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Original paper

Evaluation the therapeutic efficacy of different concentration of amphotericin B drug in mice infected with visceral leishmaniosis

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ABSTRACT. Liposomal amphotericin B (Amph B) has been used effectively to treat leishmaniosis, in spite of its high toxicity appeared in some patients. In our study, Amph B was administered in *Leishmania donovani* that infected BALB/c male mice using different concentrations to evaluate its efficacy challenge against infection as well as its effect in modulating immunity of the host. We observed that low doses with short duration of Amph B as a therapy regime significantly enhanced the induction of Th1 cytokine (INF-γ), but suppressed Th2 cytokine (IL-10) production. Groups of mice infected with *L. donovani* and treated with Amph B showed clearly increasing in INF-γ level and reduction in IL-10 level in concentration (3, 4, 5 mg/ml/kg) with best result in 5 mg/ml/kg (accumulation dose 25 mg/ml) than concentrations (6, 7 mg/ml/kg). We hypothesized that Amph B with low doses and short duration regime worked to mediate immunotherapeutic for eliminating the parasite-induced immunosuppression.

Keywords: liposomal amphotericin B, *Leishmania donovani*, leishmaniosis, immunotherapeutic

Introduction

Visceral leishmaniosis (VL) or kala-azar is a parasitic disease that second one to malaria in numbers of mortality; it affects millions of people worldwide. VL is mostly distributed in East Africa, South America, South Asia and Mediterranean Region [1].

The parasite exists in two forms. The promastigote form is found in the vector; the amastigote form is found in the vertebrate host and targets the reticulo-endothelial system in several tissues, principally infiltrating the spleen, liver, bone marrow and lymph nodes [2]. The signs of disease range from asymptomatic infection to life-threatening illness. Activation of macrophages and an intact T-helper cell type 1 (Th1) response contribute to immune control [3]. As usual, the symptomatic incubation time for VL ranges from two to six months. Viable parasites may persevere for decades, even after successful treatment, and in the situation of immunosuppression, these reactivate and cause illness [4]. Resolution of infection by *Leishmania* parasites is dependent on harmonic interactions between components of cell mediated immunity; activation of T-cell populations for suitable cytokine production and activation of infected macrophage [5]. Studies in human and murine infection leishmaniosis have showed induction of a predominant Th1 response and IFN-γ induced macrophage activation to combine with immunity and healing [6]. Interleukin 10 (IL-10) promotes intracellular infection including human VL, by preventing Th1 cell type responses and/or deactivating parasitized tissue macrophages. In human study, increased plasma levels of IL-10 during active VL have been reported and cure from disease is associated with a fall in IL-10 mRNA levels [5–7]. The above evidences suggested that IL-10 plays a major role for failure to control the growth and systemic spread of *Leishmania* parasites in human. The progressive development of splenic pathology is largely associated with high levels of IL-10 [8]. Leishmaniosis disease represents a considerable global load and a great challenge to drug invention and delivery; because of the

disseminated locations and intracellular nature of the parasite [9]. The therapy of VL has developed significantly by time as a result of arising resistance patterns and modern drug delivery systems [10]. On the other hand, the classical treatment of leishmaniosis demands the handling with toxic and poorly tolerated drugs [9]. Because there is no vaccine candidates either preventive or prophylactic are under clinical or animal trials, thus, it becomes clearly important to find an effective drug and/or a prophylactic vaccine for control leishmaniosis [5]. Amphotericin B, it is believed to be a second-line drug in Bihar following the loss of effectiveness of antimonial drugs which was the first line [11]. Universally, 3 formulations have been largely tested in VL: liposomal Amph B (L-AmB), Amph B colloidal dispersion and Amph B lipid complex. L-Amph B is the only confirmed drug by the US Food and Drug Administration for clinical use [8]. The total dose requirements of lipid formulations for treatment of VL vary by region [12].

As optimum liposomal amphotericin B dosage remains inconclusive and WHO recommended total dose 20 mg/kg administered intravenously at 5 mg/kg doses on days 0, 1, 4, and 9, we worked to determine the efficacy dose and less toxic one.

Materials and Methods

Parasite used in this research

Leishmania donovani isolate was obtained from the Laboratory of Parasitology, Department of Biology, College of Science, University of Baghdad. The parasites were grown in Novy-MacNeal-Nicolle medium (NNN medium).

Drug (*amphotericin B*) *preparation*

This drug is available as ampoules for injection (50 mg) each, which must be present by rapidly expressing 10 ml of sterile water for injection to get an initial concentration of five milligram per one milliliter according to the manufacture instruction of Affy Parenterals, India.

