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***In vivo* Application of Killed Leishmania Vaccine and Imiquimod as Adjuvant in Balb/c Mice Infected with *Leishmania major* MRHO/IR/75/ER as Iranian Strain**

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ABSTRACT

Killed Leishmania Vaccine (KLV) has been applied for its immunogenicity in hosts. Imiquimod (IMQ) as adjuvant induces immune responses during leishmaniasis. In this study, KLV/IMQ were applied to inhibit the proliferation and visceralization of *Leishmania major* in mice. Animals were treated with KLV/IMQ, then they infected by promastigotes of *L. major*. Six weeks later, a small nodule was appeared leading to a large lesion and visceralisation. KLV/IMQ effects on physiopathology, lesion sizes, lesion delay, amastigote proliferation and leishmania detection in target organs were all studied. Data analysis of body weight, hepato/splenomegaly and survival rates indicated no significant differences among experimental groups. It is concluded that KLV/IMQ represented no cytotoxic effects but they partly had effects on lesion size and impressed number of amastigotes inside macrophages. Application of KLV/IMQ decreased visceralization in liver; induced NO in liver, spleen and plasma. Although, IMQ application solely decreased visceralization in lymph nodes but KLV/IMQ increased SGOT/SGPT, however they represented no effects in concentrations of plasma Cu/Zn. Unlike IMQ topical application, its injection presented no ameliorative affects on CL, however its inhibition systemic leishmaniasis is indicated here. IMQ efficacy may be associated with route, dose and number of injection, which require more investigations.

Key words: Vaccine, *Leishmania major*, adjuvant, imiquimod, nitric oxide

INTRODUCTION

Leishmaniasis, a vector-borne disease caused by obligate intra-macrophage protozoa, is characterized by diversity and complexity. *Leishmania* are characterized by the possession of a kinetoplast, a unique form of mitochondrial DNA (Singh, 2006). More than 20 species of *Leishmania* can infect humans and thirty species of sandfly have been incriminated in transmission of the disease. In some areas, leishmaniasis is a zoonotic infection, while in other areas humans are the sole reservoir of infection (TDR, 2005). Human infections with *Leishmania* protozoan parasites, transmitted via the bite of a sandfly, which can be categorized in three major

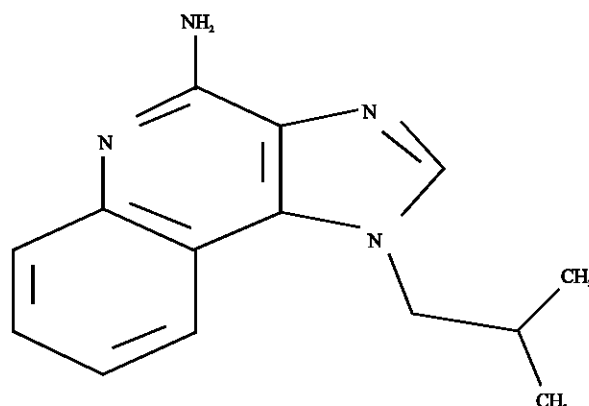


Fig. 1: Biochemical structure of IMQ (Miller *et al.*, 1999)

forms Cutaneous (CL), Mucocutaneous (MCL) and Visceral Leishmaniasis (VL) (Singh *et al.*, 2006; Davies *et al.*, 2003; Salotra and Singh, 2006). The disease symptoms range from self-healing CL caused by *L. major* and *L. tropica* to the more severe chronic MCL caused by *L. braziliensis* but the most severe form is VL which affecting internal organs (Salotra and Singh, 2006; Mukhopadhyay and Mandal, 2006; Garnier and Croft, 2002). The burden of CL is borne by Afghanistan, Pakistan, Syria, Saudi Arabia, Algeria, Iran, Brazil and Peru; this has sparked concern regarding the contribution that global warming might have on this observation (Piscopo and Azzopardi, 2006).

There is no vaccine against leishmaniasis for general human use yet. However, it should be feasible considering the experimental immunology of leishmaniasis and the availability of vaccines that can protect animals against challenge with different *Leishmania* species (Giunchetti *et al.*, 2007). A number of positive results has been reported by successful vaccination against VL using intradermal inoculation of alum-precipitated Autoclaved *L. major* (ALM) or *L. donovani* (ALD) with BCG in Indian langurs (Garg and Dube, 2006). The significant efficacies of attenuated killed *L. major* vaccine were also published in a Rhesus monkey model of the human disease (Amaral *et al.*, 2002) and in dogs vaccinated with autoclaved *L. major* promastigotes (ALM) plus BCG in Iran (Mohebbi *et al.*, 2004).

