

Immuno-Biochemical Alterations in *Leishmania major* Infected Balb/c Mice after Immunization with Killed *Leishmania* Vaccine and BCG as Adjuvant

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Abstract: BCG is an immune modulatory that inducing humoral and cellular immune responses during a number of infections including cutaneous leishmaniasis. Killed *Leishmania* Vaccine (KLV) has been applied for its immunogenicity in hosts. This study was carried out between 2010 and 2011 at the Department of Parasitology, Pasteur Institute of Iran. In the present study, alterations of Nitric Oxide (NO) levels, total content of essential trace elements (Cu, Zn) and liver enzymes (SGOT, SGPT) were investigated in Balb/c mice infected with *Leishmania major*. Results showed that BCG induced NO in liver; Zn increased in KLV/BCG and KLV test groups and Cu decreased in KLV test group, but increased in BCG test group. Moreover to decreasing SGOT level in KLV/BCG test group, SGPT decreased in BCG test group. Our findings showed that serum trace element concentrations were altered in CL infection, probably in accordance with host defense mechanisms. It is indicated that application of KLV/BCG as a combined vaccine/adjuvant could be indicated as a potent inducer of immuno-biochemical characters during infection with Leishmaniasis. In conclusion, some dependency is observed between KLV/BCG and NO levels, Cu, Zn concentrations and SGOT, SGPT production. The mechanism of KLV/BCG action may be associated more on BCG rather than KLV.

Keywords: BCG, immunization, Iran, killed leishmania vaccine, KLV, *Leishmania major*

INTRODUCTION

Leishmaniasis, caused by obligate intracellular parasites of the genus *Leishmania*, the causative agents are species of *Leishmania*, a protozoan flagellate (Manson-Bahr and Apted, 1983), which is transmitted by species of *Phlebotomine* sandflies (Liew and O'Donnell, 1993). Human leishmaniasis occurs worldwide but is mainly localized to the tropics and subtropics, with a prevalence of 12 million cases and an incidence of 0.5 million cases of Visceral Leishmaniasis (VL) and 1.5 million cases of Cutaneous Leishmaniasis (CL). Globally there are an estimated 70000 deaths each year and 350 million people are at risk of infection (Klaus *et al.*, 1999). *Leishmania* are protozoa parasites that cause cutaneous, mucocutaneous or visceral clinical manifestations in host. In the majority of patients the disease occasionally is CL. Currently the main foci of is in Sudan and India and those of CL are in Afghanistan, Syria and Brazil (Klaus *et al.*, 1999).

Acute CL, caused by *L. major*, *L. tropica* and *L. aethiops* in the Old World and *L. braziliensis*, *L. panamensis* and *L. mexicana* in the New World are the most common causes of infection. Complex and rare manifestations of CL including Mucocutaneous Leishmaniasis (MCL), Diffuse Cutaneous

Leishmaniasis (DCL) and '*Recidivans*' Leishmaniasis (RL) (Croft, 2001). It was clearly shown in animal models and humans that immunity against and recovery from leishmaniasis is associated with Th1 type of immune responses and delayed type hypersensitivity. Healing in CL is thus dependent on the host immunity (Esther, 2007) and the development of a protection is dependent on the generation of cytokines and mediators (Reiner and Locksley, 1995; Sacks and Noben-Trauth, 2002). In experimental infection of *L. major* in inbred mice, the immune response is shown to be host genotype dependent. BALB/c mice develop severe lesions and are susceptible to the *L. major* model with early T-helper-2 (Th2) responses, whereas the resistance to leishmania is conferred by Th1 type responses (Scott, 2005; Croft *et al.*, 2006). In the absence of effective vaccines and vector-control measures, the main line of defense against the disease is chemotherapy. Organic pentavalent antimonials (Sb (V)) have been the first-line drugs for the treatment of leishmaniasis for the last six decades and clinical resistance to these drugs has emerged as a primary obstacle to successful treatment and control (Ashutosh and Neena, 2007).

A safe, efficacious and affordable vaccine would be the most practical and cost effective control tool in many epidemiological situations. As such, vaccination

becomes an ideal effective method of control (Modabber, 1989) and recently, some promising safe and protective vaccines were developed. *Leishmania* vaccines have been used in South America (Mayrink *et al.*, 1979, 1986; Antunes *et al.*, 1986; Castes *et al.*, 1994; Sharples *et al.*, 1994) and Iran (Momeni *et al.*, 1998; Sharifi and Fekri, 1998) with encouraging results. The other studies describe the safety and immunogenicity of killed *L. major* plus BCG vaccine in healthy CL adult volunteers caused by *L. major* and mucosal leishmaniasis caused by *L. donovani* (Klaus *et al.*, 1999; El-Safi *et al.*, 1991; Ghalib *et al.*, 1992; El-Hassan *et al.*, 1995).

