

# EVALUATION OF ANTI-LEISHMANIAL ACTIVITY BY INDUCTION OF NITRIC OXIDE AND INHIBITION OF PROSTAGLANDIN IN BALB/C MICE INFECTED WITH *LEISHMANIA MAJOR*

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**Abstract.** Cutaneous leishmaniasis is still one of the health problems in Iran and in the region. Nitric oxide (NO) has a key mechanism in the elimination of parasite from the body by its anti-leishmanial activity. Prostaglandin (PG) is a critical inhibitory factor of infected macrophage to decrease their anti-leishmanial activity. This study was designed to induce NO by L-arginine (L-Arg) precursor and inhibit PG production by anti-inflammatory Indomethacin (INDO) in *Leishmania major* infected Balb/c mice, in order to evaluate the effects of NO and PG on delay of lesion formation, size of lesion and proliferation of amastigotes inside macrophages. Liver, spleen and lymph nodes were also studied as target organs to detect amastigotes. Serum, liver and spleen suspensions were investigated for NO induction by using Griess microassay and serum PG was determined by ELISA. The results indicated that NO production was inhibited by *Leishmania* in infected Balb/c mice as compared with naive animals. Serum NO was inhibited by a combination therapy of L-Arg and INDO. Although NO was decreased in the liver by L-Arg, however it increased in the spleen after L-Arg and INDO application. A significant decline was observed in lesion size from Week 6 after infection by INDO. Both L-Arg and INDO had significant inhibitory effects on visceralization of leishmania in target organs. Only L-Arg decreased proliferation of promastigotes in macrophages. Pathophysiological signs including hepatomegaly, splenomegaly, survival rate and body weight all were affected in this experiment. Statistical analysis of data revealed an association between NO induction and PG inhibition in leishmaniasis. These data may indicate a possible candidacy for L-Arg and INDO as novel drugs for the treatment of leishmaniasis in mouse model.

## INTRODUCTION

*Leishmania* protozoa are the etiologic agents of leishmaniasis, a group of diseases

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that currently threaten 350 million people in 88 countries around the world, with more than 15 million people known to be infected and about 1.5-2 million new cases estimated per year, many of which go unreported (WHO, 2001). These parasitic protozoa are transmitted as monoflagellated promastigotes to vertebrate hosts by the bites of sand flies of the genera *Phlebotomine* and *Lutzomyia*. In human blood, these protozoan parasites

invade phagocytic cells in which they replicate as non-flagellated amastigotes and can either lead to asymptomatic disease or present a wide variety of distinct clinical syndromes, such as cutaneous (CL), mucocutaneous (MCL) and visceral leishmaniasis (VL) (Roberts *et al*, 2000).

Although a majority of inbred strains of mice develop small, self-healing lesions following infection with *Leishmania major*, a few mouse strains, such as BALB/c, develop large, nonhealing CL lesions and ultimately succumb to disseminated disease (Li *et al*, 2002). Mice that spontaneously resolve their infections develop Th1-type responses characterized by heightened production of the macrophage (M $\phi$ )-activating cytokines, IFN- $\gamma$ , and TNF- $\alpha$ , as inducers of nitric oxide synthase 2 (NOS II) and NO synthesis from L-arginine (L-Arg), the main pathway responsible for the killing of *Leishmania*, while nonhealing BALB/c mice develop Th2-type responses in which interleukin-4 (IL-4) is the dominant cytokine produced by CD4 effector cells (Holzmuller *et al*, 2002).

Activated M $\phi$  participates in the inflammatory response by releasing chemokines and factors that recruit additional cells to the site of infection. In addition, M $\phi$  activates the expression of genes responsible for a high-output synthesis of intermediates, which contribute to the regulation of the inflammatory response (Bosca *et al*, 2005). Studies of human CL reveal that *Leishmania* killing is associated with NO production. Moreover, the capacity of canine M $\phi$  to eliminate intracellular amastigotes through a NO-dependent mechanism has been documented (Pinelli *et al*, 2000; Sisto *et al*, 2001). NO-dependent cytostatic and/or cytotoxic activities by activated M $\phi$  on various parasites have been clearly demonstrated (Mauel *et al*, 1991; Johnson, 2000).

M $\phi$ s are a major source of arachidonic acid (AA) metabolites (Lonardoni *et al*, 2000).

