Parasitology International 68 (2019) 63–72

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

A Pluronic® F127-based polymeric micelle system containing an antileishmanial molecule is immunotherapeutic and effective in the treatment against *Leishmania amazonensis* infection

Grasiele S.V. Tavares[a], Débora V.C. Mendonça[a], Carolina K. Miyazaki[a], Daniela P. Lage[a], Tauane G. Soyer[a], Lívia M. Carvalho[b], Flaviano M. Ottoni[c], Daniel S. Dias[a], Patrícia A.F. Ribeiro[a], Luciana M.R. Antinarelli[a], Fernanda Ludolf[a], Mariana C. Duarte[a,d], Elaine S. Coimbra[e], Miguel A. Chávez-Fumagalli[a], Bruno M. Roatt[b], Daniel Menezes-Souza[a,d], José Mário Barichello[f], Ricardo J. Alves[c], Eduardo A.F. Coelho[a,d,*]

[a] Faculdade de Medicina, Programa de Pós-Graduação em Ciências da Saúde: Infectologia e Medicina Tropical, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais 30130-100, Brazil
[b] Laboratório de Imunopatologia, Núcleo de Pesquisas em Ciências Biológicas, Universidade Federal de Ouro Preto, Ouro Preto, Minas Gerais 35400-000, Brazil
[c] Departamento de Produtos Farmacêuticos, Faculdade de Farmácia, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais 31270-901, Brazil
[d] Departamento de Patologia Clínica, COLTEC, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais 31270-901, Brazil
[e] Departamento de Parasitologia, Microbiologia e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Juiz de Fora, Juiz de Fora, Minas Gerais 36036-900, Brazil
[f] Laboratório de Tecnologia Farmacêutica, Centro de Ciências Químicas, Farmacêuticas e de Alimentos, Universidade Federal de Pelotas, Pelotas, Rio Grande do Sul 96900-010, Brazil

ARTICLE INFO

Keywords:
Visceral leishmaniasis
Treatment
Toxicity
Delivery systems
5-chloro-7-iodoquinolin-8-ol

ABSTRACT

Clioquinol (5-chloro-7-iodoquinolin-8-ol or ICHQ) was recently showed to presents an *in vitro* effective antileishmanial action, causing changes in membrane permeability, mitochondrial functionality, and parasite morphology. In the present study, ICHQ was incorporated into a Poloxamer 407-based polymeric micelles system (ICHQ/M), and its antileishmanial activity was *in vivo* evaluated in L. *amazonensis*-infected BALB/c mice. Amphotericin B (AmpB) and its liposomal formulation (Ambisome®) were used as controls. Parasitological and immunological evaluations were performed 30 days after the treatment. Results indicated more significant reductions in the average lesion diameter and parasite burden in ICHQ or ICHQ/M-treated mice, which were associated with the development of a polarized Th1 immune response, based on production of high levels of IFN-γ, IL-12, TNF-α, GM-CSF, and antileishmanial IgG2a antibody. Control groups´ mice produced high levels of IL-4, IL-10, and IgG1 isotype antibody. No organic toxicity was found by using ICHQ or ICHQ/M to treat the animals, although those receiving AmpB and Ambisome® have presented higher levels of renal and hepatic damage markers. In conclusion, results suggested that the ICHQ/M composition can be considered as an antileishmanial candidate to be tested against human leishmaniasis.

1. Introduction

Leishmaniasis is a disease complex considered endemic in 98 countries, with 350 million people at risk of contracting the infection by the *Leishmania* parasite [1]. It is a neglected disease that presents high morbidity and mortality in tropical and subtropical regions in the world, occurring mainly in Africa, Asia, Southern Europe, and South and Central Americas. Tegumentary leishmaniasis (TL), the more common clinical form of disease, can be caused by *Leishmania major, L. tropica, L. aethiopica, L. panamensis, L. mexicana, L. braziliensis,* and L. *amazonensis* species; whereas visceral leishmaniasis (VL) is caused by L. *donovani* and L. *infantum* species [2]. TL causes self-healing cutaneous lesions, although the uncontrolled parasite replication leading to mutilation and morbidity is observed in the patients. On the other hand, VL, which can be fatal if acute and untreated, results from an infection in phagocytic cells within the reticulum-endothelial system due to

* Corresponding author at: Faculdade de Medicina, Programa de Pós-Graduação em Ciências da Saúde: Infectologia e Medicina Tropical, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais 30130-100, Brazil.
E-mail address: eduardoferrazcoelho@yahoo.com.br (E.A.F. Coelho).

https://doi.org/10.1016/j.parint.2018.10.005
Received 11 September 2018; Received in revised form 12 October 2018; Accepted 15 October 2018
Available online 16 October 2018

metastasis of parasites and infected cells from the initial site of infection [3].

Leishmania amazonensis is one of the major etiologic agents of disease in the Americas, and it is associated with a broad spectrum of human leishmaniasis, ranging from cutaneous to visceral form [4,5]. This parasite species presents a wide geographical distribution, which overlaps with transmission areas of other species [6,7]. Murine models susceptible to the L. *amazonensis* infection develop a antileishmanial Th2 immune response, which is characterized by production of high levels of IL-4, IL-10, IL-13, among other anti-inflammatory cytokines; while the resistance has been associated with the development of Th1 response, which is characterized by production of IFN-γ, IL-2, IL-12, among other pro-inflammatory molecules [8,9].

The parasitological diagnosis of leishmaniasis is considered the gold standard to identify the infection; however, it requires an invasive procedure of collect of the samples, such as aspirates in organs as spleen and bone marrow, or lesion or mucosal fragments; being performed only in specialized health centers and by trained professionals [10]. In addition, the sensitivity of the tests is variable, depending on the presence of the parasite in the collected sample [11]. There are more sensitive molecular techniques to search for the parasite DNA; however, they are expensive and require sophisticated equipment, thus limiting their use [12]. Serological tests become an ideal diagnostic tool for detection of antileishmanial antibodies in sera of infected patients, due to its simplicity and low cost, and are considered less invasive [13]. As consequence, methods such as indirect immunofluorescence, direct agglutination test, enzyme-linked immunosorbent assay (ELISA), latex agglutination test, and immunochromatographic tests have been employed [14,15]. However, their serodiagnostic performance is hampered by variable sensitivity and/or specificity, thus leading to the occurrence of false-negative or false-positive results [16].

Among the more successfully tested antigens, the rK39 is a prominent. This 39-amino acid repetitive immunodominant B-cell epitope kinesin-related protein is conserved among viscerotropic *Leishmania* species [17]. It has been employed for the serodiagnosis of disease, as incorporated in rapid tests, such as rK39 dipstick and immunochromatographic tests. However, this antigen appears to be more suited for the detection of symptomatic cases with severe disease than those asymptomatic or in healthy people living in endemic areas of leishmaniasis [18]. Faced with these difficulties, an effective treatment against disease should be performed, aiming to allow the cure of the patients, as well as to inhibit the occurrence of spreading of the infection caused by dissemination of the parasites between sandflies and mammalian hosts.

The treatment against leishmaniasis is based on the use of pentavalent antimonials, although other drugs, such as amphotericin B (AmpB), pentamidine, miltefosine, and paramomycin, are used [19]. However, problems related with the toxicity and teratogenicity, treatment duration, high cost, administration route, and/or development of resistant strains have limited the efficacy of the therapeutics [20–22]. AmpB is able to binds to the membrane sterols of parasites, hampering their cell permeability and leading to the loss of cations, such as K^+, causing cell death. The toxicity of this drug is derived from its interaction with sterols in bilayer membranes, such as cell walls, causing pore formation in the membranes, in turn leading either to their rupture. To reduce the toxicity of this drug, AmpB-containing lipid formulations have been developed. A liposomal formulation (AmBisome®), an AmpB colloidal dispersion (Amphocil®), and an AmpB-lipid complex (Abelcet®) have been used for the treatment of leishmaniasis. The World Health Organization has recommended the use of these products, based on their higher levels of efficacy and safety, besides low toxicity when compared to the use of free drug. However, the main factor limiting the widespread use of these formulations has been their high cost [23,24]. In this context, and due to few current alternatives available on the market, the identification of new compounds to be used in the treatment against leishmaniasis should be considered, aiming to find novel effective, safe and economically feasible antileishmanial agents.

The growing interest in applying herbal extracts, essential oils, and natural products to treat against human disease has increased in the last decades, aiming to identify new antiparasitic targets derived from vegetable sources, as based on secondary metabolites, of which naphthoquinones and quinolines are examples [25,26]. This alternative is required, since the drug discovery is a long and expensive process, requiring an average of 10 to 20 years before a new product is identified and developed for human clinical applications. In the case of leishmaniasis, there are few new antileishmanial drugs in the pipeline, and drug resistance is increasing in distinct regions in the world [26].

