 8) were used as control. Bars represent the mean ± standard deviation of the groups. The letters *a–e* indicate statistically significant differences in relation to the saline, micelle, AmB, Ambisome®, and Flau-A groups, respectively ($P < 0.0001$).

of IFN-γ, IL-12 and GM-CSF. In addition, these animals produced also higher levels of antileishmanial nitrite, thus demonstrating a specific macrophage activation that can be related with the reduction in the parasite load in the infected tissue and organs of these animals. On the other hand, saline and micelle groups´ mice mounted a polarized Th2 response, which was characterized by high levels of IL-4, IL-10, and parasite-specific IgG1 antibody. Others have also found these immune correlates, when distinct biological candidates against *Leishmania* parasites are evaluated [35–37].

Plant metabolites have been investigated for their antileishmanial and antitrypanosomal activities [38]. Naphthoquinones are present in several plant families, and these products have been employed in traditional medicine as anticancer and microbicidal agents [39,40]. Due to their structural properties, quinones can participate in multiple oxidative processes. Their fundamental feature is the redox property, which is induced by means of the formation of a fully aromatic system [41]. In spite of the use of synthetic naphthoquinone derivatives, the mechanisms involved in their cytotoxicity are still unknown. The cytotoxic effects of these compounds, such as menadione, might be due to oxidative stress and arylation of cellular thiols [42]. In our model, mice that were infected and later treated with Flau-A or Flau-A/M did not present significant toxicity, when renal and hepatic damage markers were investigated. In addition, Ambisome® group mice showed also low levels of these markers, when compared with the controls; although the values have been higher in comparison to results found in Flau-A or Flau-A/M groups. Otherwise, AmB-treated mice presented significantly higher levels of AST, ALT, urea and creatinine; thus reflecting the toxicity of the drug in this mammalian model, such as showed in other studies [43–45].

Poloxamer 407 (Pluronic F127) was used as a non-ionic surfactant composed of a symmetric tri-block copolymer of propylene oxide and ethylene oxide. The polypropylene oxide block is sandwiched between the more hydrophilic polyethylene oxide blocks [46]. Micelles made of block copolymers, such as Poloxamer 407, have a hydrophobic core and a hydrophilic shell, which can effectively harbor amphiphilic moiety preventing its direct exposure to vital organs [47]. The interface formed by the hydrophilic block prevents the micellar aggregation, the protein recognition and non-specific adherence, thereby sparing body from adverse effects induced by antileishmanial molecules [48]. In addition, Poloxamer 407-based formulations have been successfully used against leishmaniasis, and they can be considered as alternative in comparison to the traditional formulations, in terms of efficacy, target orientation, toxicity, and cost [49].

Usually, these Poloxamer P407-based polymeric micelles are administered by subcutaneous route in murine models, thus turning into a semi-rigid gel when in contact with the local tissue, and creating a reservoir system aiming to maintain the product in the extracellular space to allow its action against parasites in the infection site. In course of time, as the gel matrix is diluted by body fluids and phagocytosis, the product will be gradually released into the circulation, enabling its systemic action in a controlled manner [26]. Here, Flau-A was incorporated to this delivery system, and the formed compound was showed to be highly effective against L. *amazonensis* infection. In addition, parasitological analyses demonstrated the systemic efficacy of the treatment using Flau-A/M, since significant reductions in the parasite load were also observed in spleen, liver, and dLN of the treated and infected animals. Similar results were also found when other natural or synthetic molecules were associated to delivery systems and administered in *Leishmania*-infected mice [50,51].

As limitation of our work, distinct therapeutic regimens and parasitological and immunological analyses in other periods of time were not evaluated. In addition, the products were administered by different routes in the infected animals, and it can be considered as a variable to interfere in the results obtained in our study. Nevertheless, we believe that the present work can be considered relevant, since the Flau-A-containing micelles showed an *in vivo* effective antileishmanial action, which was associated with low toxicity in our tested murine model. As a consequence, and due to its stability and easily of production, Flau-A/M can be considered in future studies as an antileishmanial agent against human leishmaniasis.

Authors' contribution

Conceived and designed the experiments: EAFC DVCM GSVT DSD DPL PAFR FL. Performed the experiments: DVCM GSVT DSD TGS LMC DSD FMO LMRA DLV MCD BMR DMS. Analyzed the data: EAFC MACF. Contributed reagents/materials/analysis tools: JMB RJA ESC. Wrote the paper: EAFC.

Conflict of interest

The authors confirm that they have no conflicts of interest in relation to this work.

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