Imiquimod (IMQ) (1-(2-methylpropyl)-1 H-imidazo [4, 5-C] quinolin-4 amine), is a small molecule as an immune response modifier (Fig. 1), demonstrates potent antiviral and antitumor activities in animal models. It could mediate a better anti-leishmania Th1 immune response that would result in the production of IFN- γ and macrophage activation, resulting in enhanced parasite killing (Miranda-Verastegui *et al.* 2009). In addition, IMQ activates Toll-like receptors (TLR) on antigen-presenting cells and mediates the production of a variety of cytokines including IFN- α , IFN- γ , TNF- α , IL-1 and IL-12 leading to the induction of enhanced Th1 immune responses. It has been also demonstrated that IMQ can directly activate macrophage (MQ) killing of leishmania amastigotes in the absence of a T-cell-mediated response. Enhancing the local immune response at the site of CL infection, therefore, may be a logical approach to enhance parasite clearance (Miranda-Verastegui *et al.*, 2009; Miller *et al.*, 1999). IMQ as an oral inducer of several other proinflammatory cytokines, has been successfully used topically as an antiviral agent for the treatment of genital warts (Bottrel *et al.*, 1999). Although, local applications of IMQ in CL patient responded no relapse of disease (Hervas *et al.*, 2011) and to enhance the Th1 immune response against *L. major* infection in Balb/c mice (Miranda-Verastegui *et al.*, 2009), however no beneficial

effects of combination therapy with IMQ were also reported in human (Firooz *et al.*, 2006) and animal (El-On *et al.*, 2007). Khalili *et al.* (2011) reported the high efficacy for treatment of *L. major* infection in BALB/c mice in a combination of IMQ with Glucantime than each drug alone. Therefore, the aim of this study is to assess the *in vivo* application of Killed *Leishmania* Vaccine (KLV) and injectable IMQ as adjuvant in Balb/c mice infected with *Leishmania major* MRHO/IR/75/ER as Iranian strain (Nahrevanian *et al.*, 2011).

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 18.2 ± 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.

***Leishmania* parasite:** 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), $292 \mu\text{g mL}^{-1}$ L-glutamine and 4.5 mg mL^{-1} glucose (all supplied by Sigma). Under these culture conditions, the stationary phase of parasite growth was obtained in 6 days as determined by 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 2×10^8 promastigotes. The animal experiments were performed once in five groups (n = 10 mice/group) considering time, budget and long-period monitoring of animals. The leishmania infection was carried out in experimental animals and terminated at week 16 after injection.

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+IMQ test groups received 100 μL of this solution per injection.

Imiquimod: Lyophilized IMQ was purchased from Tocris, Co., UK and dissolved in DMSO 100 mM as drug vehicle to make a concentration of $0.5 \mu\text{g } \mu\text{L}^{-1}$ working solution and stored at 4°C until use.

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 with 100 μL injection of KLV in each phase), Group 3 (IMQ test with 100 μL injection of IMQ solution in each phase), Group 4 (KLV+IMQ test with injection of 200 μL of KLV+IMQ solution in each phase) and Group 5 (Control *L. major* with 100 μL injection of DMSO solution in each phase). One week after the first KLV and IMQ injections, the second phase as booster with the same dose was injected subcutaneously (s.c.) in abdominal area. The challenge of mice within groups 2 to 5 was carried out one hour after the

second injection of KLV and IMQ s.c. at base of tail by inoculation of 300 μ L containing 10^6 *L. major* promastigotes obtained from stationary phase of cultivated tubes.

Griess Micro Assay (GMA): The Griess reaction was adapted to assay nitrate as described previously (Nahrevanian 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.

CRP detection by qualitative Latex Agglutination Test (LAT): C-Reactive Protein (CRP) was detected by a rapid LAT kit (Avitex CRP, Omega, UK). Briefly, 50 μ L 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 (50 μ L) 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.