Quinones are natural aromatic metabolites found in plant families, as well as in algae, fungi, and bacteria. They include anthraquinones, benzoquinones, and naphthoquinones [27,28], which present biological properties, such as microbicidal, antileishmanial and anticancer action [29–31]. Recently, a quinoline-derivate molecule, namely clioquinol, 5-chloro-7-iodoquinolin-8-ol or ICHQ, presented an *in vitro* effective antileishmanial activity against L. *amazonensis* and L. *infantum* species. The inhibition 50% of the *Leishmania* viability (IC_{50}) against *L. amazonensis* and *L. infantum* promastigotes was of 2.55 ± 0.25 and 1.44 ± 0.35 μg/mL, respectively, and of 1.88 ± 0.13 and 0.98 ± 0.17 μg/mL against the amastigote forms, respectively. On the other hand, the inhibition of 50% of the macrophage viability (CC_{50}) was of 255 ± 23 μg/mL, with selectivity index of 99.9 and 177.1 against promastigotes, respectively, and of 135.6 and 260.1 against amastigotes, respectively [32].

In this context, in the present study, the *in vivo* antileishmanial activity of ICHQ was evaluated in chronically L. *amazonensis*-infected BALB/c mice. This molecule was incorporated to a Poloxamer 407 (Pluronic® F127)-based micelle system, and the therapeutic efficacy of the product was compared with the use of free AmpB and Ambisome®. The compounds were administered in the infected mice, and the formulation efficacy was investigated 30 days after the treatment, by means of the evaluation of the immune response and parasite load in the treated and infected animals.

2. Materials and methods

2.1. Pharmaceutics and formulation of the ICHQ-containing micelle system

Poloxamer 407 (Pluronic® F127) and ICHQ were purchased from Sigma-Aldrich (St. Louis, MO, USA), with catalog numbers 16,758 and 130–26-7, respectively. AmpB (Cristália, São Paulo, São Paulo, Brazil) was resuspended (1 mg) in methanol/DMSO (9:1 *v*/v) solution, and was maintained at −80° until use. AmBisome® (Gilead Sciences, Inc. San Dimas, USA) was obtained as a lyophilized powder (50 mg), and resuspended in water to prepare a stock solution (4 mg/mL). ICHQ-containing micelles (ICHQ/M) were prepared as described [26]. Briefly, Poloxamer 407 (18% *w*/w) was diluted in phosphate buffer (PBS) pH 7.4 under magnetic agitation for 18 h, at 4 °C. Eight milligrams of ICHQ were added to 500 μL of dichloromethane PA, and solubilized by using vortex. The mixture was added to the previously prepared solution under vigorous magnetic agitation and in an ice bath, until a viscous emulsion has been obtained. The dichloromethane was evaporated using rotary evaporate (Buchi, Flawil, Switzerland), and the formulation was obtained as a transparent yellow gel at room temperature. Empty micelles (18% *w*/w) were prepared using the same technical protocol.

2.2. Mice and parasites

BALB/c mice (female, 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

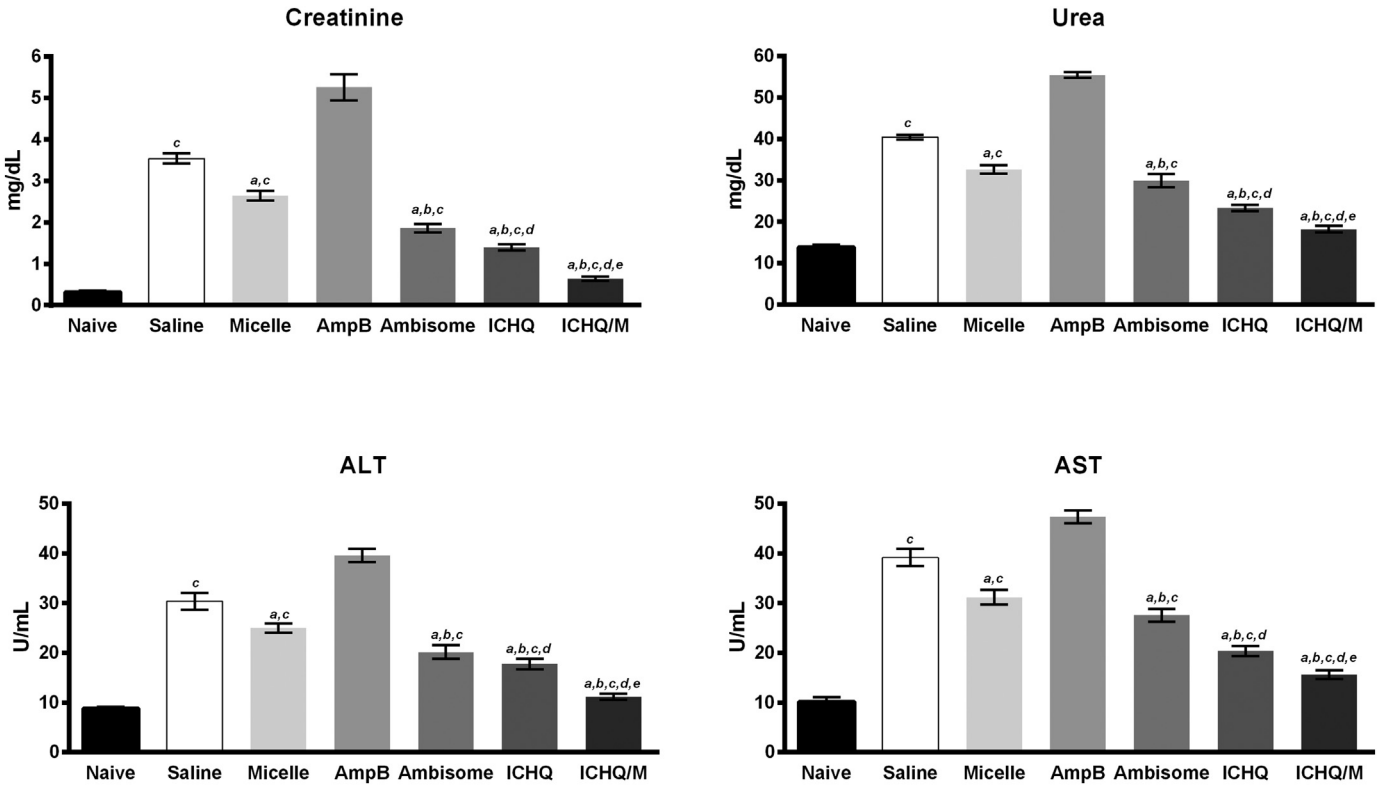

Fig. 1. Toxicity induced in the treated and infected animals. The levels of creatinine, urea, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were evaluated in sera samples of infected and treated mice (n = 10 per group), 30 days after the treatment. Samples of naive (non-infected and non-treated) mice were used as control. 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, AmpB, Ambisome®, and ICHQ groups, respectively ($P < 0.0001$).

maintained under pathogen-free conditions. The study was approved by Committee for the Ethical Handling of Research Animals (CEUA) of UFMG (protocol number 085/2017). *L. amazonensis* (IFLA/BR/1967/PH-8) stationary promastigotes were grown in complete Schneider's medium (Sigma-Aldrich, St. Louis, MO, USA), which was composed by Schneider's medium added with 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. The soluble *Leishmania* antigenic (SLA) extract was prepared as described [33].

2.3. Infection and treatment schedules

BALB/c mice (n = 10 per group) were infected with 10^7 *L. amazonensis* stationary promastigotes through subcutaneous injection in the base of the tail, after trichotomy. After the development of ulcerated lesions (at approximately 50 days post-infection), animals were divided into groups according to lesion size (2 to 3 mm), aiming to ensure similar average lesion diameter between the groups. Then, mice were treated with one of the following therapeutic schedules: (a) Control group (saline): mice received 50 µL of PBS pH 7.4 by subcutaneous route, once a day during 15 days; (b) Empty micelle (micelle) group: mice received 50 µL of non-incorporated micelle (10 mg/kg body weight) by subcutaneous route, every two days and during 15 days; (c) Amphotericin B (AmpB) group: mice received 50 µL of a solution containing 1 mg/kg body weight by intraperitoneal route, every two days and during 15 days; (d) Ambisome® group: mice received 50 µL of a solution containing 2 mg/kg body weight by intravenous route, every five days and during 15 days; (e) ICHQ group: mice received 50 µL of a solution containing 10 mg/kg body weight by subcutaneous route, every two days and during 15 days; and (f) ICHQ/micelle (ICHQ/M) group: mice received 50 µL of micelle incorporated with ICHQ (5 mg/kg body weight) by subcutaneous route, every two days and during 15 days. Further observations including the occurrence of nodules, as well as metastasis and other clinical signals in the treated and infected animals were performed. After the treatment, the lesion average diameter was measured weekly using an electronic caliper (799-6/150 model, Starrett®, Brazil). Thirty days after the treatment, animals were euthanized and parasitological and immunological parameters were evaluated to investigate the treatment efficacy.

2.4. Assays for toxicity

To evaluate the toxicity of the treatment, the hepatic function was analyzed by dosage of the alanine transaminase (ALT) and aspartate transaminase (AST), whereas the nephrotoxicity was evaluated by the levels of blood urea nitrogen and serum creatinine in serum samples of the treated and infected animals using commercial kits (Labtest Diagnostica®, Belo Horizonte). Serum samples from naive (non-treated and non-infected) mice (n = 6) were used as control.

