Investigation on Nitric Oxide and C-Reactive Protein Involvement in Anti-Leishmanial Effects of Artemisinin and Glucantim on Cutaneous Leishmaniasis

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Abstract
Cutaneous leishmaniasis (CL), a dermatological parasitic infection caused by Leishmania major and L. tropica. This disease is still one of the health problems in the tropical and sub tropical parts of world, region and Iran. Although, artemisinin (qinghaosu) is widely used as anti-malarial agent, it is also demonstrated its anti-promastigote effects on some leishmania species. Inflammatory responses against leishmania consist of chemokines, immune cells and mediators. This study investigates two immunological pathways including nitric oxide (NO) and C-reactive protein (CRP) in L. major infected Balb/c mice following treatment with artemisinin and glucantim. Plasma was investigated for NO and CRP alterations using Griess Micro Assay (GMA) and Latex
Agglutinations Test (LAT) respectively. The results indicated a significant decline in NO levels due to atemisinin treatment (P≤0.05) and in untreated group (P≤0.05). No changes in CRP were observed in experimental groups. It is indicated that L. major infection naturally decreased NO induction in Balb/c mice as a result of amastigote action; therefore artemisinin was not able to increase NO to combat parasite. It is concluded that artemisinin/glucantim action in CL was not associated with NO and CRP pathways; however more studies are needed to clarify other immunological parameters.

**Keywords:** Artemisinin, Glucantim, Leishmaniasis, Leishmani major, NO, CRP

**Introduction**

Cutaneous infections caused by protozoa the genus *Leishmania* are a major worldwide health problem, with high endemicity in developing countries including the Middle East, Africa and Latin America. According to the World Health Organization, 88 countries are affected by leishmaniasis, with approximately 350 million people at risk. Comprehending 12 million infected cases, the incidence is increasing worldwide with 1-2 million new cases registered annually, despite efforts being made to fight the disease [1]. There are three main clinical manifestations of leishmaniasis, caused by various species of leishmania, cutaneous leishmaniasis (CL, oriental sore), mucocutaneous leishmaniasis and visceral leishmaniasis (Kala-azar) [2,3].

The pentavalent antimonial drugs, sodium stibogluconate (SSG), meglumine antimonate (MA) and glucantim (GLU), are currently the first-line drugs for the treatment of leishmaniasis [4], but these drugs have serious side effects and progressive antimonial resistance [5]. Clinical reports indicate that a large proportion of the cases are becoming unresponsive to chemotherapy. Variable efficacy, toxicity, requirement of long courses of parenteral administration, or combinations of these factors, have been reported [6]. Artemisinin (ART) is isolated from the Chinese herb *Artemisia annua* L. Its derivatives have anti-malarial activities and are associated with very low toxicity to animals [7]. ART is a powerful anti-malarial drug having significant activity against strains of the disease which are resistant to chloroquine [8]. ART has been demonstrated its activities against other parasites e.g. *Schistosoma japonicum* [9] and several leishmania species [10]. The anti-leishmanial activity of an ethanolic extract of *Artemisia indica* leaves and, more specifically, ART has been demonstrated against several leishmania species [11,12] and in animal models of CL [13].

Immune responses during leishmaniasis include antibodies, cytokines, immune cells, mediators, and acute phase proteins [14]. In CL, various phagocytes predominant in the skin, including neutrophiles, macrophages (Mφ), and dendritic cells, play distinct roles for the host’s immune responses [15]. Parasite can multiply in Mφ and the clinical spectrum of the disease is due to the
severity of the immune response of the host [16]. The nitric oxide (NO) molecule is a product of L-arginine and a reactive free radical, and in the presence of oxygen, is oxidised to a variety of nitrogen oxides [17]. NO is produced in the neutrophils, lymphocytes and Mφ as part of the cytotoxic function of these cells via TNF-α and IFN-γ pathways [18,19]. NO acts as both a pro-inflammatory and an anti-inflammatory agent. The mechanisms that underline these effects remain poorly defined [20]. Chronic inflammation is known to be associated with increased levels of both NO, O₂ and H₂O₂. There are several experimental evidences that NO is involved in the microbicidal activity of Mφ against a number of intracellular pathogens including L. major, Trypanozoma cruzi and Toxoplasma gondii [21]. NO plays a pivotal role as a leishmanicidal agent in mouse Mφs and elevated level of NO within leishmania lesions could reduce parasite number [22]. In contrast, Th2 responses limit the Th1 functions, which deactivate Mφ and NO production helping growth of intracellular leishmania parasites and disease progression. Balb/c mice develop severe lesions and are susceptible to the L. major model with early Th2 responses, whereas the resistance to leishmania is conferred by Th1 type responses [23-26]. Perhaps, both endogenous and exogenous NO inhibits the development of intracellular amastigotes [26-28]. Recent studies found that apoptotic processes and several targets in organisms may be affected by NO [29,30]. Furthermore, both in vitro and in vivo immunological studies indicate that NO radicals within leishmania lesions could reduce the parasite number [31].