The eicosanoids are a family of 20-carbon fatty acid metabolites that include prostaglandins (PGs) and leukotrienes. Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) is an oxygenated metabolite of AA and is produced *via* a two-step process, beginning with the initial action of a cyclooxygenase (COX) to form PGH<sub>2</sub> followed by specific PGE synthases (Erb-Downward and Noverr 2007). COX is the rate-limiting enzyme for the conversion of AA to PGs, which include PGE<sub>2</sub>, PGD<sub>2</sub>, PGF<sub>2</sub>, PGI<sub>2</sub>, and tromboxane A<sub>2</sub> (Spinella *et al*, 2004). COX-1 is constitutively expressed in most tissues and is believed to produce the PGE<sub>2</sub> necessary for homeostasis. Although COX-2 is also constitutively expressed in some tissues and it is induced by various mitogens, hormones, and environmental stimuli (Konger *et al*, 2002). COX-2 is dramatically up-regulated in response to inflammatory signals and is therefore believed to play an important role in the PGE<sub>2</sub> production involved in pathophysiological processes (Trebino *et al*, 2003). An important role of COX-2-derived PGE<sub>2</sub> is the regulation of inflammatory processes. PGE<sub>2</sub> regulates key functions in the reproductive, gastrointestinal, neuroendocrine, immune, and central nervous system (Benneis *et al*, 2006). PGE<sub>2</sub> is a potent regulator of host immune responses, with the ability to elicit both pro- and anti-inflammatory responses, depending on the target cell. PGE<sub>2</sub> acts *via* one of four different G-protein coupled receptors. PGE<sub>2</sub> can inhibit Th1-type immune response, phagocytosis, and lymphocyte proliferation (McIlroy *et al*, 2006). PGE<sub>2</sub> can also promote Th2-type response, IgE production, and tissue eosinophilia (Shibata *et al*, 2005).

Classical COX inhibitors, also known as non-steroidal anti-inflammatory inhibitors (NSAID), are among the most frequently used analgesics. They inhibit PG synthesis through nonselective blockade of constitutive COX-1 and inducible COX-2 (Reinold

*et al*, 2005). NSAID such as INDO blocks the activity of COXs and can enhance the production of Th1-type cytokines in *L. major*-infected mice (Li *et al*, 2002). As COX is stimulated by NO and is inhibited by INDO (Hrabak *et al*, 2001), supporting an interaction between NO and PG (Griffon *et al*, 1998). The role of PGE<sub>2</sub> has been determined in experimental leishmania infection and relationship between these mediators and NO production was also confirmed (Griffon *et al*, 1998). Antileishmanial activity correlates more with PG absence than with NO presence (Lonardon *et al*, 2000).

## MATERIALS AND METHODS

### Animals

Male inbred Balb/c mice (supplied by Karaj Laboratory Animal Unit, Pasteur Institute of Iran) were used in this study. The initial body weight was 18.2 ± 1.3 g (mean ± standard error of mean, SEM) and mice were housed at room temperature (20-23°C) on a 12-hour light and 12-hour dark cycle, with unlimited access to food and tap water. Experiments with animals were approved by Ethics Committee of the Pasteur Institute of Iran.

### *In vitro* cultivation of *L. major*

The *L. major* used in this study was the standard strain MRHO/IR/75/ER. Parasites were maintained by regular passage in susceptible Balb/c mice. Parasites were cultured in 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 10 days (Farahmand *et al*, 2008).

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

Promastigotes of *L. major* were harvested from culture medium, counted and

used to infect Balb/c mice. The base of the tail was injected intradermally with inoculums of 2 × 10<sup>6</sup> promastigotes.

### Experimental groups

Balb/c mice were divided into 6 groups (8 mice/group) as follows: Group 1 (control), Group 2 (*L. major* + 0.4% ethanol), Group 3 (*L. major* + INDO), Group 4 (*L. major* + distilled water, DW), Group 5 (*L. major* + L-Arg) and Group 6 (*L. major* + INDO + L-Arg). L-Arg (100 mg/ml) was dissolved in 1,000 ml DW as drinking water for 4 weeks after infection in experimental groups. Four ml of INDO (50 mg/ml ethanol) were added to 996 ml DW as drinking water.

### Measurement of lesion size

Lesion size (mm) was measured every other week after inoculation using a digital caliper (Chuan Brand, China). Diameters at right angles to each other were measured and the average value recorded (El-On *et al*, 1986).

### Microscopic examination of smear preparation

Clinical diagnosis was confirmed by laboratory demonstration of parasites 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 smears were made of exudation material. Smears were dried in air, fixed by methanol and stained with Giemsa for detection of amastigotes by light microscopy (Nahrevanian *et al*, 2007).