Trace metals, including Zn and Cu, are directly involved in metabolic processes critical to cell differentiation and replication. The most known changes in inflammation and infections are alterations in Fe, Zn and Cu levels in association with elevated levels of acute phase proteins (Kolak *et al.*, 2001). Zn is an essential trace element for all organisms and body growth and development is strictly dependent on Zn. Zn has numerous central roles in DNA and RNA metabolism (MacDonald, 2000) and it is involved in signal transduction, gene expression and apoptosis. Zn metalloenzymes and zinc-dependent enzymes have been identified and are involved in nucleic acid metabolism and cellular proliferation, differentiation and growth (Chesters, 1978). Zn plays a regulatory role in apoptosis (Truong-Tran *et al.*, 2001), with cytoprotective functions that suppress major pathways leading to apoptosis. The immune system relies on Zn for white blood cell function. Zn also plays a role in supporting the immune system and affecting the integrity of the epithelial barrier and the function of white blood cells (Salgueiro *et al.*, 2002). The nervous, reproductive and immune systems are particularly influenced by Zn deficiency, as well as by increased levels of Zn. The relationship between Zn and the immune system is complex, since there are different types of influence associated with Zn (Rink and Gabriel, 2000). The biologic role of Zn is now recognized in structure and function of proteins, including enzymes, transcription factors, hormonal receptor sites and biologic membranes. The changes in the levels of trace elements are part of immunity of organism and are induced by different cytokines (Faryadi and Mohebbali, 2003; Barber and Cousins, 1988; Klassing, 1988).

Serum Glutamic Pyruvic Transaminase (SGPT) is an enzyme that helps metabolize protein, Serum Glutamic Oxaloacetic Transaminase (SGOT) plays a role in the metabolism of the amino acid alanine and Alkaline Phosphatase (ALP) is an enzyme needed in small amounts to trigger specific chemical reactions and to diagnose liver function and its integrity (Green and Flamm, 2002; Berk and Korenblat, 2009).

This study aimed to investigate production of NO, biochemical alterations of Zn and Cu and also the serum concentration of liver enzymes including SGOT,

SGPT during *L. major* infection in Balb/C mice after immunization with KLV and BCG and whether these variations might be involved in the immune responses against the parasite.

MATERIALS AND METHODS

This study was carried out between 2010 and 2011 at the Department of Parasitology, Pasteur Institute of Iran.

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 was 19.2 ± 1.3 g (mean \pm 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 Ethical Committee of the Pasteur Institute of Iran, in which the work was done.

In vitro cultivation of *L. major*: The *L. major* MRHO/IR/75/ER used in this study was the standard strain isolated from Iran. The infectivity of the parasite was maintained by regular passage in susceptible Balb/c mice and parasite was cultured in the RPMI-1640 medium supplemented with 10% Fetal Bovine Serum (FBS), 292 mg/mL L-glutamine and 4.5 mg/mL glucose (all supplied by Sigma). Under these conditions, the stationary phase of parasite growth was obtained in 10 days (Farahmand *et al.*, 2008; Nahrevanian *et al.*, 2007).

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.

Experiments and groups: Total number of animals used in this experiment were 50 Balb/c mice, divided into test and control animals in 5 groups (n = 10 mice/group) including Group 1 (Control naive with no injection), Group 2 (KLV test), Group 3 (BCG test), Group 4 (KLV+BCG test) and Group 5 (Control *L. major*). One week after the first KLV and BCG injections, the second phase as booster with the same dose was injected subcutaneously (s.c.) in abdominal areas.

Vaccine: The KLV used in this study was autoclaved from of *L. major* and prepared by Razi Serum and Vaccine Research Institute (RSVRI), Hesarak, Karaj, Iran and kindly donated by Department of Immunology, Pasteur Institute of Iran, Tehran, Iran. The vial content

of vaccine is diluted with the normal saline to have a volume of 10 mL. Each mouse of KLV and KLV+BCG test groups received 100 μ L of this solution per injection.