In addition to NO, C-reactive protein (CRP) is a major acute phase protein present in normal serum, which is increased significantly after most forms of tissue injuries and infections as a non-specific innate defense mechanism of the host. CRP as a protein is mainly regulated at the transcriptional level, induced by cytokines [32]. It is a marker of inflammatory reactions and cytokine activation [33], which is produced early after infection [34]. CRP is reported to be a critical element during a majority of infections [35] and there is a correlation of CRP and NO in some infections including leishmaniasis. This may clarify the co-involvement of CRP and NO as two major immune elements during infection [36]; however, it is not justified, whether the CRP / NO production is beneficial or detrimental to the host. Notwithstanding the conflicting publications, the role of CRP [37] and NO [38,39] in the immune responses to infections remains uncertain. It is suggested that NO alone or in accompany with CRP and other chemokines is involved in protective or pathogenic responses of CL [36].

Accordingly, this study aimed to investigate immuno-parasitological effects of ART in comparison with GLU on cutaneous leishmaniasis caused by L. major. This investigation has been also carried out to clarify NO and CRP hypothesis against intracellular leishmania parasites and to determine whether ART and GLU have effects on NO and CRP pathways in Balb/c mice infected with L. major, a prevalent strain of CL in Iran.
Materials and Methods

**Animals**

Female inbred Balb/c mice (supplied by the Karaj Laboratory Animal Unit, Pasteur Institute of Iran) were used in this study. The initial body weight 18.2 ± 1.3 g (mean ± standard error of mean, SEM) and mice were housed at room temperature (20–23 °C) on a 12-h light and 12-h dark cycle, with unlimited access to food and tap water. Experiments with animals were done according to the ethical standards formulated in the Declaration of Helsinki, and measures taken to protect animals from pain or discomfort. It has been approved by institutional ethical review board (*Ethical Committee of the Pasteur Institute of Iran*), in which the work was done.

**In vitro cultivation of L. major**

The *L. major* used in this study was the standard strain MRHO/IR/75/ER. The infectivity of the parasites was maintained by regular passage in susceptible Balb/c mice. The parasites were cultured in the RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 292 μg/ml l-glutamine and 4.5 mg/ml glucose (all supplied by Sigma). Under these culture conditions, the stationary phase of parasite growth was obtained in 6 days as determined previously [14].

**Infection of Balb/c mice with L. major**

Promastigotes of *L. major* were harvested from culture media, counted and used to infect Balb/c mice. The base of the tail was injected intradermally with inoculums of 2 × 10^6 promastigotes. The animal experiments were performed once in five groups (*n* = 10 mice/group) considering time, budget and long-period monitoring of animals according to the ethical issues for sample size and replication in order to protect animals from further pain or discomfort. It has been approved by institutional ethical review board in which the work was done. The leishmania infection was carried out in experimental animals and terminated at week 16 after injection.

**Experiments and groups**

The total number of animals used in this experiment was 60 Balb/c mice, divided into test and control animals in 6 groups (*n* = 10 mice/group) including Group 1 (naïve), Group 2 (*L. major* + saline), Group 3 (*L. major* + Methanol 0/5 %), Group 4 (*L. major* + GLU), Group 5 (*L. major* + ART) and Group 6 (*L. major* + without treatment).

**Anti-leishmanial drugs**

Two groups of mice were treated by interperitoneal injection of GLU (20 mg/kg/day) and ART (10 mg/kg/day) up to 3 weeks. Control groups were received saline and methanol 0.5 % as drug vehicles for GLU and artemisisnin respectively.
**Preparation of plasma**

Plasma was prepared from blood taken by cardiac puncture into a 1 ml syringe containing 50 i.u. heparin (Monoparin, CP Pharmaceuticals Ltd.), from mice terminally anaesthetized by inhalation of diethyl ether. Plasma was prepared by centrifuging blood at 1500 \( g \) for 10 min, collected and stored at \(-70 \degree C\) until assayed with Griess Microassay (GMA) [40].