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, Germany). Liver enzymes in Serum Glutamic Oxaloacetic Transaminase (SGOT) and Glutamic Pyruvic Transaminase (SGPT) were determined by Auto Analyzer Technical RA1000 (Davachi *et al.*, 2009).

Assessment of pathology

Measurement of lesion size: Lesion size was measured at every other week after inoculation in millimeters (mm) by a digital caliper (Chuan Brand, China) in two diameters (D and d) at right angles to each other and the size (mm) was determined according to the formula: $S = (D+d) \text{ divided in } 2$ (El-On *et al.*, 1986).

Microscopical examinations and smear preparation: The clinical diagnosis was confirmed by laboratory demonstration of the parasite in the lesions by making stained smears at the end of the experimental period. Lesions were cleaned with ethanol and punctured at the margins with a sterile lancet and exudation material was smeared. Impression smears were prepared from liver, spleen and lymph nodes by placing a small piece of tissue between two glass slides and pushing them in different directions. The smears were dried in air, fixed by methanol and stained with Giemsa for detection of amastigotes under light microscopy (Erel *et al.*, 1999).

Measurement of proliferation in amastigotes: The proliferation of parasite was evaluated by counting of amastigotes inside macrophages on Geimsa stained lesion smears at the end of the experimental period. Five random macrophages were selected; counted and mean percentages were calculated as indicators for the degree of proliferation of amastigotes inside each macrophage.

Assessment of degree of hepato/splenomegaly: Entire livers and spleens were removed post mortem at the end of the experimental period from mice after induction of terminal general anaesthesia by inhalation of diethyl ether (Sigma). Organ wet weights were measured and compared with controls as indices for degree of hepato/splenomegaly.

Measurement of survival rate and body weight: Survival rate was presented as percentage of surviving experimental mice at every other week after inoculation; the significance of differences was determined by ANOVA test and compared with concurrent appropriate vehicle-treated leishmania and control groups. Body weight was measured initially and at different time of experiment using a top pan balance (Ohaus Scale Corp., USA).

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

The antileishmanial effects of IMQ and KLV were evaluated by considering lesion sizes, proliferation of amastigotes inside MQ and percentage of positive impression smears among target organs (liver, spleen and lymph nodes). There is an opposite effects of IMQ and KLV on lesion size when compared with control. There was no significant indication of using IMQ and KLV to limit lesion size in CL as presented in experimental groups (Fig. 2). The proliferation of amastigotes inside MQ in lesion smears represented no significant alterations after KLV/IMQ treatment (Fig. 3). By considering visceralization in this study, although, a parasite reduction ($p<0.01$) on the forth group (KLV+IMQ Test) was indicated in liver. However, there are no significant differences based on parasite percentage in smears of spleen and lymph node (Fig. 4).

The pathophysiological effects of vaccine and adjuvant including body weight, hepato/splenomegaly and survival rate were all evaluated during the experimental period. No significant differences by statistical analysis and ANOVA test using Graph Pad Prism software were indicated between groups (Fig. 5).

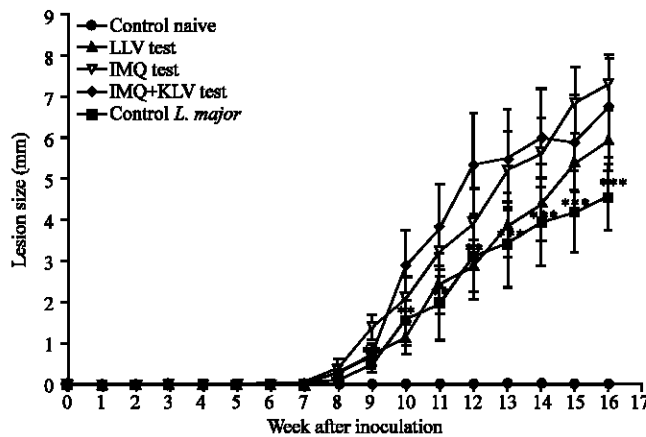


Fig. 2: Lesion size variation in experimental groups. Group 1 (Control Naive), Group 2 (KLV test), Group 3 (IMQ test), Group 4 (KLV+IMQ test), Group 5 (Control *L. major*+DMSO), (n = 10 mice/group), ** $p<0.01$, *** $p<0.001$, ANOVA, using graph pad prism software