2.5. Determination of the parasite burden

2.5.1. Limiting dilution technique

The parasitism was evaluated in the infected tissue, draining lymph node, spleen, and liver of the animals, by limiting dilution technique [34] and RT-PCR assay [35]. For this, tissue and organs were 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 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

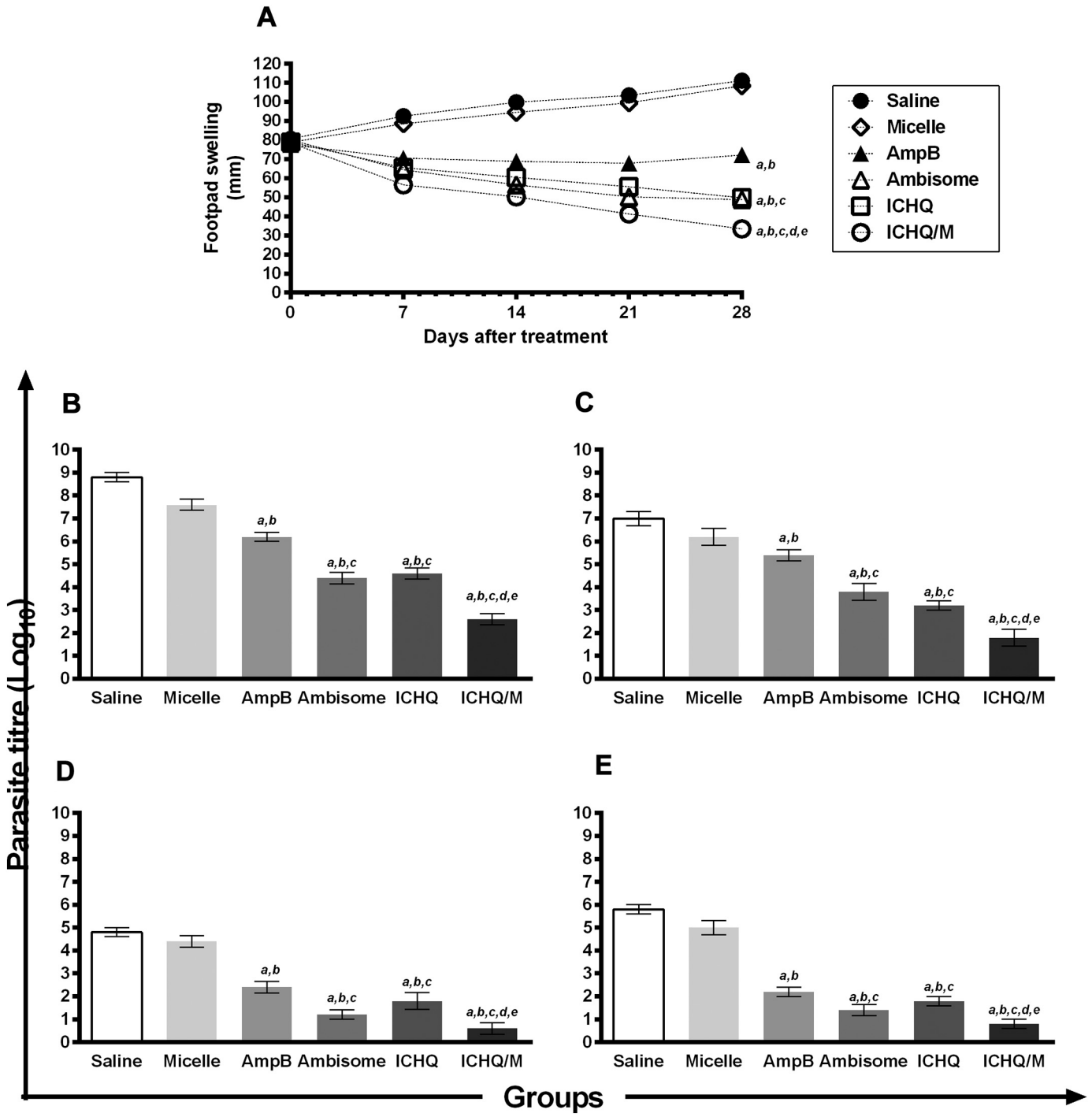

Fig. 2. Evaluation of infection and parasite burden in the treated animals. BALB/c mice were subcutaneously infected with 10^7 *L. amazonensis* stationary promastigotes. At approximately 50 days post-infection, when animals presented lesion size between 2 and 3 mm, they were divided into groups (n = 10 per group), and received saline or were treated with micelle, AmpB, Ambisome®, ICHQ or ICHQ/M. The lesion development was monitored weekly and during 4 weeks. The 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 treatments in the infected tissue (B), draining lymph nodes (C), spleen (D), and liver (E), by a limiting dilution technique. 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, AmpB, Ambisome®, and ICHQ groups, respectively ($P < 0.001$).

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.

2.5.2. RT-PCR

The parasite load was also evaluated by RT-PCR technique. For this, lesion fragment DNA was extracted using a phenol-chloroform method. Five hundred microliters of lysis buffer solution [50 mM Tris, 50 mM NaCl, and 10 mM EDTA (pH 8.0)], 1% Triton X-100 and 20 μL of proteinase K solution (20 mg/mL, Promega) were added to each fragment (20 mg). The mixture was incubated for 16 h at 37 °C, and 500 μL of phenol (Sigma-Aldrich) were added, homogenized by inversion for 10 min and centrifuged at 10,000 × *g* for 5 min. The supernatant was

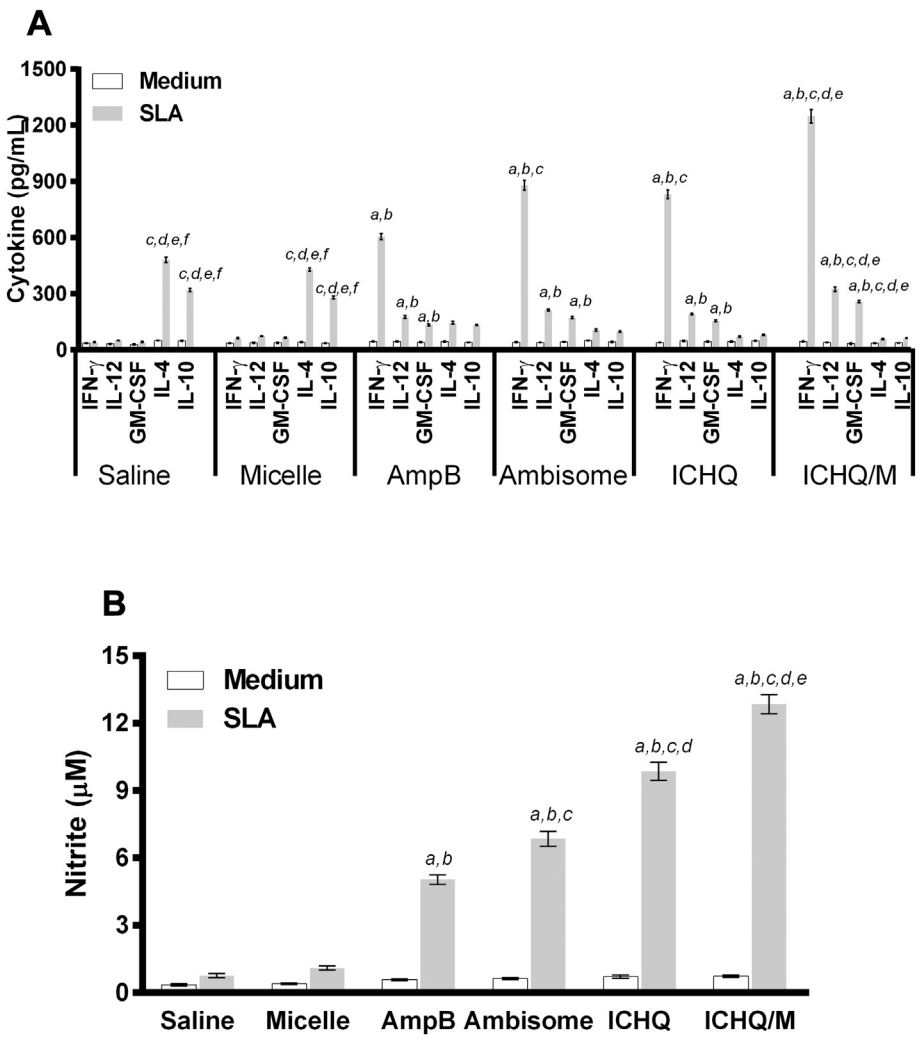

Fig. 3. Cellular response developed after the treatment. Single cells suspensions were obtained from the spleen of treated and infected mice (n = 10 per group), 30 days after the treatment. Then, cells (5×10^6) were unstimulated (medium, background control) or stimulated with L. *amazonensis* SLA (25 μg/mL), for 48 h at 37 °C in 5% CO_2. IFN-γ, IL-4, IL-10, IL-12p70 and GM-CSF levels were measured in the supernatants by a capture ELISA (A). The nitrite production was evaluated in the cell supernatant by Griess reaction (B). 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, AmpB, Ambisome®, and ICHQ groups, respectively ($P < 0.001$).

transferred to a new tube and this step was repeated. Fifty-hundred microliters of chloroform-isoamyl alcohol (24:1; Sigma-Aldrich) were added and the solution was homogenized by inversion and centrifuged at 10,000 x *g* for 5 min. The sample was transferred for a new tube, and 500 μL of ethanol 95% (*v*/v) were added. The material was incubated for 10 min in ice bath for DNA precipitation, and thus centrifuged at 14,000 x *g* for 10 min. Then, 500 μL of ethanol 70% (v/v) were added to pellet, which was resuspended and centrifuged in the same conditions. The supernatant was removed and the sample was dried at room temperature. The resulting DNA was resuspended in 100 μL of milli-Q water, and was amplified using the *Forward* (CCTATTTTACACCAACC CCCAGT) and *Reverse* (GGGTAGGGGCGTTCTGCGAAA) primers. The mouse β-actin gene (*Forward*: CAGAGCAAGAGAGGTATCC and *Reverse*: TCATTGTAGAAGGTGTGGTGC) was used as endogenous control and to verify sample's integrity. Standard curves were obtained from DNA extracted from 10^8 parasites for kDNA and from 10^8 peritoneal macrophages for β-actin, under the same conditions used to extract the other samples. PCR was performed on StepOne™ Instrument (48 wells-plate; Life Technologies) using 2× 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 (in log) per nucleated cell (multiplied by one thousand to facilitate visualization).