**Griess Micro Assay (GMA)**

The Griess reaction was adapted to assay nitrite. Briefly, nitrate was determined indirectly by the Griess assay. Standard curve for sodium nitrate (Sigma) were prepared. Samples were treated with Griess reagent (5% phosphoric acid, 1% sulfanilic acid and 0.1% \( N \) (1-naphthyl-1)-ethylendiaminedihydrochloride (NED), all from Sigma, dissolved in deionised water), then proteins precipitated by 10% trichloroacetic acid (Sigma). Tube contents were vortex mixed then centrifuged (Eppendorf centrifuge 5415 C, Germany). Duplicate samples of supernatants were transferred to a 96-well flat-bottomed microplate (Costar, USA) and absorbances read at 510 nm using a microplate reader (Bio-TEK, power wave XS, USA). Values for the concentration of nitrate were calculated from standard calibration plot [40,41].

**CRP detection by Latex Agglutination Test (LAT)**

CRP was detected by a Qualitative rapid LAT kit (Avitex CRP, Omega, UK). Briefly, 50µl of the sample and one drop of each positive and negative control reagents was placed into separate circles on the slide test. The CRP latex reagent was swirled gently before using and one drop (50µl) added next to the sample tested. The drop was mixed with a stirrer; spread them over the entire circle. The presence of visible agglutination was examined macroscopically immediately after removing the slide [36, 42].

**Statistical analysis**

Values are presented as the mean ± SEM for groups of \( n \) samples. The significance of differences was determined by Analysis of Variances (ANOVA) and Student’s \( t \)-test using Graph Pad Prism Software (Graph Pad, San Diego, CA, USA).

**Results**

Plasma levels of NO were measured for all six groups (Fig. 1). The results indicated that production of NO was inhibited in infected Balb/c mice by \textit{L. major} (Group 6) \( (P < 0.05) \) as compared with naïve animals (Group 1). Production of NO was inhibited in both ART and GLU (Group 2) when compared with their related controls. However, ART (Group 5) presented more ability to increase NO levels \( (P < 0.05) \) in compare with GLU (Group 5). Leishmania probably had some inhibitory effects on NO pathway (Fig. 1). The results indicated no changes in CRP levels among entire experimental groups. The ART and GLU were not
able to alter NO and CRP in order to combat parasite proliferation; therefore, their mechanism of action is not associated with NO and CRP pathways.

![Graph showing NO production in plasma of six control and test groups of Balb/c mice.](image)

**Fig. 1.** NO production in plasma of six control and test groups of Balb/c mice. NO levels were assayed in plasma by GMA at the end of the experimental period. Entire animals used in this experiment were divided in 6 groups (n = 10 mice/group) including Group 1 (naïve), Group 2 (L.major + saline), Group 3 (L. major + Methanol 0/5 %), Group 4 (L.major + GLU ), Group 5 (L. major + ART ) and Group 6 (L.major + without treatment ). Significance of differences (*P < 0.05) was determined by an unpaired Student’s t-test and ANOVA using Graph Pad Prism.

**Discussion**

This investigation determined whether ART and GLU have effects on NO and CRP pathways in Balb/c mice infected with L. major, a prevalent strain of CL in Iran. This experiment was a part of a continuous study on the role of NO, CRP and other immune chemokines against intracellular parasite e.g. leishmania. We have previously published the detailed applications of NO modulators, time courses and the associated changes in plasma [42-46]. ART presented its ability to increase NO in susceptible BALB/c mice. The results indicated that Anti-leishmanial effects of ART and GLU were not associated with NO and CRP pathways. Notwithstanding the conflicting publications, the role of CRP and NO in the immune responses to leishmania remains uncertain. It is suggested that NO is involved in protective and/or pathogenic responses of human CL. However, there are substantial data in the literature on CRP and NO suggesting a potent antimicrobial role for both of them individually or acting together. Increased
CRP/NO synthesis might have a protective rather than pathological role in other parasitic diseases e.g. malaria [36, 42].

It is indicated that L. major infection solely decreased NO induction in Balb/c mice as a result of amastigote action; therefore ART and GLU were not able to increase NO to combat parasite development. Therefore, it is concluded that ART and GLU actions in CL were not associated with NO and CRP pathways. Although, CRP/NO play an important role in infections, their actual functions and interactions in the treatment of disease remain unclear. It is indicated other immunological pathways may be involved in therapeutic mechanisms of ART and GLU, which requires further investigations.

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References
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