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Partial Immunotherapy of Leishmaniasis by *in vivo* Trial of L-Arginine in Balb/c Mice Infected with *Leishmania major* via Nitric Oxide Pathway

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ABSTRACT

Leishmaniasis is a vector-borne disease in tropical and subtropical regions. It presents a wide spectrum of clinical manifestations; Cutaneous Leishmaniasis (CL) is the most common form leading to a skin nodule. L-Arginine (L-Arg) is an important amino acid involved in many metabolic pathways in host macrophages (MΦs) including NO synthesis. The L-Arg pathway is relevant to leishmaniasis due to its role in regulating MΦ functions. Activated MΦs can produce leishmaniacidal molecules, such as Nitric Oxide (NO) and oxidative mediators to kill parasite. Different concentrations of L-Arg were used; their toxicities assessed in naïve Balb/c mice. The highest concentration with lowest toxicity was selected to apply in *Leishmania major* infected mice. Test group was injected with three types of L-Arg (oral, local, injection) and control group received normal saline. Then, the lesion size, the measurement of amastigotes proliferation in MΦ, the pathophysiology of mice (hepato/spelenomegaly, survival rate, body weight) was all evaluated. In addition, plasma and tissue suspensions were investigated for NO induction using Griess Micro Assay (GMA). This is the first application of oral, local and injection forms of L-Arg against Iranian strain *L. major* MRHO/IR/75/ER. Results indicated L-Arg had ability to elevate NO in murine host. No pathological effects were observed in oral, local and injection types of L-Arg. Moreover, a significant decrease in parasite proliferation was observed only in oral group which presented anti-leishmanial activity by reduction liver and spleen positive smears. In injection form, percentage of positive smears was reduced only in spleen; a reduction observed in lesion sizes after treatment with oral and injection form of L-Arg; no significant alteration of local L-Arg to limit lesion size in CL was indicated here. The L-Arg is NO precursor and has been used safely for decades to treat physiological condition and used as food supplementary with no toxicity. In this study, L-Arg presented its partial ability to induce NO and treat animals. It also has limited potential therapeutic effects against CL by alteration of NO in host; therefore, it may be indicated *solo* as an anti-leishmanial agent or in a combination therapy for CL in mice. This may be the first immunotherapy trial against CL in Iran.

Key words: L-arginine, nitric oxide, NO, cutaneous leishmaniasis, *Leishmania major*

INTRODUCTION

Leishmaniasis, caused by parasites of the genus *Leishmania* (L.), affects millions of people in tropical and subtropical countries. *Leishmania* spp., lives as an intracellular parasite within mammalian hosts. Three forms of leishmaniasis exist; visceral (VL), cutaneous (CL) and mucocutaneous (MCL) (WHO., 2014; Manson-Bahr and Apted, 1983; El-Safi and Peters, 1991; Ghalib *et al*., 1992; El-Hassan *et al*., 1995). The CL is the most common form of infection that leads to a development of skin papules or nodules (Bogitsh *et al*., 2013). Since tissue macrophages (MΦs) are major target cells for parasite replication, the outcome of infection depends largely on the activation status of these cells (Green *et al*., 1990a).

