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Therapeutic efficacy of artemisinin-loaded nanoparticles in experimental visceral leishmaniasis

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ABSTRACT

Visceral leishmaniasis (VL) is a fatal vector-borne parasitic syndrome attributable to the protozoa of the *Leishmania donovani* complex. The available chemotherapeutic options are not ideal due to their potential toxicity, high cost and prolonged treatment schedule. In the present study, we conjectured the use of nano drug delivery systems for plant-derived secondary metabolite; artemisinin as an alternative strategy for the treatment of experimental VL. Artemisinin-loaded poly lactic co-glycolic acid (ALPLGA) nanoparticles prepared were spherical in shape with a particle size of 220.0 ± 15.0 nm, 29.2 ± 2.0% drug loading and 69.0 ± 3.3% encapsulation efficiency. ALPLGA nanoparticles administered at doses of 10 and 20 mg/kg body weight showed superior antileishmanial efficacy compared with free artemisinin in BALB/c model of VL. There was a significant reduction in hepatosplenomegaly as well as in parasite load in the liver (85.0 ± 5.4%) and spleen (82.0 ± 2.4%) with ALPLGA nanoparticles treatment at 20 mg/kg body weight compared to free artemisinin (70.3 ± 0.6% in liver and 62.7 ± 3.7% in spleen). In addition, ALPLGA nanoparticle treatment restored the defective host immune response in mice with established VL infection. The protection was associated with a Th1-biased immune response as evident from a positive delayed-type hypersensitivity reaction, escalated IgG2a levels, augmented lymphoproliferation and enhancement in proinflammatory cytokines (IFN-γ and IL-2) with significant suppression of Th2 cytokines (IL-10 and IL-4) after *in vitro* recall, compared to infected control and free artemisinin treatment. In conclusion, our results advocate superior efficacy of ALPLGA nanoparticles over free artemisinin, which was coupled with restoration of suppressed cell-mediated immunity in animal models of VL.

1. Introduction

Visceral leishmaniasis (VL) or kala-azar is a debilitating disease caused by protozoa of the *Leishmania donovani* complex (*L. donovani* and *L. infantum*) that imperils 200 million people in 62 countries with an estimated 500,000 new cases and 60,000 deaths annually [1]. VL leads to substantial health problems and death with more than 90% cases occurring in India, Nepal, Bangladesh, Sudan and Brazil [2]. *Leishmania* parasites are digenetic having two morphological forms: promastigotes in the digestive organs of the sand fly vector and amastigotes, a clinically relevant form in the mammalian host [3]. The parasite infects cells of the reticuloendothelial system and results in hepatosplenomegaly, anemia, hypergammaglobulinemia and chronic immunosuppression. Successful treatment is associated with restoration of Th1-biased response and suppression of Th2 cytokines that aid in parasite clearance [4,5]. Due to unavailability of vaccines, chemotherapy remains the only option for the treatment of VL. The present regimen for VL includes pentavalent antimonials, amphotericin B (AmB), liposomal amphotericin B, miltefosine and paromomycin that are unsatisfactory owing to high cost, toxicity and resistance in some cases.

Artemisinin is a secondary metabolite of the herb *Artemisia annua*, which has shown tremendous potential in the treatment

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of experimental cutaneous and visceral leishmaniasis; however, its efficacy is compromised due to serious drawbacks such as low bioavailability and short half-life [6–8]. These limitations can be partly overcome by encapsulating in colloidal nanoparticles that can potentiate the therapeutic effectiveness of artemisinin. Earlier, we have shown enhanced efficacy of artemisinin in colloidal nanoparticles as delivery system against *L. donovani* amastigotes *ex vivo* with reduced toxicity on mammalian macrophages [9]. The present study is aimed at investigating the immunomodulatory and therapeutic efficacy of these nanoparticles in experimental VL.

2. Materials and methods

2.1. Materials

Artemisinin was procured from Baoji Herbest Bio-Tech Ltd, China; poly (D,L-lactide-co-glycolide) (PLGA Resomer® 503H, lactide:glycolide 50:50), carboxyfluorescein succinimidyl ester (CFSE), AmB, RPMI-1640 medium, M-199 medium, penicillin, streptomycin, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), anti-mouse IgG and isotype antibodies, o-phenylenediamine dihydrochloride (OPD) were procured from Sigma-Aldrich (St Louis, MO, USA). Fluorochrome-conjugated antibodies such as anti-mouse CD4-phycoerythin, anti-mouse CD8-fluorescein isothiocyanate, anti-mouse CD80-allophycocyanin, anti-mouse CD86-phycoerythin cyanine dye 7 and anti-mouse CD40-fluorescein isothiocyanate, isotype controls and cytokine bead array kit (CBA) were procured from BD Pharmingen, USA. Aspartate aminotransaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), creatinine and urea kits were obtained from Span Diagnostics Ltd (Surat, Gujarat, India). All the other reagents were of analytical grade.