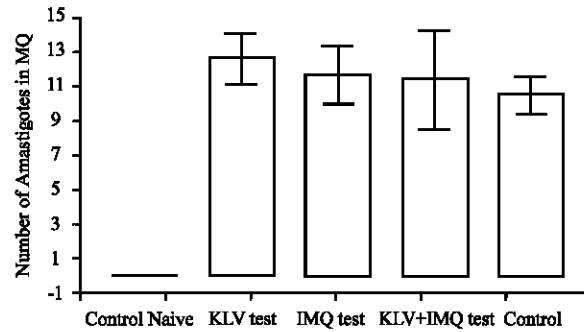


Fig. 3: Percentage of proliferation of amastigotes inside MQ in positive lesion smears in experimental groups. Group 1 (Control Naive), Group 2 (KLV test), Group 3 (IMQ test), Group 4 (KLV+IMQ test), Group 5 (Control *L. major*+DMSO), (n = 10 mice/group), ANOVA, using graph pad prism software

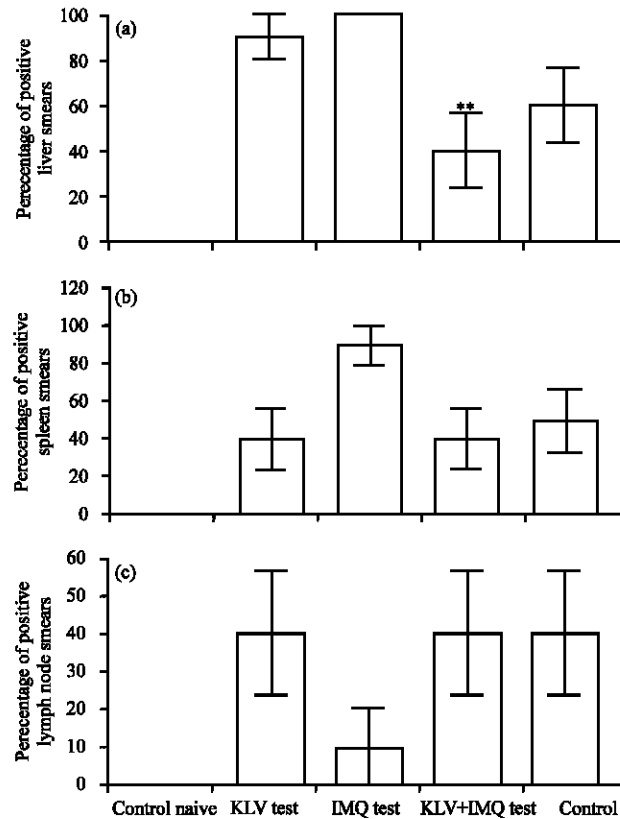


Fig. 4(a-c): Percentage of leishmania visceralization in target organs (Liver, Spleen, lymph node) in experimental groups. Group 1 (Control Naive), Group 2 (KLV test), Group 3 (IMQ test), Group 4 (KLV+IMQ test), Group 5 (Control *L. major*+DMSO), (n = 10 mice/group), **p<0.01, ANOVA, using graph pad prism software

In order to evaluate the immunological effects of KLV and IMQ, NO and CRP was determined in plasma samples and tissue suspensions by GMA and LAT respectively. The data emphasized no significant differences among experimental groups (Fig. 6).