The L-Arginine (L-Arg) is an important amino acid involved in many crucial pathways in host MΦs and *Leishmania* parasites. Activated MΦs can produce leishmaniacidal molecules, such as Nitric Oxide (NO) and oxidative mediators to kill parasite (Green *et al*., 1990b; Liew *et al*., 1991). L-Arg metabolic pathways not only participate in the regulation of iNOS-mediated parasite killing and arginase-mediated parasite growth, but also are involved in the regulation of other immune components, including; T-cells, B-cells, dendritic cells and neutrophils (Li *et al*., 2007). Although, several anti-leishmanial reagents which target L-Arg metabolic pathways have been studied, more research in this area is still needed. Although, the role of L-Arg in Leishmania-specific T-cell responses is unclear, the crucial roles of L-Arg in T-cell proliferation and activation have been demonstrated in tumors (Rodriguez *et al*., 2004). In MΦs lacking L-Arg transporter, NO synthesis was reduced when cells were stimulated with Th1 and Th2 cytokines (Stevens *et al*., 1996; Yeramian *et al*., 2006), suggesting that L-Arg availability is the bottle-neck control step for both iNOS and arginase. Therefore, L-Arg transport from the extracellular milieu is critically important for parasite growth and killing within an infected cell (Krassner and Flory, 1971; Steiger and Steiger, 1977). Since, L-Arg is essential for the survival of intracellular parasites, its reduced concentration may exert a detrimental effect on these parasites. Research focusing on L-Arg functions in *Leishmania* may provide a novel and effective therapeutic approach for the control of leishmaniasis (Wanasen and Soong, 2008). Moreover, administration of synthetic arginase inhibitor to *L. major*-infected Balb/c mice during infection significantly reduced lesion sizes and parasite burdens (Kropf *et al*., 2005). As L-Arg has long been identified as an essential amino acid for *Leishmania* growth, promastigotes could not be maintained in L-Arg-free media (Krassner and Flory, 1971), suggesting mechanisms of L-Arg uptake and utilization in *Leishmania* parasites (Shaked-Mishan *et al*., 2006). This study is a continuous research of authors previous publications (Nahrevanian, 2004, 2006; Nahrevanian and Amini, 2009; Nahrevanian *et al*., 2006, 2008, 2009a; 2009b). Therefore, the aim of this study is to investigate the partial immunotherapy of leishmaniasis by *in vivo* trial of L-Arg in Balb/c mice infected with *Leishmania major* via nitric oxide pathway.

MATERIALS AND METHODS

Animals: Male Balb/c mice were supplied by the Karaj Animal Production Unit, Pasteur Institute of Iran, used in this study. Their initial body weights were 18.2±1.3 g (Mean±SEM). Mice were housed at room temperature (20-23°C) on a 12 h light and dark cycle, with unlimited access to food and tap water. Experiments with animals were done according to the ethical standards 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:** *Leishmania major* MRHO/IR/75/ER was used as standard Iranian strain. The infectivity of the parasites was maintained by regular passage in Balb/c mice. The parasites were cultured in the RPMI 1640 medium supplemented with 10% Fetal Bovine Serum (FBS), 292 μg mL^{-1} L-glutamine and 4.5 mg mL^{-1} glucose (all supplied by Sigma). Under these conditions, the stationary phase of parasite growth was obtained in 6 days as determined previously (Nahrevanian *et al*., 2007).

Infection of mice with *Leishmania major*: Promastigotes of *L. major* were harvested from culture media, counted and used to infect the mice. The base of the tail was injected intradermally with inoculums of 2×10^6 promastigotes. The experiment was performed once *in vivo* and terminated at week 16 after injection.

Drugs and types: The L-Arg (Sigma) was prepared for three types applications; since 100 mg was dissolved in 1 L Distilled Water (DW) with stirrer (Thermal Stirrer, USA) to make a concentration of 0.1 mg mL^{-1} which was used for oral (200 μL day^{-1} by gavage) and injection (200 μL day^{-1}, ip). For making local L-Arg lotion, at first step, 5 mL ethanol was poured in a glass container then 3 g hydroxyl ethyl cellulose was added to it with additional of 30 mL DW and mixed gently to make a suspension. In second step, glycerol 3 g, propylene glycol 2 g and phenoxy ethanol 0.9 g were mixed together at 60ºC (all from Sigma). The contents of first and second steps were merged and 1 g L-Arg was added to it and mixed thoroughly.

Experiments and groups: Entire animals were divided into test and control animals in 7 groups (n = 10 mice/group) including Group 1 (naive with no injection), Group 2 (local test), Group 3 (local control), Group 4 (oral test), Group 5 (oral control), Group 6 (Injection test) and Group 7 (Injection control).