### Assay of amastigote proliferation

Proliferation of parasites was evaluated by counting amastigotes inside Mø on Geimsa-stained lesion smears at the end of the experimental period. Five random Møs were selected, amastigotes counted and mean percentages were calculated as indicators of the degree of proliferation of amastigotes inside Møs (Nahrevanian *et al*, 2007).

### 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 anesthesia by inhalation of diethyl ether (Sigma). Organ wet weights were measured and compared with controls as indices for degree of hepatomegaly and splenomegaly (Nahrevanian *et al*, 2007). Impression smears were prepared from liver, spleen and lymph nodes by placing a small piece of tissue between two glass slides. Smears were examined for presence of amastigotes as described above.

### Measurement of survival rate

Survival rate was presented as the percentage of surviving experimental mice at every week after inoculation.

### Body weight

Body weight was measured initially and at different times of experiment using a top pan balance (OHAUS Scale Corp, USA).

### Preparation of serum

Serum was prepared from blood taken by cardiac puncture from mice terminally anesthetized as described above. Serum was prepared by sedimenting blood at 6,000g for 5 minutes, and stored at -70°C until assayed with Griess microassay (Nahrevanian and Dascombe, 2001).

### Griess microassay (GMA)

GMA was applied as described previously (Nahrevanian and Dascombe, 2001). In brief NO was determined indirectly as nitrite produced from nitrate when incubated with nitrate reductase. Standard curves for sodium nitrite and nitrate were prepared. Samples were treated with nitrate reductase (NAD[P]H and NADPH  $\beta$ -nicotinamide adenine dinucleotide phosphate. Griess reagent was then added and proteins subsequently precipitated by trichloroacetic

acid. Tubes were vortex mixed and centrifuged, and supernatants were transferred to a flat-bottom microplate and absorbances read at 520 nm (Bio-Tek-Powerwave, XS). Values for the concentration of nitrite assayed were calculated from standard calibration plots of sodium nitrite and nitrate.

### Assessment of serum PGE<sub>2</sub>

PGE<sub>2</sub> was determined in serum by enzyme-linked immunosorbent assay (ELISA) kit (DRG Instruments, Germany) according to manufacturer's instructions. Standard dilutions were prepared with PGE<sub>2</sub> concentrations of 2,500, 1,250, 625, 313, 156, 78.1 and 39.1 pg/ml. Aliquots of 100  $\mu$ l of samples were added to 50  $\mu$ l of assay buffer, conjugate and antibody. The plate was incubated at room temperature on a plate shaker for 2 hours at ~500 rpm. Wells were washed 3 times with 400  $\mu$ l of wash solution, then 200  $\mu$ l of substrate solution were added, incubated at room temperature for 45 minutes without shaking. The reaction was stopped by adding 50  $\mu$ l of stop solution and absorbance was read immediately at 405 nm using a microplate reader (Bio-Tek-Powerwave, XS).

### Statistical analysis

Values for NO and PG are presented as mean  $\pm$  SEM. Significance of difference was determined by Analysis of Variances (ANOVA) and Student's *t*-test using Graph Pad Prism Software (Graph Pad, San Diego, CA).

## RESULTS

Results of serum NO levels for all six groups indicated that production of NO was inhibited in infected Balb/c mice by *L. major* as compared with naive animals. INDO as PG inhibitor showed its ability to elevate NO levels in infected animals (Fig 1).

NO production was measured in suspensions of the liver and spleen. Both L-Arg

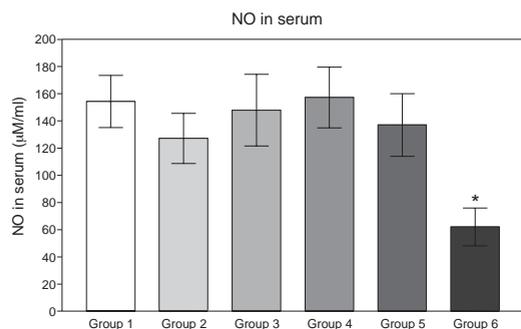


Fig 1—NO production in serum of control and test groups of Balb/c mice. NO levels were assayed in serum by GMA at the end of the experimental period. All animals used in this experiment were 48 Balb/c mice, in six groups ( $n=8$  mice/group) including Group 1 (naive), Group 2 (*L. major* + 0.4% ethanol), Group 3 (*L. major* + INDO), Group 4 (*L. major* + DW), Group 5 (*L. major* + L-Arg) and Group 6 (*L. major* + INDO + L-Arg). Significance of differences ( $p < 0.05$ ) was determined by an unpaired Student's *t*-test using Graph Pad Prism.

and INDO elevated NO levels ( $p < 0.05$ ). Leishmania probably had some inhibitory effects, in spite of immune activation by L-Arg and INDO. Despite NO inhibition by leishmania parasite, L-Arg as a putative NO inducer showed its strong ability to elevate NO ( $p < 0.05$ ) (Fig 2).