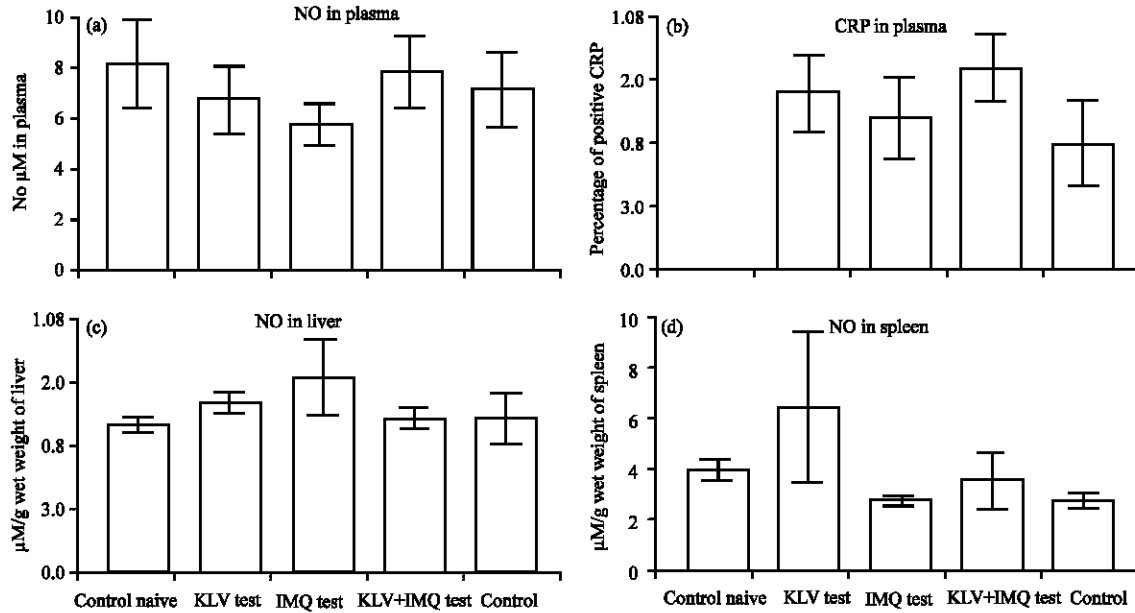


Fig. 5: Plasma NO concentrations in target organs and CRP in plasma in experimental groups. Group 1 (Control naive) Group 2 (KLV test), Group 3 (IMQ test) Group 4 (KLV+IMQ test), Group 5 (Control *L. major* + DMSO), (n = 10 mice/group), ANOVA, Using Graph Pad Prism software

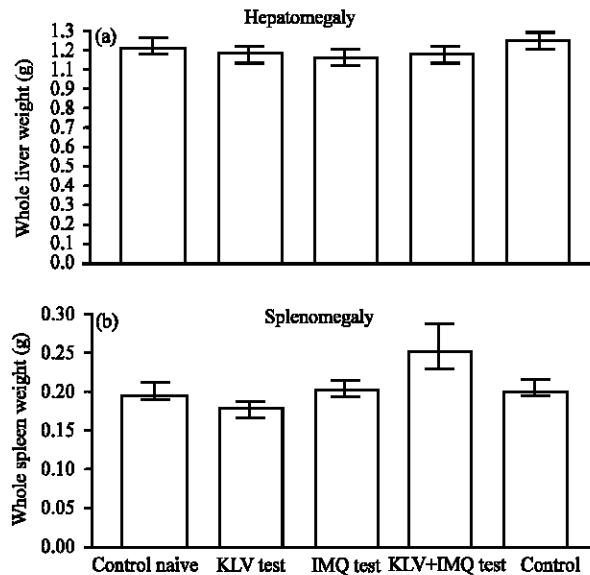


Fig. 6: Pathophysiology of KLV/IMQ; hepato/splenomegaly variation in experimental groups. Group 1 (Control Naive), Group 2 (KLV test), Group 3 (IMQ test), Group 4 (KLV+IMQ test), Group 5 (Control *L. major*+DMSO), (n = 10 mice/group), ANOVA, using graph pad prism software

Biochemical alterations were also evaluated after KLV and IMQ injections by measurement of microelements (Zn, Cu) by FAAS and liver enzymes (SGPT, SGOT) in plasma by