Griess Micro Assay (GMA): The Griess reaction was adapted to assay nitrate as described previously (Nahrevanian and Dascombe, 2001, 2002, 2003). Briefly, standard curves for sodium nitrate (Sigma) were prepared and 100 μL samples were poured in microtubes, then 100 μL Griess reagent was added and proteins subsequently precipitated by 100 μL trichloroacetic acid (Sigma). Tube contents were vortex mixed then centrifuged at 13,400 RCF (Eppendorf centrifuge 5415 C, Germany). Duplicate 100 μL supernatants were transferred into a flat-bottomed microplate (Costar, USA) and absorbance read at 520 nm using microplate reader (Bio-TEK, power wave XS, USA). Values for the concentration of nitrate were calculated from standard calibration plots.

Assessment of pathology

Lesion size: Lesion size was measured at every other week after inoculation 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) divided in two (El-On *et al*., 1986).

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 also 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 amastigotes proliferation: The proliferation of parasite was evaluated by counting of amastigotes inside MΦs on Geimsa stained lesion smears. Five random MΦs were selected; counted and mean percentages were calculated as indicators for the degree of proliferation of amastigotes inside each MΦ.

Assessment of hepato/splenomegaly: Entire livers and spleens were removed post mortem from mice after induction of terminal 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 existing mice at every week after inoculation; the significant differences were determined by ANOVA test and compared with concurrent appropriate vehicle-treated *Leishmania* and control groups. Body weight was measured initially at different time of experiment using a top pan balance (Ohasus Scale Corp., USA).

Assay procedures: The experiments were applied in two sections. The first part assessed toxicity of L-Arg using different doses in naïve Balb/c mice. The pathophysiological effects of L-Arg on host including body weight, hepato/splenomegaly and survival rate were all evaluated. The NO levels in plasma and tissue homogenates of mice were measured. In the second part, three different types of L-Arg (oral, local and injection) were applied *in vivo* and compared to reveal the actual role of L-Arg.

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, California, USA).

RESULTS

The results indicated that L-Arg had ability to elevate ($p<0.001$) NO levels in test animals (93.9±3.4) in comparison with control group (28.3±4.6), which was determined by Student's t-test using Graph Pad Prism. The results showed no pathological effects on survival rate, hepato/splenomegaly and body weight in L-Arg groups (Fig. 1). In order to evaluate the immunological effects of L-Arg, NO was determined in plasma samples and tissue suspensions by GMA. The result showed significant differences among oral group in plasma ($*p<0.05$) and in liver and spleen NO levels reduced significantly ($*p<0.05$) in both injection and oral groups (Fig. 2). The results of second part of experiment on pathophysiological effects using *in vivo* L-Arg as oral, local and injection in Balb/c mice revealed an increase in degree of splenomegaly ($p<0.001$, by injection) and hepatomegaly ($p<0.05$, by local application). No significant differences were indicated in body weight and survival rates among these groups by ANOVA test using Graph Pad Prism software (Fig. 3). The antileishmanial effects of L-Arg were evaluated by lesion sizes, proliferation of