2.6. Cellular response

2.6.1. Capture ELISA

Splenocytes were collected to evaluate the cellular response 30 days after the treatment. For this, cells (5×10^6) were plated in 24-well plates (Nunc) in duplicate and incubated in complete DMEM (medium), which was composed by the medium plus 20% FBS and 20 mM L-glutamine pH 7.4. Then, cells 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, IL-10, IL-12p70 and GM-CSF levels were measured in the culture supernatants by capture ELISA, by 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, according described [35]. Aiming to block the IL-12, $CD4^+$ and $CD8^+$ mediated T cell cytokine release, splenocytes of treated mice were *in vitro* stimulated with L. *amazonensis* SLA (25 μg/mL) and incubated with 5 μg/mL of monoclonal antibodies (mAb) against mouse IL-12 (C17.8), CD4 (GK 1.5) or CD8 (53–6.7). Appropriate isotype-matched controls – rat IgG2a (R35–95) and rat IgG2b (95–1) – were employed in the assays. Antibodies (no azide/low endotoxin™) were purchased from BD (Pharmingen®).

2.6.2. Flow cytometry

A flow cytometry assay was also performed to evaluate the IFN-γ, TNF-α and IL-10-producing $CD4^+$ and $CD8^+$ T cell frequency. For this, splenocytes (5×10^6) were incubated in complete RPMI 1640 medium

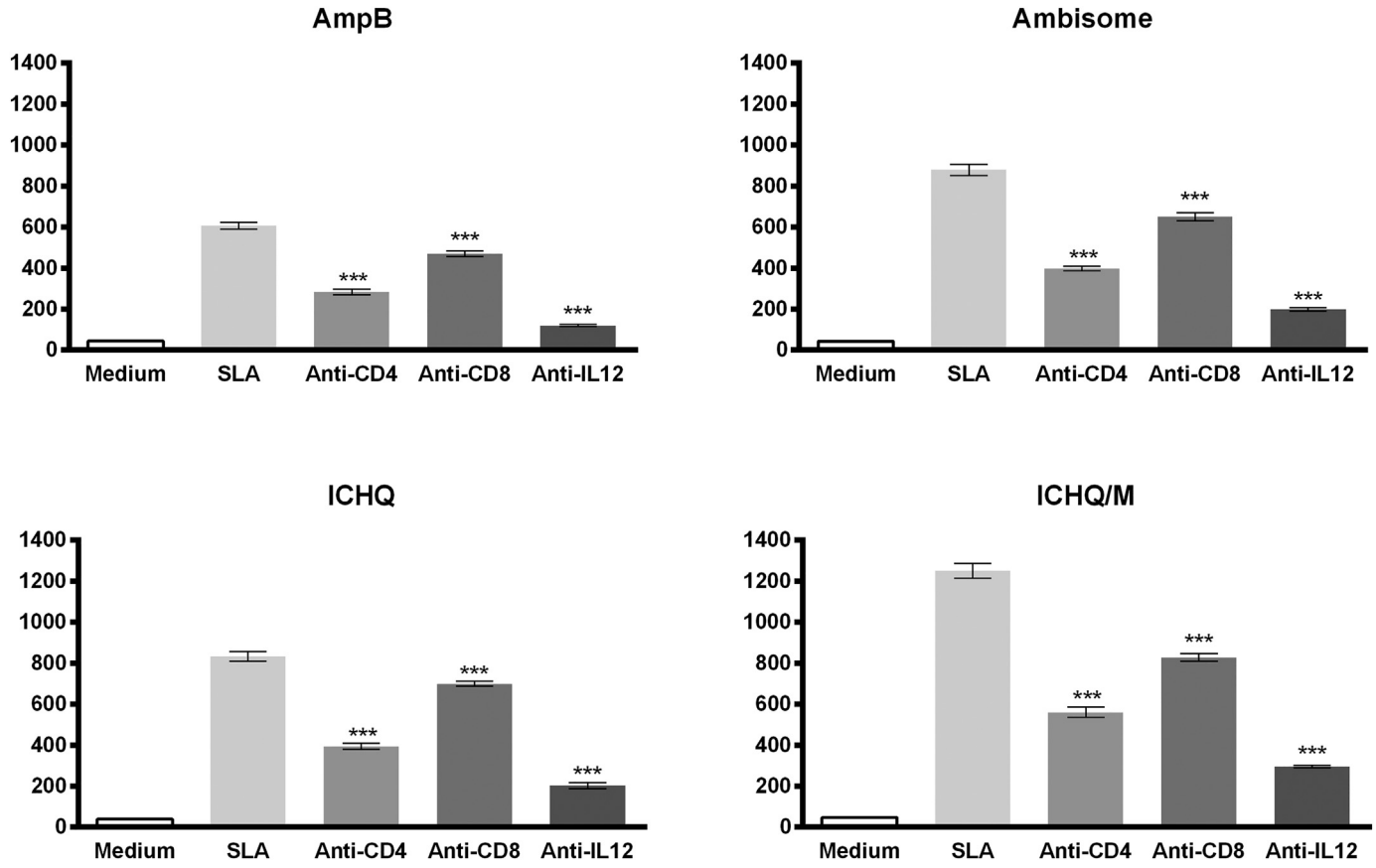

Fig. 4. Evaluation of the IFN-γ production by $CD4^+$ and $CD8^+$ T cells in the treated and infected animals. Spleen cells (5×10^6 cells) of AmpB, Ambisome®, ICHQ or ICHQ/M groups´ mice (n = 10 per group) were stimulated with SLA (25 μg/mL), for 48 h at 37 °C in 5% CO_2, in the absence (control) or presence of monoclonal antibodies (mAb) against mouse IL-12, $CD4^+$ or $CD8^+$. The supernatants were collected, and the IFN-γ production was analyzed by capture ELISA. Bars represent the mean ± standard deviation of the groups. (***) indicates the existence of statistically significant difference in relation to the unstimulated or SLA-stimulated cultures ($P < 0.001$).

in polypropylene tubes (Pharmingen®), and were unstimulated (medium) or stimulated with L. *amazonensis* SLA (25 μg/mL), for 48 h at 37 °C in 5% CO_2. Then, cells were incubated with phorbol myristate acetate (PMA, 25 ng/mL) and ionomycin (1 μg/mL), which were diluted in complete RPMI 1640 medium. The IFN-γ, TNF-α and IL-10-producing $CD4^+$ and $CD8^+$ T cell frequency was evaluated following an analysis based on their relative flow cytometry size (forward laser scatter – FSC) and granularity (side laser scatter – SSC) graphs. After the selection of the interest region R1 containing FSCLow and SSCLow phenotype cells, graphs of density plot distribution of CD4/FL1 or CD8/FL1 *versus* IFN-γ/$FL2^+$, TNF-α/$FL2^+$, and IL-10/$FL2^+$ cells were performed to determine the IFN-γ^+, TNF-α^+, and IL-10^+ T cell frequency in the cultures. Results were expressed as indexes, which were calculated by the ratio between the cytokine-producing T cell percentages *versus* the values found in the unstimulated cultures (SLA/CC ratio).

2.7. Humoral response

The antileishmanial IgG1 and IgG2a isotypes production was evaluated in serum samples from the treated and infected animals. For this, SLA was used as an antigen (1.0 μg per well), and serum samples were diluted at 1:100 in PBS-T (PBS 1× plus 0.05% Tween 20), with an incubation occurring for 1 h at 37 °C. After washing plates seven times, anti-mouse IgG total, IgG1 and IgG2a horseradish-peroxidase conjugated antibodies (Sigma-Aldrich) were added (in 1:10,000, 1:5000 and 1:10,000 dilutions, respectively, in PBS-T), and reaction was 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.

2.8. 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 with $P < 0.05$. The experiments were repeated, and the results were similar between them. Data shown in this study are representative of the first experiment.

