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Treatment of murine visceral leishmaniasis using an 8-hydroxyquinoline-containing polymeric micelle system

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ABSTRACT

New therapeutics are urgently needed to treat visceral leishmaniasis (VL). Due to the fact that drug discovery is a long and expensive process, the development of delivery systems to carry old and toxic drugs could be considered, as well as the evaluation of new molecules that have already shown to present biological activity. In this context, the present study evaluated the *in vitro* and *in vivo* antileishmanial activity of an 8-hydroxyquinoline (8-HQN)-containing polymeric micelle (8-HQN/M) system against *Leishmania infantum*, the main causative agent of VL in the Americas. The experimental strategy used was based on the evaluation of the parasite load by a limiting-dilution technique in the spleen, liver, bone marrow and draining lymph nodes of the infected and treated animals, as well as by a quantitative PCR (*q*PCR) technique to also assess the splenic parasite load. The immune response developed was evaluated by the production of IFN-γ, IL-4, IL-10, IL-12 and GM-CSF cytokines, as well as by antileishmanial nitrite dosage and antibodies production. Hepatic and renal enzymes were also investigated to verify cellular injury as a result of treatments toxicity. In the results, 8-HQN/M-treated mice, when compared to the other groups: saline, free amphotericin B (AmpB, as a drug control), 8-HQN and B-8-HQN/M (as a micelle control) showed more significant reductions in their parasite burden in all evaluated organs. These animals also showed an antileishmanial Th1 immunity, which was represented by high levels of IFN-γ, IL-12, GM-CSF and nitrite, associated with a low production of IL-4 and IL-10 and *anti-Leishmania* IgG1 isotype antibodies. In addition, any hepatic or renal damage was found in these treated animals. In conclusion, 8-HQN/M was effective in treating *L. infantum*-infected BALB/c mice, and can be considered alone, or combined with other drugs, as an alternative treatment for VL.

1. Introduction

Visceral leishmaniasis (VL) is an important disease worldwide leading to nearly 50,000 deaths annually [1–3]. The disease has gained greater importance in HIV co-infected patients as an opportunistic infection in areas where both infections are endemic [4]. In Brazil, VL is caused by *Leishmania infantum* species, where dogs are the main domestic reservoirs of the parasites and play an important role in the transmission cycle between humans and sandflies [5,6]. Symptomatic VL is characterized by clinical manifestations such as irregular fever, weight loss, pallor, splenomegaly, pancytopenia and it also carries a high risk of mortality in the absence of an adequate treatment [7,8].

Chemotherapy based on the administration of pentavalent antimonials has been used to treat the disease [9]. Although these products are considered effective [10], there are evidences that their efficacy depends on the *Leishmania* species, geographic region, presence of resistant strains, therapeutic schemes and other factors [11–13]. In addition, the high incidence of side effects associated with toxicity in

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the liver, kidneys and spleen of the patients contributes to limit their use in clinical practice [14,15]. Other drugs have been also used to treat VL, although they also present problems related to their side effects and/or high cost [16]. Since the drug discovery is a very long and expensive process, an alternative way could be based on the development of technologies to improve the drugs delivery for treating this disease [17–19]. In addition to reducing the toxicity of conventional drugs, these systems can also diminish the number of doses administered to the patients, due to a slower liberation of the therapeutics, as well as exert a synergistic effect with the pharmaceutics employed [20]. In this context, studies for the development of polymeric micelle as delivery systems have been developed, based on the physicochemical and immune stimulatory properties of these products, which make them suitable to be used as drug delivery [21].

8-hydroxyquinoline (8-HQN) and its derivates have been experimentally evaluated in different studies as chemotherapeutic agents [22,23], presenting antimicrobial activity [24,25] and associated in the treatment of neurodegenerative diseases, such as Alzheimer's and Parkinson's disease [26]. In leishmaniasis, previous studies have shown that 8-HQN inhibited the growth of *L. panamensis, L.* tropica and *L. major* promastigotes [27,28]. In addition, in a recent study developed by our group, the *in vitro* antileishmanial activity of 8-HQN against stationary promastigotes and intra-macrophage amastigotes of *L. amazonensis, L. braziliensis* and *L. infantum* species was shown, and it was associated with a low toxicity in murine macrophages and in human red cells [29].

In this context, the purpose of the present study was to evaluate an 8-HQN-containing polymeric micelle (8-HQN/M) system developed to treat VL. BALB/c mice were infected with *L. infantum* stationary promastigotes and the parasite load in different organs was evaluated using the limiting dilution and quantitative PCR (*q*PCR) techniques. In addition, the immune response developed in the infected and treated animals was also assessed, determining the cytokine production by a capture ELISA and by flow cytometry; as well as investigating the nitrite production and humoral response in these animals. In addition, the treatments toxicity was evaluated by the measurement of renal and hepatic damage markers. Clinical symptoms and signals were also assessed.