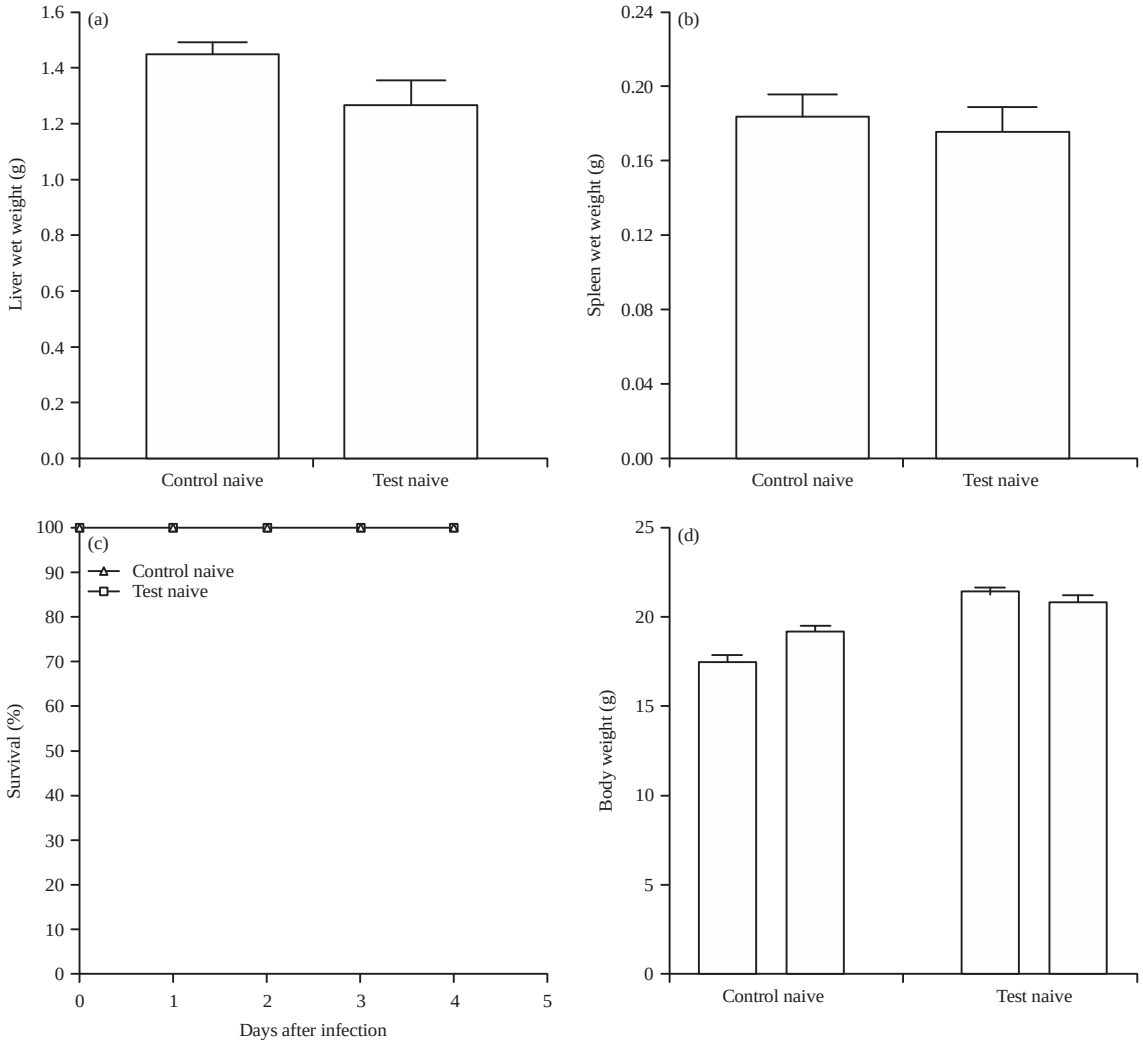

Fig. 1(a-d): Pathophysiological evaluation of L-Arg on naïve Balb/c mice, (a) Hepatomegaly, (b) Splenomegaly, (c) Survival rate and (d) Body weight were evaluated in test and control groups of naïve Balb/c mice. Control animals received saline, as drug vehicle and test group treated with L-arginine, n = 10 mice/groups, student's t-test and one-way ANOVA test using Graph Pad Prism software

amastigotes inside MΦ and percentage of positive impression smears among target organs (liver, spleen, lymph nodes). Comparative proliferation of amastigotes inside MΦs was made by observation of Geimsa-stained smears of cutaneous lesions in *Leishmania* groups and calculations of amastigotes. A significant decrease in parasite proliferation was observed in L-Arg oral group (Fig. 4). Percentages of positive smears from liver, spleen and lymph nodes of mice infected with *L. major* were counted. Oral L-Arg presented anti-leishmanial activity by significant reduction of positive smears from liver and spleen, whereas, local L-Arg increased positive smears from spleen and liver. Injection of L-Arg reduced percentage of positive smears in spleen, but this in liver did not have significant effect and no amastigote was observed in smears of lymph nodes (Fig. 5).