3. Results

3.1. Toxicological study in the treated and infected mice

A toxicological study was performed in the treated and infected animals 30 days after the treatment. Results showed significant increase in serum enzymes, which were associated with renal and hepatic damage, since significantly higher levels of ALT, AST, urea, and creatinine were found in the AmpB-treated animals, when compared to the values found in the ICHQ and ICHQ/M groups. These groups presented also lower values of these enzymes in comparison to results obtained in the Ambisome® group (Fig. 1). Clinical symptoms such as ataxia and weakness were observed in the AmpB group, with 10% reduction in the

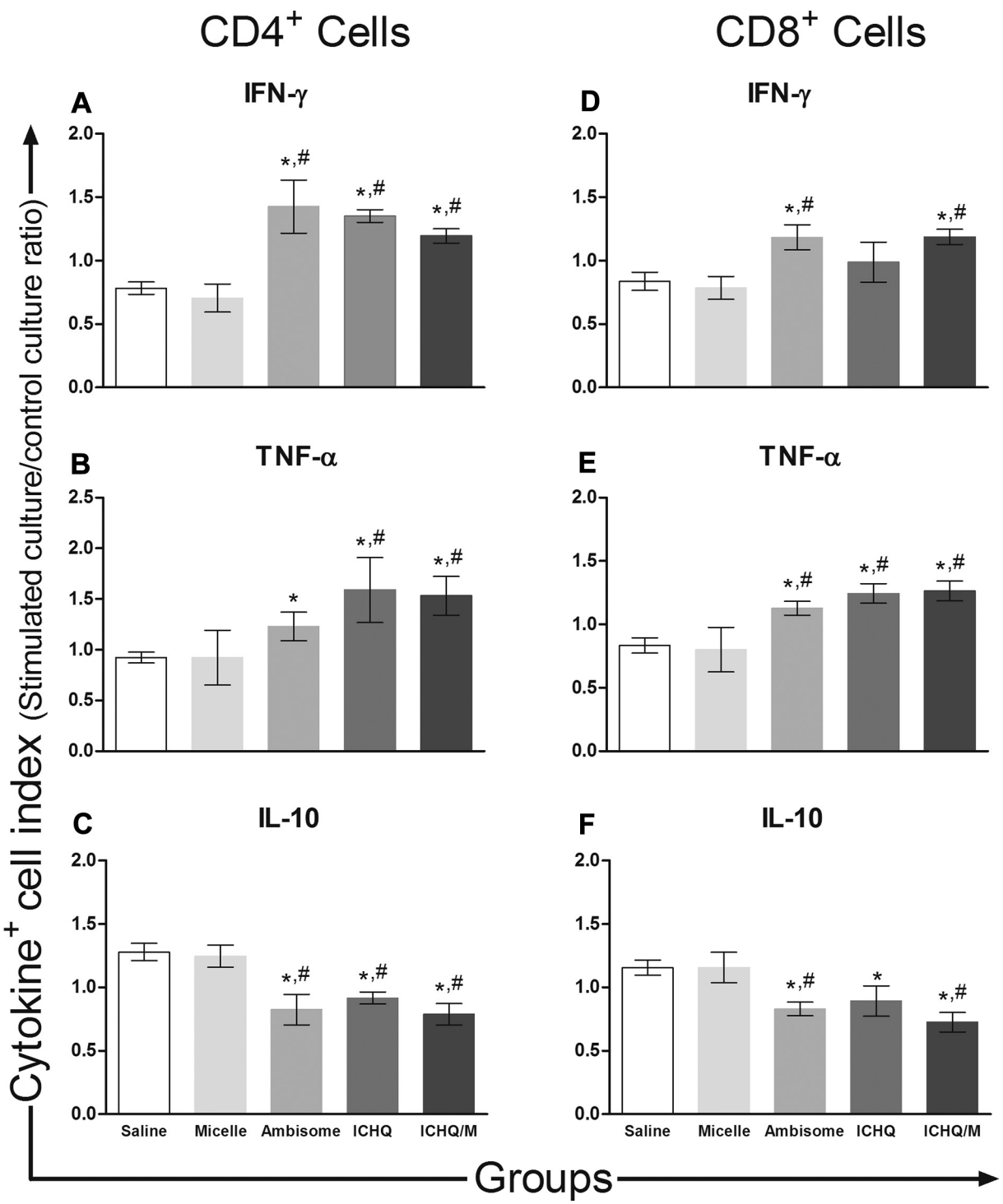

Fig. 5. Profile of intracytoplasmic cytokine-producing T cells. BALB/c mice were subcutaneously infected with 10^7 *L. amazonensis* stationary promastigotes, and after 50 days they received saline or were treated with micelle, Ambisome®, ICHQ or ICHQ/M. The cytokine (IFN-γ, TNF-α, and IL-10)-producing $CD4^+$ and $CD8^+$ T cell frequency in their spleen cells (n = 10 per group) was evaluated 30 days after the treatment. Results were reported as cytokine indexes, which were calculated by the ratio of cytokine$^+$-cells present in the SLA-stimulated cultures *versus* those found in the unstimulated (control) cultures (SLA/CC ratio). Bars represent the mean ± standard deviation of the groups for $CD4^+$ (panels A, B and C) and $CD8^+$ (panels D, E and F) T cells. * and # indicate the existence of statistically significant difference in relation to the saline and micelle groups, respectively ($P < 0.05$).

animals´ body weight, possibly reflecting the *in vivo* toxicity of the drug. On the other hand, ICHQ and ICHQ/M-treated mice showed a positive variation in their body weight, in the order of 4% and 8%, respectively (data not shown).

3.2. Parasitological evaluation in the treated and infected animals

The efficacy of the therapeutics was evaluated 30 days after the treatment of L. *amazonensis*-infected BALB/c mice, by measuring the average lesion diameter and parasite burden in the infected tissue, liver, spleen and draining lymph nodes of the animals. Results showed that

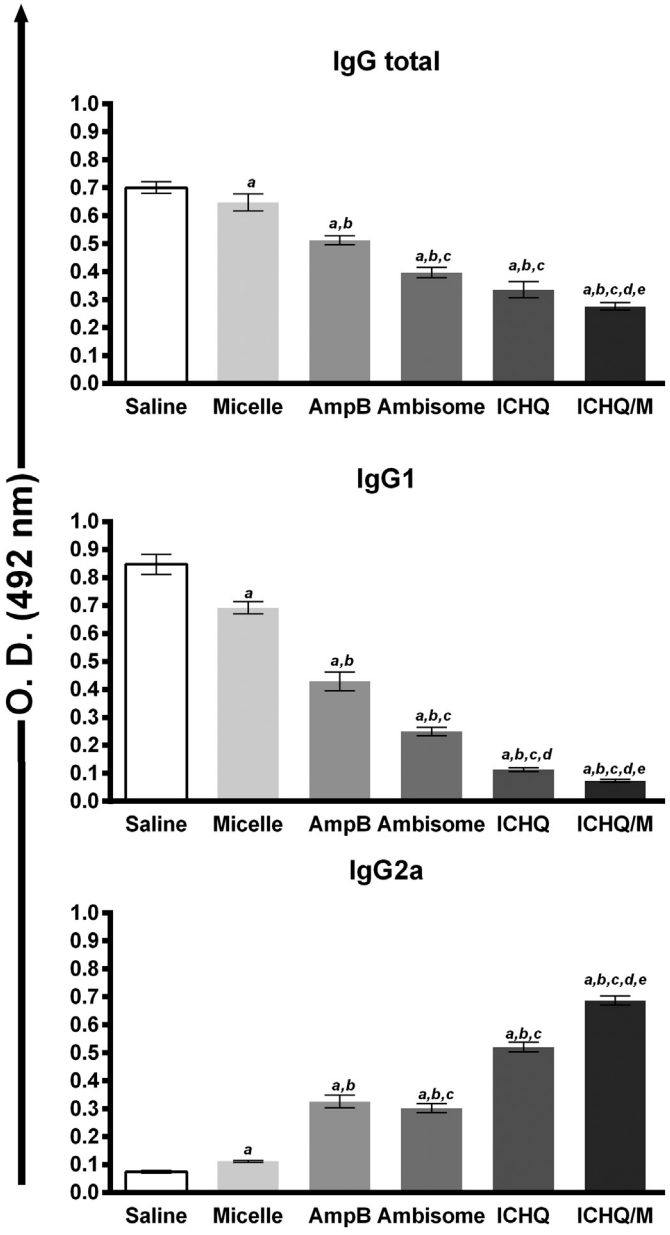

Fig. 6. Humoral response developed after the treatment. Serum samples were obtained of infected and treated mice (n = 10 per group) 30 days after the treatments, and the antileishmanial IgG total, IgG1 and IgG2a production was investigated. Bars represent the mean ± standard deviation of the groups. The letters *a*, *b*, *c*, *d*, and *e* indicate statistically significant differences in relation to the saline, micelle, AmpB, Ambisome®, and ICHQ groups, respectively ($P < 0.0001$).