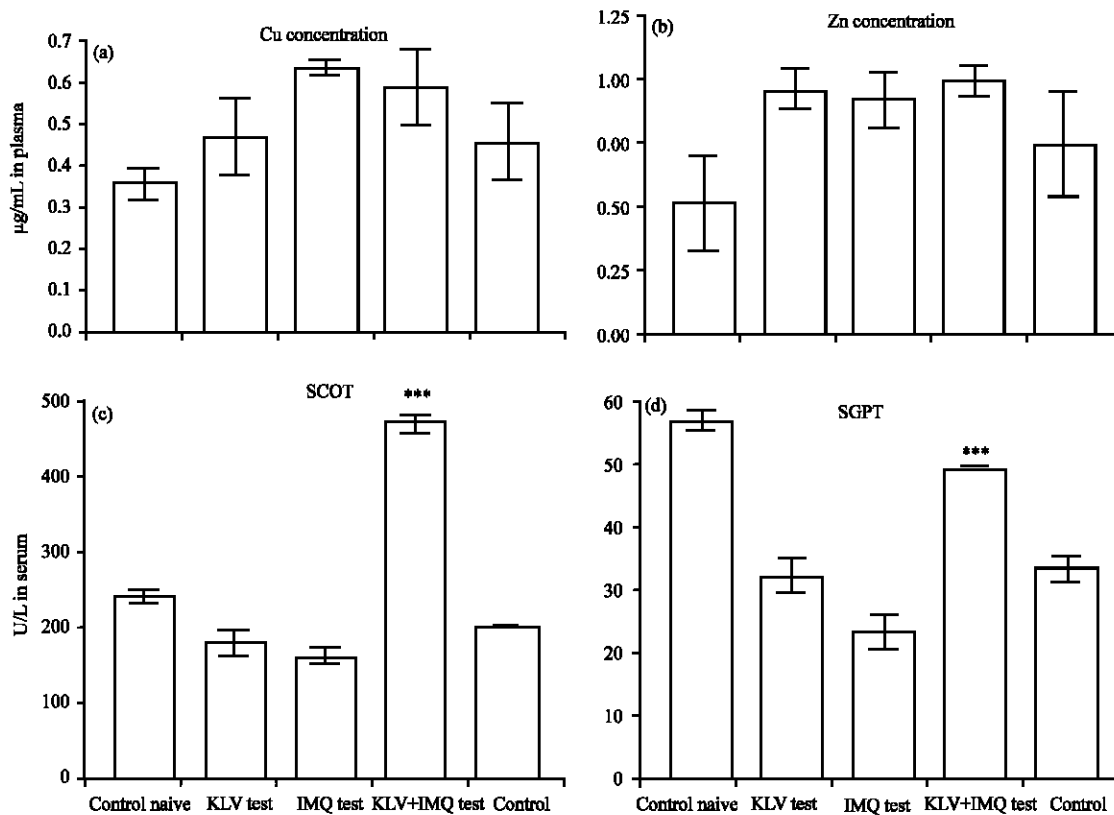


Fig. 7: Microelements and liver enzymes in sera of experimental groups. Group 1 (Control naive), Group 2 (KLV test), Group 3 (IMQ test), Group 4 (KLV+IMQ test), Group 5 (Control *L. major*+DMSO), (n = 10 mice/group), ***p<0.001, ANOVA, using graph pad prism software

TAA. Results show no significant differences between microelements among experimental groups. Results shows a significant increase (p<0.001) of SGOT and SGPT as compared with control groups (Fig. 7).

DISCUSSION

Physiological parameters including body weight, hepatomegaly, splenomegaly and survival rate represented no pathological consequences on the host after vaccine (KLV) and adjuvant (IMQ) injections, in separate or in accompany. Therefore they can be applied due to the minimum cytotoxic effects made by KLV and IMQ in Balb/c model of leishmaniasis.

In majority of publications regarding local application of IMQ the results are satisfactory (Hervas *et al.*, 2011; Miranda-Verastegui *et al.*, 2009). However, no beneficial effects of combination therapy with IMQ were also reported in human (Firooz *et al.*, 2006) and animal (El-On *et al.*, 2007). According to the lesion size and in opposite to previous reports on using local IMQ in animals and human CL (Arevalo *et al.*, 2001, 2007; Buates and Matlashewski, 1999; Nahrevanian *et al.*, 2008), there is no significant indications of injectable IMQ on *L. major* in Balb/c mice in current study. This may be different from results of Khalili *et al.* (2011) reported high efficacy for treatment of *L. major* infection in Balb/c mice than each drug alone using a

combination therapy of Glucantime with IMQ. The oral IMQ therapy in other diseases rather than leishmaniasis, encourages further exploration of the immune modifying properties of this novel molecule (Slade *et al.*, 1998).

In addition, there is an observable inhibition of visceralization using KLV and IMQ in the liver and spleen, however IMQ solely decreased the visceralization in the lymph nodes. Conclusively, this evident highlights the important role of vaccine (KLV) and/or adjuvant (IMQ) in control of visceral form of infections; complementary studies are required to explain this concept.