BCG: The lyophilized BCG vaccine (*Mycobacterium bovis*, Bacillus Calmette Guerin) was prepared from Karaj Research and Production, Pasteur Institute of Iran and stored at 4°C until use. Just before injection, it was suspended in diluted solution SSI solvent (125 mg MgSO₄, 125 mg K₂HPO₄, 1 mg L-asparagines, H₂O, 12.5 mg Fe ammonium citrate, 18.4 mg 85% glycerol, 0.5 mg citric acid, H₂O 1 mL for injection /manufacturer's insert) with 0.1 mg BCG. All doses were injected intradermally in susceptible Balb/c mice.

Griess Micro Assay (GMA): The Griess reaction was adapted to assay nitrate as described previously (Nahrebanian and Dascombe, 2001). Standard curves for sodium nitrate (Sigma) were prepared. One hundred microliter samples were poured in microtubes, then 100 μ L Griess reagent (5% phosphoric acid, 1% sulfanilic acid and 0.1% N (1-Naphthyl-1)-Ethylendiamine Dihydrochloride (NED), all from Sigma, dissolved in 100 mL deionised water) was added and proteins subsequently precipitated by 100 μ L 10% trichloroacetic acid (Sigma). Tube contents were vortex mixed then centrifuged at 13, 400 RCF (Eppendorf centrifuge 5415 C, Germany). Duplicate 100 μ L supernatants were transferred to a 96-well flat-bottomed microplate (Costar, USA) and absorbances read at 520 nm using a microplate reader (Bio-TEK, power wave XS, USA). Values for the concentration of nitrate assayed were calculated from standard calibration plots.

Measurements of blood trace elements and liver enzymes: Serum was removed by centrifugation and stored in metal-free tubes at -70°C. Zn and Cu were determined by direct aspiration of 1:10 dilution of serum in deionized water into the Flame Atomic Absorption Spectrophotometer (FAAS, Thermo Jarrel Ash and Germany). Serum SGOT, SGPT was determined by Auto Analyzer Technical RA1000 (Green and Flamm, 2002).

Statistical analysis: Values are presented as the Mean \pm 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, California, USA).

RESULTS

In order to evaluate the immunological effects of KLV and BCG, NO production was measured in

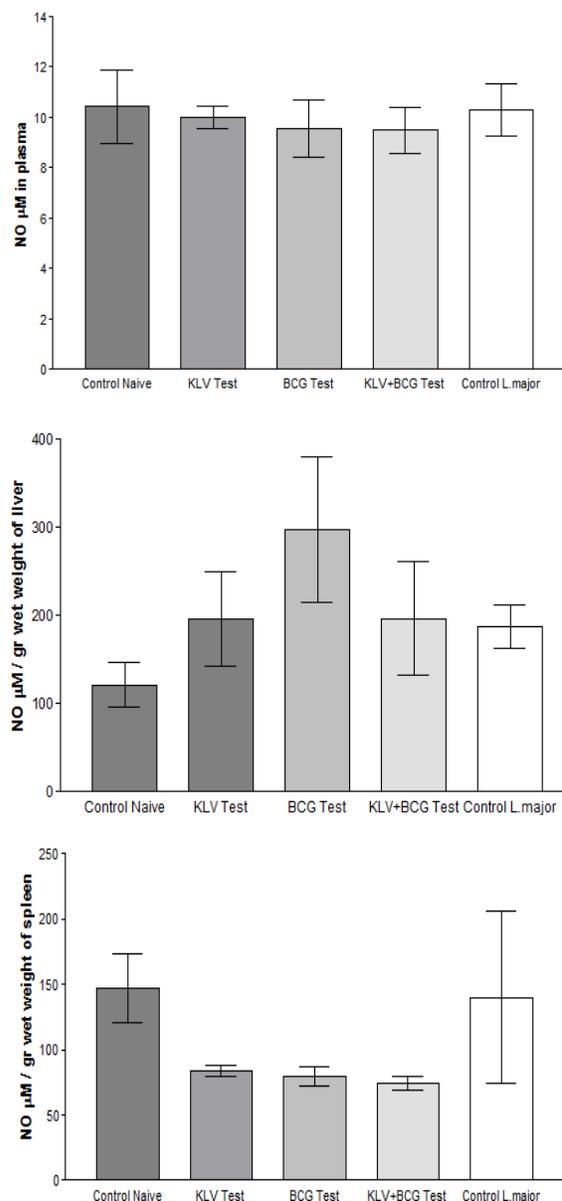


Fig. 1: NO concentrations in plasma and target organs Group 1 (Control naive) Group 2 (KLV test), Group 3 (BCG test) Group 4 (KLV+BCG test), Group 5 (Control *L. major* + DMSO); Statistical analysis as ANOVA was applied using Graph Pad Prism software ($n = 10$ mice/group)

plasma and suspensions of liver and spleen of all five groups by GMA. The data emphasized no significant differences among experimental groups (Fig. 1).