ICHQ and ICHQ/M-treated mice displayed significant reductions in their average lesion diameter, when compared to values found in the saline, micelle and AmpB groups (Fig. 2A). Data obtained in the ICHQ and Ambisome® groups were similar; however, animals that were treated with ICHQ/M presented lower results in the average lesion diameter, when compared to the others. The parasite burden showed also that ICHQ/M group´ mice presented the best protective result, while the ICHQ and Ambisome® groups showed similar parasitism found in the tissue and organs (Fig. 2B). The RT-PCR assay showed also significant reductions in the parasite load in the Ambisome®, ICHQ, and ICHQ/M groups, in the order of 55.0%, 64.0%, and 88.0%, respectively, when compared with the saline group (data not shown).

3.3. Cellular and humoral response generated in the treated and infected mice

The immune response developed in the treated and infected animals was evaluated 30 days after the treatment. Results showed a antileishmanial Th1 profile generated in all treated animals, based on significantly higher levels of IFN-γ, IL-12 and GM-CSF cytokines, when compared to the saline and micelle groups. Between the treated animals, those receiving ICHQ or ICHQ/M presented a more polarized Th1 response, when compared to the results found in the AmpB and Ambisome® groups (Fig. 3A). In addition, saline and micelle groups´ mice produced higher levels of antileishmanial IL-4 and IL-10. The nitrite production was also measured in the culture supernatant, and results showed that ICHQ or ICHQ/M-treated animals presented higher levels of this molecule; in comparison to the values obtained in AmpB and Ambisome® groups´ mice (Fig. 3B). The involvement of $CD4^+$ and $CD8^+$ T cells in the IFN-γ production was evaluated. Results showed this cytokine was significantly inhibited when anti-CD4 antibody was added to the *in vitro* cultures. Otherwise, the addition of anti-CD8 antibody decreased also the IFN-γ production in the cultures; although the levels of this molecule have been higher, when compared to the use of anti-CD4 antibody (Fig. 4).

The flow cytometry assay showed also that ICHQ or ICHQ/M-treated mice presented the higher IFN-γ and TNF-α-producing $CD4^+$ and $CD8^+$ T cell frequency, when compared to the other groups (Fig. 5). On the other hand, saline and micelle groups´ mice showed higher $IL\text{-}10^+$ T cell frequency. The humoral response was also evaluated, and results showed that ICHQ or ICHQ/M-treated mice produced higher levels of antileishmanial IgG2a isotype, when compared to the IgG1 levels, thus indicating also a polarized Th1 response in these animals (Fig. 6).

4. Discussion

This work represents a continuation of a previous study developed to identify new antileishmanial molecules, where ICHQ showed a selective *in vitro* antileishmanial action against L. *infantum* and L. *amazonensis* species, leading to alterations in the membrane permeability, mitochondrial function, and parasite morphology [32]. ICHQ was also effective in reduce the infection in treated and infected macrophages, as well as to inhibit the infection of these cells by using pretreated parasites. In the present study, the *in vivo* antileishmanial activity of ICHQ, which was incorporated to a Poloxamer 407-based micelle system, was evaluated against L. *amazonensis* infection in BALB/c mice. Immunological and parasitological analyses showed that this composition was highly effective in treat chronically infected mice, based on significant reductions in the parasite burden analyzed in different organs of the animals.

The inherent disadvantages related with the toxicity of traditional drugs have spurred the pharmaceutical industry to identify new delivery systems, since the design and development of new products is a long and expensive task [36,37]. In this context, AmpB has been applied in the treatment of leishmaniasis [38,39], since it interacts with the ergosterol in the parasite membrane causing leishmanicidal action, although it also forms pores in cholesterol-containing membranes present in mammalian hosts, thus explaining the cytotoxic activity of this drug [40]. AmpB-containing lipid formulations are less toxic to the patients; however, their high cost limits their application in underdeveloped countries [21,41,42]. As a consequence, new studies developed to identify new delivery systems to old or new candidates will to reduce their toxicity, as well as allow to the production of formulations with a lower cost [43,44].

Copolymers based on combinations between hydrophilic and hydrophobic products have been used to incorporate antileishmanial molecules [26,45–47]. In particular, a Poloxamer 407-based copolymer was recently used in association with a quinoline derivate, namely 8-

hydroxyquinoline (8-HQN), and the formed compost was well-successfully used to treat L. *amazonensis*-infected BALB/c mice [46]. In the present work, ICHQ was incorporated to this delivery system and applied in a lower dose in infected animals. Our results pointed out to most significant reductions in the lesion average diameter and parasite load in the treated animals, when compared to values found in the controls. Although Ambisome® has been also effective in treat infected animals; the parasite burden was higher in comparison to the values found in the ICHQ/M group. This fact can be considered relevant, since ICHQ-containing polymeric micelles are cheaper to produce, with an estimated value of ten times less in comparison for the production of AmpB-containing liposomal formulations.

Parasitological and immunological analyses were developed 30 days after the treatment, since our purpose was to wait the end of the administration of the therapeutics to investigate their long-term efficacy [48]. Higher periods of time were not possible to be investigated, due to the severity of the infection in the controls, as well as by the Ethical aspects in our University. Interestingly, despite the heavy infection in the saline group, ICHQ or ICHQ/M-treated mice showed significantly lower lesion diameter, which was correlated with significant reductions in the parasite burden in infection site (infected tissue and popliteal lymph nodes) and organs (liver and spleen) of the animals, when compared to the results found in the other groups. Although free AmpB or Ambisome®-treated mice have also presented reduction in the parasitism, it was less significant in comparison to the values found in the ICHQ and ICHQ/M groups. Similar results were found when other natural or synthetic molecules were associated to delivery systems and administered in *Leishmania*-infected mice, in comparison to the use of old and/or conventional drugs [49–51].

An important problem related to the use of AmpB is the organic toxicity found in the treated hosts. Alterations in renal and hepatic functions are important dose-limiting side effects, and the dosage of organic markers, such as AST, ALT, urea and creatinine, could help to evaluate internal damages caused by this product [52–54]. In our study, higher levels of AST, ALT, urea and creatinine were found in AmpB-treated animals. These mice showed also reductions in their body weight, possibly reflecting the toxicity of the drug. On the other hand, upon the treatment with ICHQ or ICHQ/M, the enzymatic markers were found in low levels, characterizing the absence or low toxicity induced by these products in the treated mice. Although Ambisome® group mice have showed also low levels of these enzymes, the values were higher in comparison to those found in the ICHQ and ICHQ/M groups.

The immune response evaluated after the treatment showed that ICHQ or ICHQ/M groups´ mice produced higher levels of Th1 cytokines (IFN-γ, IL-12, TNF-α, and GM-CSF), besides high levels of parasite-specific IgG2a isotype and antileishmanial nitrite, whereas control groups´ animals produced higher levels of Th2 cytokines (IL-4 and IL-10). In this context, our results pointed out to the profile of the development of an immune stimulation induced by ICHQ and ICHQ/M through Th1 cytokine-driven and macrophage-mediated mechanisms, which were NO-dependent, to treat L. *amazonensis*-infected animals. Similar immunological correlates using other antileishmanial molecules were also found by others [9,55,56].

Works evaluating antileishmanial molecules have showed that mitochondria are important targets in *Leishmania* parasites, which are responsible by their mechanism of death. Since this organelle is unique in these protozoa, the collapse of the mitochondrial membrane potential can causes the *Leishmania* death by means of events related with apoptosis and/or necrosis [55–57]. In fact, quinolines, such as ICHQ, have showed to alter the *Leishmania* membrane potential, thus leading to an increase in the production of reactive oxygen species and parasite's death. Previous results using ICHQ showed that it induced significant permeabilization of the *Leishmania* membrane, when compared to the untreated cells, corroborating the findings related with other quinoline derivates [58].

As a limiting factor of the present study, distinct therapeutic regimens, such as the reduction of the number of doses, parasitological investigation in other periods of time after the treatment, and the inclusion of other antileishmanial molecules as additional controls were not performed. Nevertheless, we believe that the present work can be considered adequate, since the ICHQ-containing micellar composition showed an *in vivo* effective action against *Leishmania*, as well as did not cause toxicity in the treated animals. As a consequence, and due to its stability, low cost, and easily of production, the ICHQ/M formulation could be considered as an antileishmanial candidate to be tested against human disease.

Conflict of interest

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

Acknowledgments

The authors thank the Program for Technological Development in Tools for Health-PDTIS-FIOCRUZ for use of its facilities. This work was supported by grants from CAPES, FAPEMIG (CBB-APQ-00819-12 and CBB-APQ-01778-2014) and CNPq (APQ-482976/2012-8, APQ-488237/2013-0, and APQ-467640/2014-9). MACF is a grant recipient of FAPEMIG/CAPES. EAFC, BMR and DMS are grants recipient of CNPq.