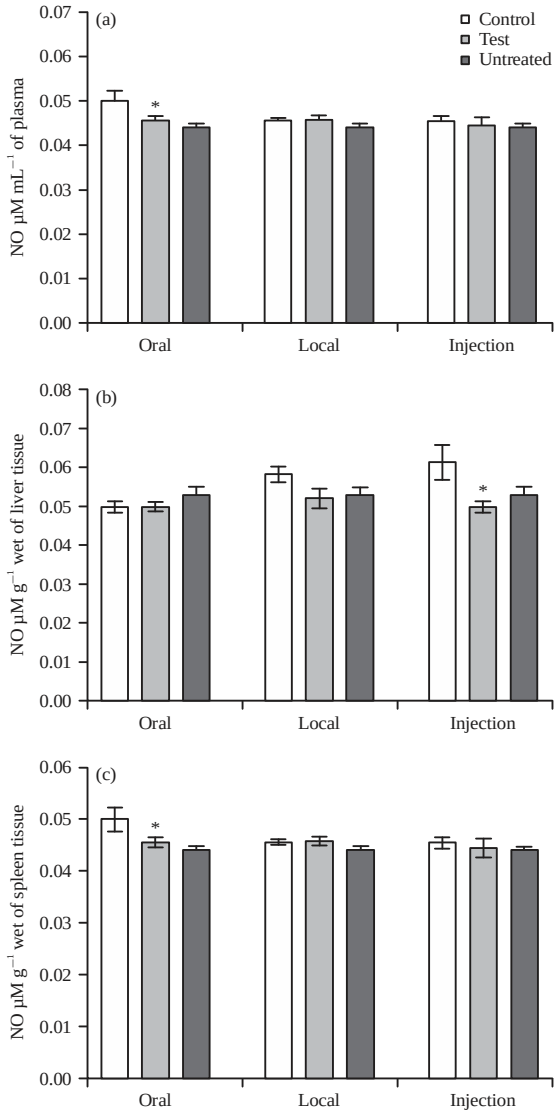

Fig. 2(a-c): Comparative NO concentrations in plasma and homogenates from liver and spleen of Balb/c mice infected with *Leishmania major* after treatment with three types of L-Arg. The NO levels were assayed in (a) Plasma, (b) Hemogenates of liver and (c) Spleen by GMA at the ultimate day of experimental period. *Leishmania* infected control and test groups received saline and L-Arg, respectively and untreated *Leishmania major* mice received no treatment. Significance of differences (*$p<0.05$) was determined by student's t-test using Graph Pad Prism software (n = 10 mice/group)

There is reduction in lesion sizes after treatment with oral and injection L-Arg, when compared with controls. However, there was no significant indication of using L-Arg as local to limit lesion size in CL as presented in experimental groups (Fig. 6).

DISCUSSION

This experiment was a part of continuous studies on the role of NO against intracellular parasites. We have previously published the detailed applications of NO modulators, time course