2.2. Methods

2.2.1. Preparation of artemisinin-loaded nanoparticles

ALPLGA nanoparticles were prepared by solvent displacement method, as described earlier [9]. In brief, solution of 12.8 mg PLGA (MW 24,000–38,000) and 1.69 mg artemisinin (in 2 ml acetone) was added drop wise to a specified volume of polyvinyl alcohol (0.47% w/v in 10 ml water). The nanoparticles were then precipitated and washed three times to purify the particles from the excess of polyvinyl alcohol and non-encapsulated artemisinin by centrifugation (45,000 × *g*, 20 min, 10 °C) (Hermle Labor Technik, Germany). Empty nanoparticles were prepared in the same manner, except that the drug was not added. These nanoparticles were lyophilized (Martin Christ, Germany) and stored at 4 °C until use to the prevent nanoparticles from degradation.

2.3. Characterization of nanoparticles

The morphology, shape and size distribution of nanoparticles were assessed by transmission electron microscopy (FEI Technai™ TF20, USA) at 200 kV. Briefly, 1 mg of nanoparticles was reconstituted in Milli Q water and diluted to approximately 5–20 μg/ml, such that the sample was transparent. A drop of the diluted sample was placed on a 300-mesh carbon-coated grid (Applied Biosystems, India). The sample was dried under vacuum and analyzed microscopically without staining. At least three samples were assessed for the determination of morphology and size of nanoparticles.

2.4. Drug loading and encapsulation efficiency

The lyophilized nanoparticles were used for determining the amount of drug loading and encapsulation efficiency, as described earlier [9].

2.5. Efficacy of artemisinin-loaded nanoparticles in experimental VL

2.5.1. Animals

Female BALB/c mice (25–30 g) maintained at the Central Animal House Facility of Jamia Hamdard were used for all experiments. The animal studies were approved by the Jamia Hamdard Animal Ethics Committee (JHAEC, Approval Number 458), and experiments were performed in accordance with the guidelines of Council for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

2.5.2. Parasite culture

The WHO strain of *L. donovani* (MHOM/IN/1983/AG83) was maintained by serial passage in BALB/c mice and hamsters [10]. These parasites were subcultured every 5 days at 22 °C in Medium 199 supplemented with 20% heat-inactivated fetal bovine serum, 25 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin G and 100 μg/ml streptomycin.

2.5.3. Infection and treatment of BALB/c mice for antileishmanial activity in vivo

Female BALB/c mice at 6–8 weeks of age were infected intravenously (i.v.) with 2.5×10^7 late log or stationary phase promastigotes/mouse in PBS. At 8 weeks, at least three animals were euthanized, and the infection was validated in giemsa-stained tissue imprints of spleen and liver by counting the number of amastigotes per 500 macrophages under oil immersion (100×) using a microscope (Nikon Eclipse 80i, Japan). The infected mice were then assigned into seven groups. Group I comprised normal mice and Group II infected control. Animals of Groups III–VIII were infected and subsequently treated for 10 days: artemisinin daily intraperitoneally (i.p.), 10 mg/kg bw (Group III) or 20 mg/kg bw (Group IV), ALPLGA nanoparticles were administered alternately (i.p.), 10 mg/kg bw (Group V) or 20 mg/kg bw (Group VI) to reduce the dosing frequency, empty nanoparticles alternately (i.p.), equivalent to the highest concentration in nanoparticles (Group VII) and AmB alternately (i.v.) at 5 mg/kg bw (Group VIII).

2.5.4. Assessment of hepatosplenomegaly and parasite burden after treatment

Infected mice were administered artemisinin daily, whereas ALPLGA nanoparticles and AmB were given alternatively for a period of 10 days. One week post treatment, animals were euthanized and weights of spleen and liver taken. Splenic and hepatic impression smears were made, amastigotes counted and parasite burden as Leishman Donovan Units (LDUs) and percentage inhibition of parasites were calculated in accordance with the formula [11].

$$\text{LDU} = \text{Number of amastigotes per 500 nuclei} \times \text{organ weight (mg)}$$

$$\text{Percentage inhibition (PI)} = \frac{\text{LDU}_{(\text{Infected control})} - \text{LDU}_{(\text{Treated group})}}{\text{LDU}_{(\text{Infected control})}} \times 100$$

2.6. Preparation of antigen

Leishmanial antigen was prepared as reported previously with modifications [12]. In brief, stationary phase *L. donovani* promastigotes at third or fourth passage in liquid culture were harvested and washed four times with cold PBS. For freeze thawed (FT) antigen,