References

[1] World Health Organization, Leishmaniasis, http://www.who.int/topics/leishmaniasis/en/, (2016) , Accessed date: 13 August 2018.
[2] J. Alvar, I.D. Vélez, C. Bern, M. Herrero, P. Desjeux, J. Cano, J. Jannin, M. den Boer, W.H.O. Leishmaniasis Control Team, Leishmaniasis worldwide and global estimates of its incidence, PLoS One 7 (2012) e35671.
[3] I. Kevric, M.A. Cappel, J.H. Keeling, New World and Old World Leishmania Infections: A Practical Review, Dermatol. Clin. 33 (2015) 579–593.
[4] A. Barral, D. Pedral-Sampaio, H. Momen, D. Mahon-Pratt, A.R. Jesus, R. Almeida, R. Badaró, M. Barral-Neto, E.M. Carvalho, W.D. Johnson, G.Jr. Grimaldi, Leishmaniasis in Bahia, Brazil: evidence that *Leishmania amazonensis* produces a wide spectrum of clinical disease, Am. J. Trop. Med. Hyg. 44 (1991) 536–546.
[5] L. Soong, C.A. Henard, P.C. Melby, Immunopathogenesis of non-healing American cutaneous leishmaniasis and progressive visceral leishmaniasis, Semin. Immunopathol. 34 (2012) 735–751.
[6] G. Grimaldi, R.B. Tesh, Leishmaniasis of the New World: current concepts and implications for future research, Clin. Microbiol. Rev. 6 (1993) 230–250.
[7] A.P. Fernandes, E.A.F. Coelho, G.L.L. Machado-Coelho, G. Grimaldi, R.T. Gazzinelli, Making an anti-amastigote vaccine for visceral leishmaniasis: rational, update and perspectives, Curr. Opin. Microbiol. 15 (2012) 476–485.
[8] P. Scott, Development and regulation of cell-mediated immunity in experimental leishmaniasis, Immunol. Res. 27 (2003) 489–498.
[9] K.F. Nascimento, F.R. de Santana, C.R.V. da Costa, V. Kaplum, H. Volpato, C.V. Nakamura, L.V. Bonamin, D. de Freitas Buchi, D, M1 homeopathic complex trigger effective responses against Leishmania (L) amazonensis in vivo and in vitro, Cytokine 99 (2017) 80–90.
[10] H. Sakkas, C. Gartzonika, S. Levidiotou, Laboratory diagnosis of human visceral leishmaniasis, J. Vector Borne Dis. 53 (2016) 8–16.
[11] C.M. Ruiter, C. Van der Veer, M.M. Leeflang, S. Deborggraeve, C. Lucas, E.R. Adams, Molecular tools for diagnosis of visceral leishmaniasis: systematic review and meta-analysis of diagnostic test accuracy, J. Clin. Microbiol. 52 (2014) 3147–3155.
[12] H. Goto, L. Lindoso, Current diagnosis and treatment of cutaneous and mucocutaneous leishmaniasis, Expert, Rev. Anti Infect. Ther. 8 (2010) 419–433.
[13] O.P. Singh, S. Sundar, Developments in diagnosis of visceral leishmaniasis in the elimination era, J. Parasitol. Res. 2015 (2015) 239469.
[14] E.K. Elmahallawy, A. Sampedro-Martinez, J. Rodriguez-Granger, Y. Hoyos-Mallecot, A. Agil, J.M. Navarro-Mari, J. Gutierrez-Fernandez, Diagnosis of leishmaniasis, J. Infect. Dev. Ctries. 8 (2014) 961–972.
[15] N.G. Schwarz, U. Loderstaedt, A. Hahn, R. Hinz, A.E. Zautner, D. Eibach, M. Fischer, R.M. Hagen, H. Frickmann, Microbiological laboratory diagnostics of neglected zoonotic diseases (NZDs), Acta Trop. 165 (2017) 40–65.
[16] M. Farahmand, H. Nahrevanian, H, Application of recombinant proteins for serodiagnosis of visceral leishmaniasis in humans and dogs, Iran Biomed, J. 20 (2016) 128–134.
[17] G. Matlashewski, V.N. Das, K. Pandey, D. Singh, S. Das, A.K. Ghosh, R.N. Pandey, P. Das, Diagnosis of visceral leishmaniasis in Bihar India: comparison of the rK39 rapid diagnostic test on whole blood versus serum, PLoS Negl. Trop. Dis. 7 (2013) e2233.
[18] K. Ritmeijer, Y. Melaku, M. Mueller, S. Kipngetich, C. O'Keeffe, R.N. Davidson, Evaluation of a new recombinant K39 rapid diagnostic test for Sudanese visceral