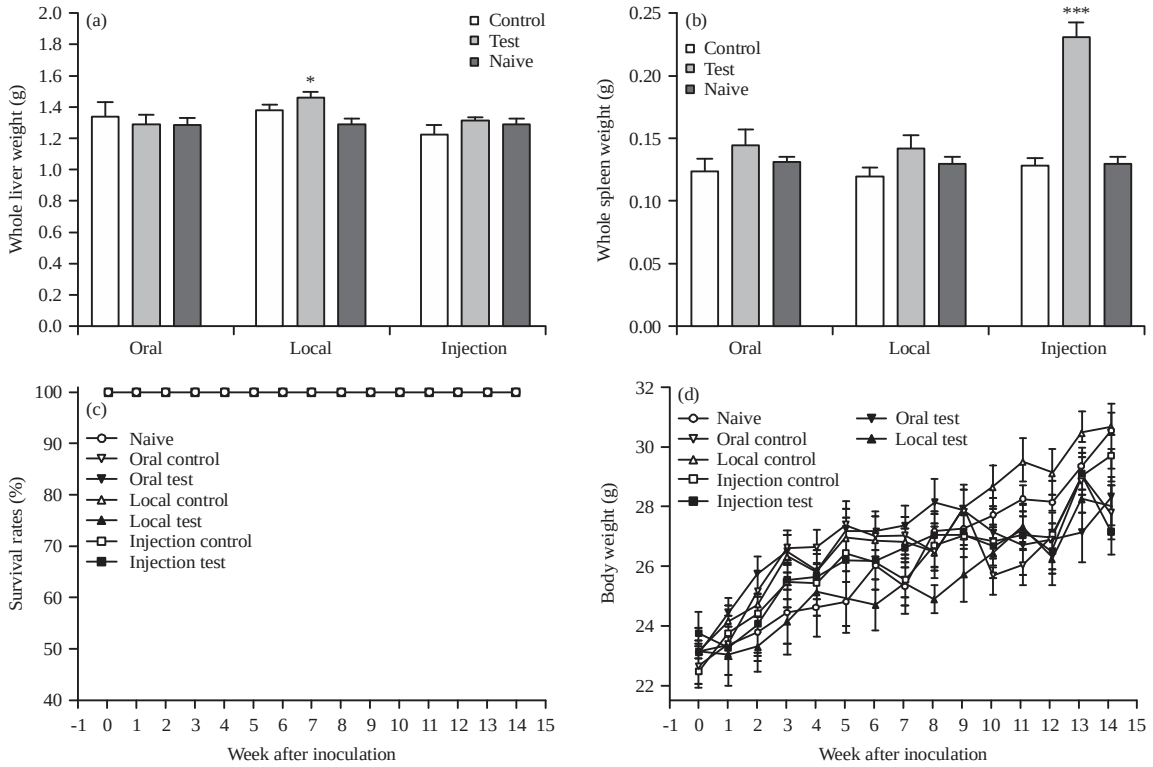

Fig. 3(a-d): Pathophysiological evaluation on groups of *Leishmania major* infected Balb/c mice and treated by three types of L-Arg, (a) Hepatomegaly, (b) Splenomegaly, (c) Survival rate and (d) Body weight were evaluated in test and control groups of *Leishmania major* infected Balb/c mice. Significance of differences (*p<0.05, ***p<0.001) was determined by student's t-test and one-way anova test using Graph Pad Prism software (n = 10 mice/group)

and the associated changes in spleen, as well as plasma, brain and liver concentrations of NO in intracellular parasites including plasmodium and *Leishmania* (Nahrevanian *et al*., 2007, 2009a, 2009b, 2012; Nahrevanian and Dascombe, 2001; Nahrevanian, 2009; Nemati *et al*., 2013). This is the first application of oral, local and injection forms of L-Arg against Iranian strain *L. major* MRHO/IR/75/ER. In current study, naïve and leishmania infected hosts represented no negative consequences after treatment with L-Arg; this confirm wide applications of this amino acid as a food supplement and medication for a variety of purposes (Green *et al*., 1990a; Li *et al*., 2007; Stevens *et al*., 1996; Yeramian *et al*., 2006). In addition to this study, which confirm the minimum cytotoxic effects of L-Arg application in Balb/c model of *Leishmania*, there are some reports indicated no or low toxicity due to L-Arg applications in animals and human (Moncada and Higgs, 1993; Bronte and Zanovello, 2005; Wanasen and Soong, 2008; Munder *et al*., 2009). Therefore, L-Arg may be used for treatment of both CL and VL in different hosts. These data indicated that L-Arg had ability to induce NO in treated animals which are confirmed by several publications *in vitro* and *in vivo* (Green *et al*., 1990b; Krassner and Flory, 1971; Wanasen and Soong, 2008; Kropf *et al*., 2005; Shaked-Mishan *et al*., 2006; Nahrevanian *et al*., 2007; El-On *et al*., 1986).