Animals

Sixty one male BALB/c mice with the age of 6–8 weeks old were obtained from National Center for Drug Control and Research in Baghdad. Animals were used in different steps to determine the drugs lethal doses and then in the main research steps by infected them intraperitonially with 2×10^7 *L. donovani* promastigotes and treated them with different doses of the used drugs.

Detection of visceral leishmaniosis infection in mice

Leishmania donovani (2×10^7 promastigotes/ml) was used to perform systemic infection in BALB/c males' mice intraperitoneally. In addition to five male mice left without infection as a negative control. After 21 days post-infection, samples of blood were collected from animals. At the beginning, to detect and confirm the occurrence of VL systemic infection, three mice were injected intraperitoneally with combination of xylazine 2% and ketamine 10% (Alfasan, Netherlands) as anesthesia [13]. The intracardiac blood samples were collected in Eppendorf tubes for serum separation. Serum was screened for anti-VL antibodies rapid diagnostic tests (RDTs) or dipstick fast test (InBios International, USA).

Treatment of visceral leishmaniosis infected mice

Five groups (6 mice each) were treated were treated intraperitoneally injection with Amph B drug with the concentrations (3, 4, 5, 6, 7 mg/kg/ml) once a day for nine days with interval [14,15].

Serum level of cytokines

After 20 days of the end of the treatment duration, mice from each group were anesthetized and blood was drawn from intracardiac to collect serum for the detection of cytokines levels. Serum level of two cytokines (IFN-γ and IL-10) was determined by ELISA method using (Elabscience, USA) that was designed for *in vitro* quantitative measurement of cytokine in mice sera according to the manufacture instructions.

Statistical analysis

The Statistical Analysis System-SAS (2012) program was used to detect the effect of difference factors in study parameters. The least significant difference (LSD) test (Analysis of Variation-ANOVA) was used to significant compare between means in this study [16].

Ethical statement

This study was approved from the College of Science Research Ethics Committee**,** Approval No (CSEC/1019/0005).

Results

The results obtained from this study with using

Table 1. Comparison between VL infected mice and treated with different concentrations of Amph B drug on the level of IFN-γ

Group	Mean ± SE IFN-γ
Infected without treatment	29.50 ±1.07 b
Not infected	6.73 ± 0.37 d
Amph B: 3 mg/ml	27.20 ± 0.84 b
Amph B: 4 mg/ml	27.04 ± 0.92 b
Amph B: 5 mg/ml	39.23 ±1.08 a
Amph B: 6 mg/ml	20.51 ± 0.57 c
Amph B: 7 mg/ml	22.77 ± 0.63 c
LSD value	4.982*

Explanations: means having with the different letters in same column differed significantly, * $P \leq 0.05$

Table 2. Comparison between VL infected mice and treated with different concentrations of Amph B drug on the level of IL-10

Group	Mean ± SE IL-10
Infected without treatment	19.70 ± 0.82a
Not infected	14.41 ± 0.56 c
Amph B: 3 mg/ml	14.59 ± 0.44 c
Amph B: 4 mg/ml	16.61 ± 0.74 bc
Amph B: 5 mg/ml	14.43 ± 0.27c
Amph B: 6 mg/ml	17.45 ± 0.86 b
Amph B: 7 mg/ml	17.00 ± 0.06 b
LSD value	2.586*

Explanations: see table 1

different concentrations of Amph B drug revealed a significantly differences ($P \leq 0.05$) between groups of mice infected and treated with Amph B drug, and groups of positive and negative controls. Amph B drug induced IFN-γ production that led to rise the level of this cytokine at the concentration (3, 4 and 5 mg/ml/kg) of Amph B with best result revealed in 5 mg/ml dose. The higher concentrations (6, 7 mg/ml/kg) of Amph B have shown lower level of IFN-γ than other concentrations and positive control group, as shown in table 1.

Although IL-10 level reduced than its level in positive control group with statistical ($P \leq 0.05$) differences, as illustrated in table 2. This result shows clearly that Amph B drug has outstanding bioactivity against *Leishmania* but, its use was initially limited due to toxicity.

Discussion

The Special Programme for Research and Training in Tropical Diseases (TDR) of the WHO together with many laboratory studies aim to prevent and control leishmaniosis by 2030, through showing that host-protective immunity is achievable as an effective IFN-γ, T-helper Th1 mediated immune response can aid in controlling the infection and enhancing treatment [17].