The results indicated significant differences in Cu levels among experimental groups. Concentration of Cu increased in BCG test group compared to other groups (0.53 ± 0.03 μ g/mL (Group 3, Test), with $***p < 0.001$), in contrast Cu decreased in KLV test group (0.36 ± 0.01 μ g/mL (Group 2, Control)). Concentration of Zn increased in KLV/BCG and KLV test groups as

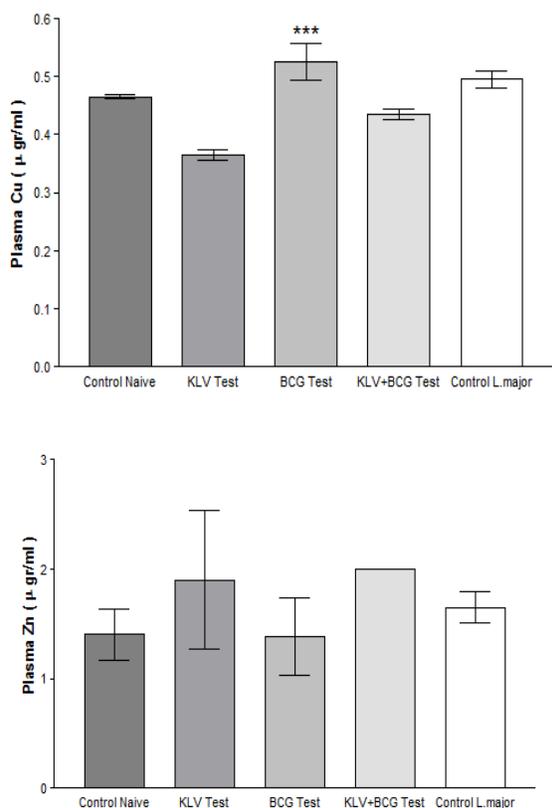


Fig. 2: Plasma Cu and Zn concentrations

Plasma Cu and Zn were determined by FAAS; Group 1 (Control naive), Group 2 (KLV test), Group 3 (BCG test), Group 4 (KLV+BCG test), Group 5 (Control *L. major*+DMSO); Significance of differences (** $p < 0.001$) was determined by One-Way ANOVA test using Graph Pad Prism software ($n = 10$ mice/group)

compared with control uninfected group (1.4 ± 0.2 µg/mL (Group 1, Control Naive); 1.9 ± 0.6 µg/mL (Group 2, KLV Test) and 2 ± 0 µg/mL (Group 3, KLV/BCG Test), not significant differences (NSD) (Fig. 2).

In spite of variations in levels of SGOT and SGPT, statistically no significant differences were observed among entire groups. SGOT decreased in KLV/BCG test groups compared to control uninfected group (214.7 ± 12.1 U/L (Group 1, Control Naive); 201.3 ± 28.8 U/L (Group 3, KLV/BCG Test)) and also SGOT increased in BCG and KLV test groups compared to control groups (289 ± 4.619 U/L (Group 3, BCG Control); 325.6 ± 35.6 U/L (Group 2, KLV Test)). SGPT increased in KLV/BCG test groups compared to control uninfected mice (60.5 ± 12.4 U/L (Group 1, Control Naive); 90 ± 67.7 U/L (Group 4, KLV/BCG Test), however SGPT decreased in BCG test groups compared to control infected mice (21 ± 6.3 U/L (Group 3, BCG Test) 28 ± 6.6 U/L (Group 5, Control infected); 36.5 ± 5.5 U/L (Group 2, KLV Test)) (Fig. 3).