The data of this study indicated that oral and injection form of L-Arg presented to be more effective than local L-Arg. These two forms slightly limited lesion size, decreased visceralization

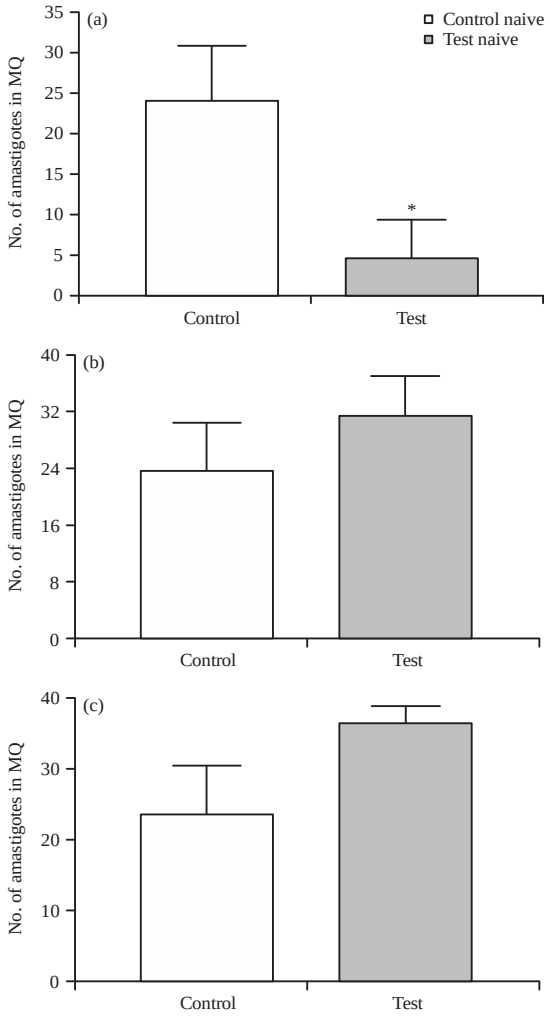

Fig. 4(a-c): Comparative proliferation of amastigotes inside MΦs of *Leishmania major* infected Balb/c mice after treatment with three types of L-Arg. Proliferation of amastigotes inside MΦs was made by observation of geimsa-stained smears of cutaneous lesions in *Leishmania* groups at the end of the experimental period. The proliferation of parasite was evaluated by calculation of mean amastigotes inside MΦs on lesion smears. Significance of difference (*$p<0.05$) was determined by Student's t-test using Graph Pad Prism (n = 10 mice/group), (a) Oral, (b) Local and (c) Injection

of parasite in target organs and declined proliferation of amastigotes inside MΦs. Consequently, it is conceivable that the partial protection induced by L-Arg against *L. major* may be attributable to the NO. Indications are that NO mediate antiparasitic effects, with the overall benefit to the host depending on the location and quantity of NO. It is concluded that there is a need for compounds with a pharmacological profile *in vivo* more conducive to the exploitation of the role of NO in *Leishmania*, including longer biological action and greater selectivity (Nahrevanian *et al*., 2007). These results demonstrated that L-Arg is not only well recognized as a NO precursor with a safely use to treat physiological condition (Fallahi *et al*., 2011; Vahedian *et al*., 2011; Hosseini *et al*., 2010;