2. Materials and METHODS

2.1. Preparation of the 8-HQN-incorporated polymeric micelle and AmpB

The 8-HQN-containing polymeric micelle system was prepared as described [30]. Briefly, P407 poloxamer (18% w/w) was diluted in a phosphate buffer pH 7.4 (PBS 1×) under moderate magnetic agitation for 18 h at 4 °C. Then, 8 mg of 8-HQN (ACS reagent 99%, catalog 252565, Sigma-Aldrich, St. Louis, MO, USA) were added to a new tube containing 500 μL of dichloromethane PA, and solubilized using vortex. The solution was immediately added to the prepared P407 poloxamer, under vigorous magnetic agitation and in an ice bath, until a viscous emulsion was obtained. The dichloromethane was evaporated by rotary evaporation (Buchi, Flawil, Switzerland), and the final concentration of the formulation (8 mg/mL) was adjusted using purified water. This product is a transparent yellow gel, at room temperature. Empty micelle (18% *w*/w) were prepared using the same protocol, but without adding 8-HQN. All products were maintained at room temperature, until their use. Free AmpB (1 mg; Cristália, São Paulo, São Paulo, Brazil) was resuspended in 2 mL of a methanol/DMSO (9:1 v/v) solution, and this preparation was maintained at −80 °C, until use.

2.2. Animals

This study was approved by the Committee on the Ethical Handling of Research Animals (CEUA) from UFMG, Belo Horizonte, Minas Gerais, Brazil, with the protocol number 182/2012. 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); and were maintained under specific pathogen-free conditions.

2.3. Parasites

L. infantum (MHOM/BR/1970/BH46) strain was used. Parasites were grown in complete Schneider's medium (Sigma-Aldrich), which was composed by Schneider's medium plus 20% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich), 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 *L. infantum* antigenic (SLA) extract was prepared as described previously [31]. The amastigote-like forms were prepared following a technical protocol described by [32] and modified by [33]. Briefly, 1×10^9 stationary-phase promastigotes were washed in sterile PBS 1×. Then, parasites were incubated in the presence of 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 Neubauer chamber, and their morphology was evaluated after staining by Giemsa [34].

2.4. In vitro antileishmanial activity

The inhibition of the *Leishmania* growth was assessed *in vitro* by cultivating *L. infantum* stationary promastigotes (1×10^6 cells) in the presence of serial dilutions of 8-HQN/M or B-8-HQN/M (700, 350, 175, 87.5, 43.75, 21.88, 10.93, 5.47, 2.74, 1.37 and 0.68 μM) in 96-well culture plates (Nunc, Nunclon®, Roskilde, Denmark), for 48 h at 24 °C. AmpB (10.8, 5.4, 2.7, 1.35, 0.68, 0.34, 0.17, 0.08 and 0.04 μM) was used as control. Cell viability was assessed by MTT method, according previously described [34]. The 50% inhibitory concentration (IC_{50}) of the compounds was determined by applying the sigmoidal regression of the concentration-response curves. Data shown are representative of three different experiments, performed in triplicate, which presented similar results.

2.5. Cytotoxicity assay

The cytotoxicity was evaluated in murine macrophages and in human red cells. 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 in the presence of serial dilutions of 8-HQN/M or B-8-HQN/M (700, 350, 175, 87.5, 43.75, 21.88, 10.93, 5.47, 2.74, 1.37 and 0.68 μM) in 96-well plates (Nunc), for 48 h at 37 °C. The inhibition of 50% of the macrophage viability (CC_{50}) was assessed by cleavage of 2 mg/mL of MTT. AmpB (10.8, 5.4, 2.7, 1.35, 0.68, 0.34, 0.17, 0.08 and 0.04 μM) was used as control. The selectivity index (SI) was determined by calculating the ratio between the CC_{50} and IC_{50} values. The hemolytic activity was investigated by incubating serial dilutions of 8-HQN/M or B-8-HQN/M (700, 350, 175, 87.5, 43.75, 21.88, 10.93, 5.47, 2.74, 1.37 and 0.68 μM) with a 5% human red blood cell suspension, for 1 h at 37 °C, according a technical protocol described [34]. The compounds concentration needed to cause 50% of hemolysis in human red cells (RBC_{50}) was determined by applying a sigmoidal regression using the concentration-response curves. Data shown are representative of three independent experiments, performed in triplicate, which presented similar results.

2.6. In vivo infection and treatment

BALB/c mice (n = 12 per group) were subcutaneously infected into the right hind footpad with 1×10^7 stationary promastigotes of *L. infantum*. Forty-five days after infection, animals were treated daily, from day 0 to day 15 after the first administration of the dose, by

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