The joint and separate applications of KLV and IMQ revealed no significant effects on the proliferation rate of amastigotes inside macrophages.

NO molecule as a key mediator produced by induction of immune response, presented no significant alterations during this experiment. CRP also as an acute phase protein is expressed in many infectious diseases. Here in this study, despite minor changes in CRP values, significant changes have not been observed in different groups. Conclusively, it is indicated here that KLV/IMQ antileishmanial function could be NO/CRP independent pathways.

Although, application of KLV and IMQ solely show small effects on microelements and liver enzymes, simultaneous inoculation of both KLV/IMQ made significant alterations of elements (Zn, Cu) and enzymes (SGPT, SGOT). Therefore, more complementary studies are needed to confirm this concept and to draw attention for other immuno-biochemical parameters.

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REFERENCES

- Amaral, V.F., A. Teva, M.P. Oliveira-Neto, A.J. Silva and M.S. Pereira *et al.*, 2002. Study of the safety, immunogenicity and efficacy of attenuated and killed *Leishmania* (*Leishmania*) major vaccines in a rhesus monkey (*Macaca mulatta*) model of the human disease. *Mem. Inst. Oswaldo Cruz.*, 97: 1041-1048.
- Arevalo, I., B. Ward, R. Miller, T.C. Meng and E. Najar *et al.*, 2001. Successful treatment of drug-resistant cutaneous leishmaniasis in humans by use of imiquimod an immunomodulator. *Clin. Infect. Dis.*, 33: 1847-1851.
- Arevalo, I., G. Tulliano, A. Quispe, G. Spaeth, G. Matlashewski, A. Llanos-Cuentas and H. Pollack, 2007. Role of imiquimod and parenteral meglumine antimoniate in the initial treatment of cutaneous leishmaniasis. *Clin. Infect. Dis.*, 44: 1549-1554.
- Bottrel, R.L., Y.L. Yang, D.E. Levy, M. Tomai and L.F. Reis, 1999. The immune response modifier imiquimod requires STAT-1 for induction of interferon, interferon-stimulated genes and interleukin-6. *Antimicrob. Agents Chemother.*, 43: 856-861.
- Buates, S. and G. Matlashewski, 1999. Treatment of experimental leishmaniasis with the immunomodulators imiquimod and S-28463: Efficacy and mode of action. *J. Infect Dis.*, 179: 1485-1494.
- Davachi, S., H. Nahrevanian, E. Omidinia, R. Hajhosseini and M. Amini *et al.*, 2009. Biochemical alterations of liver enzymes and microelements during *Leishmania* major infection in BALB/c mice after treatment with Paromomycin. *Pharmacologyonline*, 3: 424-436.
- Davies, C.R., P. Kaye, S.L. Croft and S. Sundar, 2003. Leishmaniasis: New approaches to disease control. *Brit. Med. J.*, 326: 377-382.