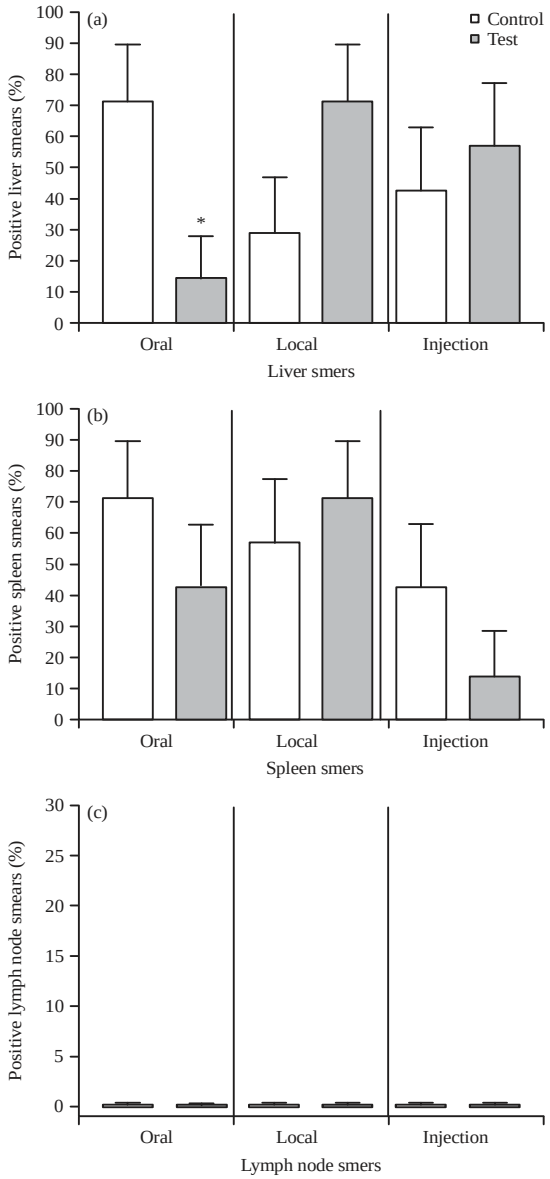

Fig. 5(a-c): Inhibition of parasite visceralization among target tissues of Balb/c mice infected with *Leishmania major* after treatment with three types of L-Arg. Positive Geimsa-stained smears of target tissues from control and test groups of mice infected with *Leishmania major* were counted at the ultimate day of experimental period. Impression smears were prepared from (a) Liver, (b) Spleen and (c) Lymph nodes by placing a small piece of tissue between two glass slides and pushing them in different directions. Smears were dried, fixed and stained with giemsa for examination by light microscopy. Significance of difference (*$p<0.05$) was determined by student's t-test using Graph Pad Prism software (n = 10 mice/group)

Nabavizadeh *et al*., 2010; Colotti and Ilari, 2011; Jowkar *et al*., 2012), but also it has potential therapeutic effects and could be used for its anti-leishmanial activity. In addition to MΦs,

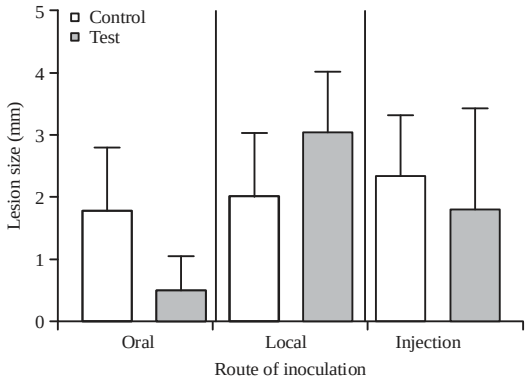

Fig. 6: Comparison of lesion sizes among Balb/c mice infected with *Leishmania major* after treatment with three types of L-Arg. Lesion sizes were measured by a digital caliper in two diameters at right angles to each other and the size (mm) was determined according to the formula: S = (D+d) divided in 2. Data presented as Mean±SEM and analysis of significance was determined by student's t-test using Graph Pad Prism software (n = 10 mice/group)

NO comes from other cellular sources which can contribute towards the protective immune responses against *Leishmania*. Further investigation in defining these sources will be important for the understanding of cell-mediated defense mechanism(s) in *Leishmania*. This is the first application of oral, local and injection forms of L-Arg against Iranian strain *L. major* MRHO/IR/75/ER and it may be the first immunotherapy trial against CL in Iran.

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