Amph B has been displayed to have immunomodulatory effects as it induces IFN-γ which leads to accumulate NO (nitric oxide) and ROS (reactive oxygen species), also stimulates surface receptors and production of multiple immune mediators (cytokines, chemokines, and prostaglandins) [18]. Accordingly, to boost host Th1 responses many studies have declared to joint treatment of Amph B and immunological mediators (IL-12 or anti-IL-10 receptor) to significantly enhanced the activity of Amph B against *L. donovani* [19].

As to many previous studies, the efficacious reduction of IL-10 correlated with the up-regulation of IFN-γ. This correlation acts as a key factor for well successfully therapeutics and shows that VL patients that were treated with an Amph B-lipid formulation presented low levels of IL-10 in their plasma [20–23].

The total dose requirements of lipid formulations for treatment of VL vary by region according to parasite load and the temperature needed [24]. Cold chain is also required for Amph B, since high or low temperature (<4° or >25°C) may alter the liposomal characteristics theoretically, affecting the drug's toxicity and effectiveness [25,26].

In 2005, WHO made recommendations on using Amph B: – in the Mediterranean, the Middle East and Brazil zoonotic foci – a maximum dose of 20 mg/kg liposomal amphotericin B with variable dosing regimens;

– in South Asia and Horn of Africa anthroponotic foci – when unresponsiveness to antimony exceeds

a threshold (to be decided by each region) consider a first-line alternative, either an amphotericin B formulation or a combination treatment [27].

Our results coined with low doses recommended by WHO in Middle East and with other study done by Sinha et al. in Bihar in India [28]. Sundar et al. [29] showed that 15 mg/kg of Amph B (3 mg/kg on each of 5 injections) cured 96% of patients. Liposomal amphotericin B: 3–5 mg/kg per daily dose by infusion given over 3–5 days period up to a total dose of 15 mg/kg by infusion was recommended for VL in Indian subcontinent [29]. In Sudan, 4 mg/kg of a total dose of 30 mg/kg given in six doses gave a cure rate of 88% to 94% with 7–10% relapse [30]. In a survey done for VL patients in Ethiopia, a dose of Amph B (24–35 mg/kg) was found to have high cure rate of 96.7% at the end of treatment and 71–100% at 6 months follow-up [31,32].

In a study done in Brazil on mucosal leishmaniosis a total of 30–35 mg/kg with median length of treatment was 12 days with an average daily dose of 2.5 mg/kg/day achieved maximum effectiveness of the drug [33]. Amph B or liposomal amphotericin B at higher dose should be used as rescue treatment in case of non-response [34].

Liposomal amphotericin B has an extremely dissimilar pharmacokinetic profile, after intravenous doses (single and multiple), the medication distributed broadly and quickly. After multiple doses of concentration 1–7.5 mg/kg/day and within four days, the drug reaches a steady-state plasma concentration [35]. The final half-life of liposomal Amph B in plasma is longer than other formulas (about 152 hours). So, a high percentage of circulating Amph B is possibly inactive, since a high percentage of it fixed in the liposome and not biologically effective. By direct connect with protozoan or fungal cell walls; the biologically active drug is unchained and freed [35,36].

This may explain the reason of poor results obtained from the concentrations (6 and 7 mg/kg/ml) of Amph B after 5 intraperitoneal injections. Also, some toxic adverse effects are attributed to Amph B after long term of injection. Many studies advice a single dose or twice doses with high concentration as a recommended treatment especially in endemic area like India. Sinha et al. [27] in a previous study recommended that 10 mg/kg single-dose first-line treatment should be preferred for implementation in all endemic areas in India. Thakur et al. [36] cleared in a separated study and other studies done by Sundar et al. in different years [38–40] the effectiveness and safety of Amph B achieving activity rates in more than 90% in a single dose of 5–15 mg/kg for VL in India. Other demonstrated study used dose 7.5 mg/kg daily for 2 days showed 100% cure rate [40,41]. Amph B with single dose: 15 mg/kg was found highly effective and safe for treatment of VL [42].

Presumably, there is another reason for the dissimilarity in the efficacy and safety doses of Amph B, is the differences in the manufacturing process and formulations of this medicine from that of the original drug [43,44]. This problem illustrates the major challenges posed by the large-scale manufacturing of highly complex nanodrugs [45]. This, regulatory pathways to confirm bioequivalence for generic liposomal Amph B products should be carefully estimated [46].

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