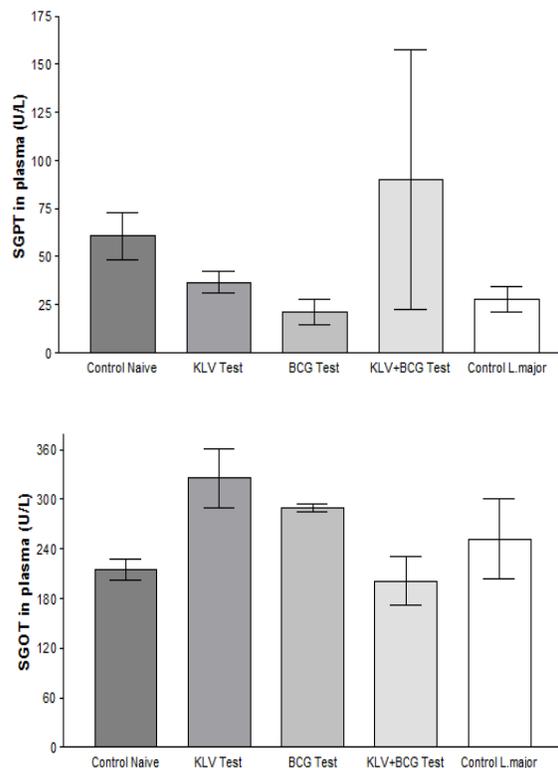


Fig. 3: SGPT and SGOT concentration

Liver enzymes SGPT and SGOT were determined by Auto Analyzer Technical RA1000 in plasma of experimental groups; Group 1 (Control naive), Group 2 (KLV test), Group 3 (BCG test), Group 4 (KLV+BCG test), Group 5 (Control *L. major*+DMSO); Statistical analysis as ANOVA was applied using Graph Pad Prism software ($n = 10$ mice/group)

DISCUSSION

Plasma levels of NO were measured for entire groups. The results indicated no changes significantly in NO levels among entire experimental groups but BCG presented more ability to increase NO levels in comparison with others group.

Zn is a cofactor in more than 300 enzymes influencing various organ functions having a secondary effect on the immune system (Rink and Gabriel, 2000). Zn influences the function of immune-stimulants used in the experimental systems. It is also known that Cu plays a role in the biochemical pathways of mammals. In the present study, we have demonstrated that Zn concentration was decreased while Cu concentrations were increased in CL mice after immunization with KLV/BCG. These Zn and Cu alterations are depending on several factors and may probably on several mediators and cytokines (Pirmez *et al.*, 1993; Wagner *et al.*, 1991; Cillarie *et al.*, 1989). In summary, we suggested that the serum redistribution of essential trace elements took place during the course of CL infections

as a part of the defense strategies of the organism, induced by the hormone-like substances or other mechanisms.

An interesting application of Zn as an adjuvant may be indicated in vaccination (Kreft *et al.*, 2000). Possible mechanisms for this relationship are an increase in IFN- λ production, or impaired T-cell functions (Sandstead *et al.*, 1982); however, high-dose Zn supplementation *in vitro* inhibited T-cell functions and reduced IFN- λ production, therefore Zn can be used as an immunosuppressant with low toxicity (Rink and Gabriel, 2000). It is proposed that trace Zn and Cu levels should be taken into account for CL treatment and vaccine strategies. Increased Cu may be attributable to inflammation associated with the disease (Pirmez *et al.*, 1993); whereas, increased levels of Zn and low values of Cu might be correlated to anti-parasitic pattern of host immunity.

Micro-elements play a major role in leishmania disease and their increasing rates could affect disease progress and its pathologies. It is concluded that the concentrations of essential trace elements including Zn and Cu were probably altered by the *leishmania* parasite during CL infection (Davachi *et al.*, 2009). The results of this study revealed an increasing pattern of Zn in KLV/BCG test and KLV test groups. It is concluded that Zn was reported to increase the immune responses. It also showed a decrease in Cu levels in KLV test groups, while a decrease in Cu levels in BCG Test was observed, which is reported to be attributable to the disease induction. The alternations of Zn and Cu may probably depend on mediators and cytokines.

In addition, liver plays a key role in the metabolic conversion and elimination of many enzymes (Jonker *et al.*, 2009). Trace elements were probably altered by the some cytokines as a host defense strategy during CL infection (Kocyigit *et al.*, 1998). Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) are enzymes located in liver cells that leak out into the general circulation when liver cells are injured. They are markedly elevated in acute liver infections and liver damages. However, this study indicated low SGOT level in KLV/BCG test group and SGPT in BCG test group in compare to other groups.

CONCLUSION

In conclusion, some dependency is observed between KLV/BCG and NO levels, Cu, Zn concentrations and SGOT, SGPT production. The mechanism of KLV/BCG action may be associated more on BCG rather than KLV.

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