- El-On, J., A. Witztum and L.F. Schnur, 1986. Protection of Guinea pigs against cutaneous leishmaniasis by combined infection and chemotherapy. *Inf. Immunit.*, 51: 704-706.
- El-On, J., E. Bazarsky and R. Sneir, 2007. *Leishmania major*: *In vitro* and *in vivo* anti-leishmanial activity of paromomycin ointment (Leshcutan) combined with the immunomodulator Imiquimod. *Exp. Parasitol.*, 116: 156-162.
- Erel, O., A. Kocyigit, V. Bulut and M.S. Gurel, 1999. Reactive nitrogen and oxygen intermediates in patients with *cutaneous leishmaniasis*. *Mem. Inst. Osw. Cruz.*, 94: 179-183.
- Firooz, A., A. Khamesipour, M.H. Ghooch, M. Nassiri-Kashani and S.E. Eskandari *et al.*, 2006. Imiquimod in combination with meglumine antimoniate for cutaneous leishmaniasis: A randomized assessor-blind controlled trial. *Ach. Dermatol.*, 142: 1575-1579.
- Garg, R. and A. Dube, 2006. Animal models for vaccine studies for visceral leishmaniasis. *Ind. J. Med. Res.*, 123: 439-454.
- Garnier, T. and S.L. Croft, 2002. Topical treatment for cutaneous leishmaniasis. *Cur. Opin. Invest. Drugs*, 3: 1472-1474.
- Giunchetti, R.C., R. Correa-Oliveira, O.A. Martins-Filho, A. Teixeira-Carvalho and B.M. Roatt, 2007. Immunogenicity of a killed *Leishmania* vaccine with saponin adjuvant in dogs. *Vaccine*, 25: 7674-7686.
- Hervas, J.A., A. Martin-Santiago, D. Hervas, E. Rojo, A. Mena, V. Rocamora and J. Duenas, 2011. Old world *Leishmania infantum* cutaneous leishmaniasis unresponsive to liposomal Amphotericin B treated with topical Imiquimod. *Ped. Inf. Dis. J.*,
- Khalili, G., F. Dobakhti, H. Mahmoudzadeh-Niknam, V. Khaze and F. Partovi, 2011. Immunotherapy with Imiquimod increases the efficacy of glucantime therapy of *Leishmania major* infection. *Iran. J. Immunol.*, 8: 45-51.
- Miller, R.L., J.F. Gerster, M.L. Owens, H.B. Slade and M.A. Tomai, 1999. Imiquimod applied topically: A novel immune response modifier and new class of drug. *Int. J. Immunopharmacol.*, 21: 1-14.
- Miranda-Verastegui, C., G.F. Tulliano, T.W. Gyorkos, W. Calderon and E. Rahme *et al.*, 2009. First-line therapy for human cutaneous *Leishmaniasis* in Peru using the TLR7 agonist Imiquimod in combination with pentavalent antimony. *Neg. Trop. Dis.*, 3: 1-10.
- Mohebbi, M., A. Khamesipour, I. Mobedi, Z. Zarei and R. Hashemi-Fesharki, 2004. Double-blind randomized efficacy field trial of alum precipitated autoclaved *Leishmania major* vaccine mixed with BCG against canine visceral leishmaniasis in Meshkin-Shahr district, I.R. Iran. *Vaccine*, 22: 4097-4100.
- Mukhopadhyay, S. and C. Mandal, 2006. Glycobiology of *Leishmania donovani*. *Indian J. Med. Res.*, 123: 203-220.
- Nahrevanian, H. and M.J. Dascombe, 2001. Nitric oxide and reactive nitrogen intermediates during lethal and nonlethal strains of murine malaria. *Parasite Immunol.*, 23: 491-501.
- Nahrevanian, H., J. Gholizadeh, M. Farahmand and M. Assmar, 2008. Patterns of co-association of C-reactive protein and nitric oxide in malaria in endemic areas of Iran. *Mem. Inst. Osw. Cruz.*, 103: 39-44.
- Nahrevanian, H., M. Farahmand, Z. Aghighi, M. Assmar and A. Amirkhani, 2007. Pharmacological evaluation of anti-leishmanial activity by *in vivo* nitric oxide modulation in Balb/c mice infected with *Leishmania major* MRHO/IR/75/ER: An Iranian strain of cutaneous leishmaniasis. *Exp. Parasitol.*, 116: 233-240.

- Nahrevanian, H., E. Salehizadeh, M. Farahmand, R. Hajihosseini, M.H. Alimohammadian, R. Saghiri and S. Javadian, 2011. Application of killed *Leishmania* vaccine's efficacy with imiquimod as adjuvant in inhibition of visceralization of *L. major* in BALB/c mice. *Trop. Med. Int. Health*, 16: 230-230.
- Piscopo, T.V. and C.M. Azzopardi, 2006. Leishmaniasis. *J. Postgraduate Med.*, 82: 649-657.
- Salotra, P. and R. Singh, 2006. Challenges in the diagnosis of post kala-azar dermal leishmaniasis. *Indian J. Med. Res.*, 123: 295-310.
- Singh, R.K., H.P. Pandey and S. Sundar, 2006. Visceral leishmaniasis (kala-azar): Challenges ahead. *Indian J. Med. Res.*, 123: 331-344.
- Singh, S., 2006. New developments in diagnosis of leishmaniasis. *Indian J. Med. Res.*, 123: 311-330.
- Slade, H.B., M.L. Owens, M.A. Tomai and R.L. Miller, 1998. Imiquimod 5% cream (AldaraTM). *Exp. Opin. Inves. Drugs*, 7: 437-449.
- TDR, 2005. Leishmaniasis. Seventeenth Programme Report, Progress Report 2003-2004.