leishmaniasis, Am. J. Trop. Med. Hyg. 74 (2006) 76–80.
[19] S. Sundar, A. Singh, Recent developments and future prospects in the treatment of visceral leishmaniasis, Ther. Adv. Infect. Dis. 3 (2016) 98–109.
[20] S. Kapil, P.K. Singh, O. Silakari, An update on small molecule strategies targeting leishmaniasis, Eur. J. Med. Chem. 157 (2018) 339–367.
[21] S. Sundar, A. Singh, Chemotherapeutics of visceral leishmaniasis: present and future developments, Parasitology 145 (2018) 481–489.
[22] B. Zulfiqar, T.B. Shelper, V.M. Avery, Leishmaniasis drug discovery: recent progress and challenges in assay development, Drug Discov. Today 22 (2017) 1516–1531.
[23] K. Pandey, B. Pal, N.A. Siddiqui, V.N. Rabi Das, K. Murti, C.S. Lal, N. Verma, R. Babu, V. Ali, R. Kumar, P. Das, Efficacy and safety of liposomal amphotericin B for visceral Leishmaniasis in children and adolescents at a tertiary care Center in Bihar, India, Am. J. Trop. Med. Hyg. 97 (2017) 1498–1502.
[24] V. Mosimann, A. Neumayr, D.H. Paris, J. Blum, Liposomal amphotericin B treatment of Old World cutaneous and mucosal leishmaniasis: a literature review, Acta Trop. 182 (2018) 246–250.
[25] M. Soosaraei, M. Fakhar, S. Hosseini Teshnizi, H. Ziaei Hezarjaribi, E.S. Banimostafavi, Medicinal plants with promising antileishmanial activity in Iran: a systematic review and meta-analysis, Ann. Med. Surg. (Lond). 21 (2017) 63–80.
[26] D.V.C. Mendonça, V.T. Martins, D.P. Lage, D.S. Dias, P.A.F. Ribeiro, A.M.R.S. Carvalho, A.L.T. Dias, C.K. Miyazaki, D. Menezes-Souza, B.M. Roatt, C.A.P. Tavares, J.M. Barichello, M.C. Duarte, E.A.F. Coelho, Comparing the therapeutic efficacy of different amphotericin B-carrying delivery systems against visceral leishmaniasis, Exp. Parasitol. 186 (2018) 24–35.
[27] S.L. Castro, F.S. Emery, E.N. da Silva Júnior, Synthesis of quinoidal molecules: strategies towards bioactive compounds with an emphasis on lapachones, Eur. J. Med. Chem. 69 (2013) 678–700.
[28] E. Moreno, J. Schwartz, E. Larrea, I. Conde, M. Font, C. Sanmartín, J.M. Irache, S. Espuelas, Assessment of β-lapachone loaded in lecithin-chitosan nanoparticles for the topical treatment of cutaneous leishmaniasis in L. major infected BALB/c mice, Nanomedicine 11 (2015) 2003–2012.
[29] E. Vargas, F. Echeverri, Y.A. Upegui, S.M. Robledo, W. Quiñones, Hydrazone derivatives enhance antileishmanial activity of thiochroman-4-ones, Molecules 23 (2017) E70.
[30] S.K. Ghosh, A. Ganta, R.A. Spanjaard, Discovery and cellular stress pathway analysis of 1,4-naphthoquinone derivatives with novel, highly potent broad-spectrum anticancer activity, J. Biomed. Sci. 25 (2018) 12.
[31] X. Li, X. Jia, H. Niu, Nanostructured lipid carriers co-delivering lapachone and doxorubicin for overcoming multidrug resistance in breast cancer therapy, Int. J. Nanomedicine 13 (2018) 4107–4119.
[32] G.S.V. Tavares, D.V.C. Mendonça, D.P. Lage, J.D.T. Granato, F.M. Ottoni, F. Ludolf, M.A. Chávez-Fumagalli, M.C. Duarte, C.A.P. Tavares, R.J. Alves, E.S. Coimbra, E.A.F. Coelho, Antileishmanial activity, Cytotoxicity and Mechanism of Action of Clioquinol against Leishmania infantum and Leishmania amazonensis Species, Basic Clin. Pharmacol. Toxicol. 123 (2018) 236–246.
[33] E.A. Coelho, C.A.P. Tavares, F.A.A. Carvalho, K.F. Chaves, K.N. Teixeira, R.C. Rodrigues, H. Charest, G. Matlashewski, R.T. Gazzinelli, A.P. Fernandes, Immune responses induced by the Leishmania (Leishmania) donovani A2 antigen, but not by the LACK antigen, are protective against experimental Leishmania (Leishmania) amazonensis infection, Infect. Immun. 71 (2003) 3988–3994.
[34] M.C. Duarte, L.M. Lage, D.P. Lage, V.T. Martins, A.M. Carvalho, B.M. Roatt, D. Menezes-Souza, C.A. Tavares, R.J. Alves, J.M. Barichello, E.A. Coelho, Treatment of murine visceral leishmaniasis using an 8-hydroxyquinoline-containing polymeric micelle system, Parasitol. Int. 65 (2016) 728–736.
[35] D.S. Dias, P.A.F. Ribeiro, V.T. Martins, D.P. Lage, F.F. Ramos, A.L.T. Dias, M.R. Rodrigues, A.S.B. Portela, L.E. Costa, R.B. Caligiorne, B.T. Steiner, M.A. Chávez-Fumagalli, B.C.S. Salles, T.T.O. Santos, J.A.G. Silveira, D.F. Magalhães-Soares, B.M. Roatt, R.A. Machado-De-Ávila, M.C. Duarte, D. Menezes-Souza, E.S. Silva, A.S. Galdino, E.A.F. Coelho, Recombinant prohibitin protein of Leishmania infantum acts as a vaccine candidate and diagnostic marker against visceral leishmaniasis, Cell. Immunol. 323 (2018) 59–69.
[36] A. Machado-Silva, P.P. Guimarães, C.A. Tavares, R.D. Sinisterra, New perspectives for leishmaniasis chemotherapy over current anti-leishmanial drugs: a patent landscape, Expert Opin. Ther. Pat. 25 (2015) 247–260.
[37] L. Almeida, A. Terumi Fujimura, M.L. Del Cistia, B. Fonseca-Santos, K. Braga Imamura, P.A.M. Michels, M. Chorilli, M.A.S. Graminha, Nanotechnological strategies for Treatment of Leishmaniasis–a Review, J. Biomed. Nanotechnol. 13 (2017) 117–133.
[38] C. Bern, J. Adler-Moore, J. Berenguer, M. Boelaert, M. den Boer, R.N. Davidson, C. Figueras, L. Gradoni, D.A. Kafetzis, K. Ritmeijer, E. Rosenthal, C. Royce, R. Russo, S. Sundar, J. Alvar, Liposomal amphotericin B for the treatment of visceral leishmaniasis, Clin. Infect. Dis. 43 (2006) 917–924.
[39] M.M. Borges, M.C. Pranchevicius, E.F. Noronha, G.A. Romero, C.O. Carranza-Tamayo, Efficacy and safety of amphotericin B deoxycholate versus N-methylglucamine antimoniate in pediatric visceral leishmaniasis: an open-label, randomized, and controlled pilot trial in Brazil, Rev. Soc. Bras. Med.Trop. 50 (2017) 67–74.
[40] A.H. Mohamed-Ahmed, S. Brocchini, S.L. Croft, Recent advances in development of amphotericin B formulations for the treatment of visceral leishmaniasis, Curr. Opin. Infect. Dis. 25 (2012) 695–702.
[41] P.K. Singh, V.K. Pawar, A.K. Jaiswal, Y. Singh, C.H. Srikanth, M. Chaurasia, H.K. Bora, K. Raval, J.G. Meher, J.R. Gayen, A. Dube, M.K. Chourasia, Chitosan coated PluronicF127 micelles for effective delivery of Amphotericin B in experimental visceral leishmaniasis, Int. J. Biol. Macromol. 105 (2017) 1220–1231.
[42] G.J. Wijnant, K. Van Bocxlaer, V. Yardley, A. Harris, M. Alavijeh, R. Silva-Pedrosa, S. Antunes, I. Mauricio, S. Murdan, S.L. Croft, Comparative efficacy, toxicity and biodistribution of the liposomal amphotericin B formulations Fungisome® and AmBisome® in murine cutaneous leishmaniasis, Int. J. Parasitol. Drugs Drug Resist. 8 (2018) 223–228.
[43] G. Carneiro, M.G. Aguiar, A.P. Fernandes, L.A. Ferreira, Drug delivery systems for the topical treatment of cutaneous leishmaniasis, Expert. Opin. Drug. Deliv. 9 (2012) 1083–1097.
[44] M.A. Chávez-Fumagalli, T.G. Ribeiro, R.O. Castilho, S.O. Fernandes, V.N. Cardoso, C.S. Coelho, D.V. Mendonça, M. Soto, C.A. Tavares, A.A. Faraco, E.A. Coelho, New delivery systems for amphotericin B applied to the improvement of leishmaniasis treatment, Rev. Soc. Bras. Med. Trop. 48 (2015) 235–242.
[45] R. Tyagi, S. Lala, A.K. Verma, A.K. Nandy, S.B. Mahato, A. Maitra, M.K. Basu, Targeted delivery of arjunglucoside I using surface hydrophilic and hydrophobic nanocarriers to combat experimental leishmaniasis, J. Drug Target. 13 (2005) 161–171.
[46] L.M. Lage, J.M. Barichello, D.P. Lage, D.V. Mendonça, A.M. Carvalho, M.R. Rodrigues, D. Menezes-Souza, B.M. Roatt, R.J. Alves, C.A. Tavares, E.A. Coelho, M.C. Duarte, An 8-hydroxyquinoline-containing polymeric micelle system is effective for the treatment of murine tegumentary leishmaniasis, Parasitol. Res. 115 (2016) 4083–4095.
[47] V.P. Torchilin, T.S. Levchenko, K.R. Whiteman, A.A. Yaroslavov, A.M. Tsatsakis, A.K. Rizos, E.V. Michailova, M.I. Shtilman, Amphiphilic poly-N-vinylpyrrolidones: synthesis, properties and liposome surface modification, Biomaterials 22 (2001) 3035–3044.
[48] D.M. Casa, D.B. Scariot, N.M. Khalil, C.V. Nakamura, R.M. Mainardes, Bovine serum albumin nanoparticles containing amphotericin B were effective in treating murine cutaneous leishmaniasis and reduced the drug toxicity, Exp. Parasitol. 192 (2018) 12–18.
[49] E. Sobarzo-Sánchez, P. Bilbao-Ramos, M. Dea-Ayuela, H. González-Díaz, M. Yañez, E. Uriarte, L. Santana, V. Martínez-Sernández, F. Bolás-Fernández, F.M. Ubeira, Synthetic oxoisoaporphine alkaloids: in vitro, in vivo and in silico assessment of antileishmanial activities, PLoS One 8 (2013) e77560.
[50] J.L. Ulloa, R. Spina, A. Casasco, P.B. Petray, V. Martino, M.A. Sosa, F.M. Frank, L.V. Muschietti, Germacranolide-type sesquiterpene lactones from Smallanthus sonchifolius with promising activity against Leishmania mexicana and Trypanosoma cruzi, Parasit. Vectors 10 (2017) 567.
[51] N. Tajuddeen, M.B. Isah, M.A. Suleiman, F.R. van Heerden, M.A. Ibrahim, The chemotherapeutic potential of chalcones against leishmaniases: a review, Int. J. Antimicrob. Agents 51 (2018) 311–318.
[52] T.G. Ribeiro, J.R. Franca, L.L. Fuscaldi, M.L. Santos, M.C. Duarte, P.S. Lage, V.T. Martins, L.E. Costa, S.O. Fernandes, V.N. Cardoso, R.O. Castilho, M. Soto, C.A. Tavares, A.A. Faraco, E.A. Coelho, M.A. Chávez-Fumagalli, An optimized nanoparticle delivery system based on chitosan and chondroitin sulfate molecules reduces the toxicity of amphotericin B and is effective in treating tegumentary leishmaniasis, Int. J. Nanomedicine 9 (2014) 5341–5353.
[53] L.R. Caldeira, F.R. Fernandes, D.F. Costa, F. Frézard, L.C. Afonso, L.A. Ferreira, Nanoemulsions loaded with amphotericin B: a new approach for the treatment of leishmaniasis, Eur. J. Pharm. Sci. 70 (2015) 125–131.
[54] D.C.M. Santos, M.L. Lima, J.S. Toledo, P.A. Fernandes, M.M.G. Aguiar, Á. López-Gonzálvez, L.A.M. Ferreira, A.P. Fernandes, C. Barbas, Metabolomics as a tool to evaluate the toxicity of formulations containing amphotericin B, an antileishmanial drug, Toxicol. Res. (Camb). 5 (2016) 1720–1732.
[55] L.M. Antinarelli, R.M. Dias, I.O. Souza, W.P. Lima, J. Gameiro, A.D. Silva, E.S. Coimbra, 4-aminoquinoline derivatives as potential antileishmanial agents, Chem. Biol. Drug Des. 86 (2015) 704–714.
[56] L.M. Fidalgo, L. Gille, Mitochondria and trypanosomatids: targets and drugs, Pharm. Res. 28 (2011) 2758–2770.
[57] E.S. Coimbra, R. Carvalhaes, R.M. Grazul, P.A. Machado, M.V. Souza, A.D. Silva, Synthesis, cytotoxicity and antileishmanial activity of some N-(2-(indol-3-yl)ethyl)-7-chloroquinolin-4-amines, Chem. Biol. Drug Des. 75 (2010) 628–631.
[58] G.S.V. Tavares, D.V.C. Mendonça, D.P. Lage, J.D.T. Granato, F.M. Ottoni, F. Ludolf, M.A. Chávez-Fumagalli, M.C. Duarte, C.A.P. Tavares, R.J. Alves, E.S. Coimbra, E.A.F. Coelho, Antileishmanial activity, cytotoxicity and mechanism of action of clioquinol against Leishmania infantum and Leishmania amazonensis species, Basic Clin. Pharmacol. Toxicol. 123 (2018) 236–246.