There were significant changes in liver NO after activation with INDO, probably the liver as an organ was not directly involved with NO induction. Some alterations were observed in NO levels in liver suspensions of Groups 2–3, which may be a result of water uptake. However, NO production in the liver was inhibited by *L. major* infection ( $p < 0.05$ ), when compared with control animals in Group 2. Healing of CL lesions was studied by measurement of lesion sizes in both control and test groups. INDO showed

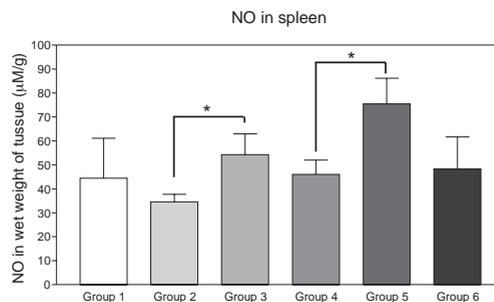
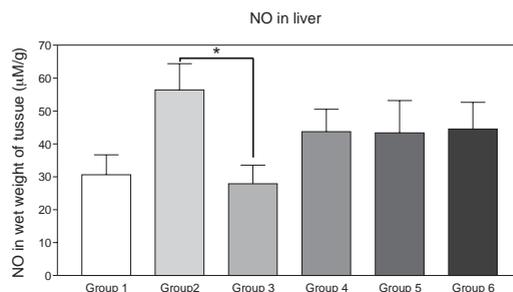


Fig 2—NO production in suspensions of the liver and the spleen of control and test groups of Balb/c mice. NO production was measured by GMA in suspensions of the liver and the spleen of all groups at the end of the experimental period. Significance of differences ( $p < 0.05$ ) was determined by an unpaired Student's *t*-test using Graph Pad Prism ( $n=8$  mice/group).

anti-leishmanial activity by reducing the lesion sizes ( $p < 0.001$ ) after Week 11 (Fig 3).

Percentages of positive Geimsa-stained smears were counted from lesion, liver, spleen and lymph nodes of mice infected with *L. major*. L-Arg had anti-leishmanial activity by reduction positive smears from the liver and spleen, whereas INDO indicated significant effects in spleen and lymph nodes during the healing stage of disease. L-Arg decreased percentage of positive smears in the spleen from 25% (Group 4, Control) to 12.5% (Group 5, Test). They also reduced positive liver smears from 100% (Group 4, Control) to 87.5% (Group 5, Test),

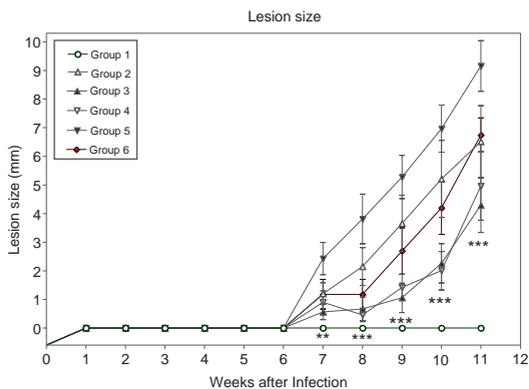


Fig 3—Progress of lesion sizes of CL in groups of Balb/c mice infected with *L. major*. Lesions were studied by making stained smears at different weeks after infection. Lesion size was measured in mm by a digital caliper in two diameters (D and d) at right angles to each other, and the size was determined according to the formula:  $S = (D + d)$  divided in two. Significance of differences ( $p < 0.001$ ) was determined by ANOVA test using Graph Pad Prism ( $n=8$  mice/group).

but positive smears of lymph nodes remained unchanged at 12.5% (Group 4 and Group 5). However, INDO reduced positive smears from 25% (Group 2, Control) to 0.2% (Group 3, Test) only in lymph nodes smears. Combination therapy by L-Arg + INDO presented a significant reduction in liver positive smears in Group 6 from 100% (Group 4) and 87.5% (Group 2) to 25% (Group 6) and in lymph nodes from 12.5% (Group 4) and 25% (Group 2) to 0% (Group 6) (Fig 4).

Proliferation of amastigotes inside Mø was compared by microscopic examination of Geimsa-stained smears of lesions in leishmania groups. Although, no putative efficacy of NO-induction was evidenced to reduce parasite proliferation, a significant decline in number of amastigotes was observed inside Møs after INDO therapy (Fig 5).

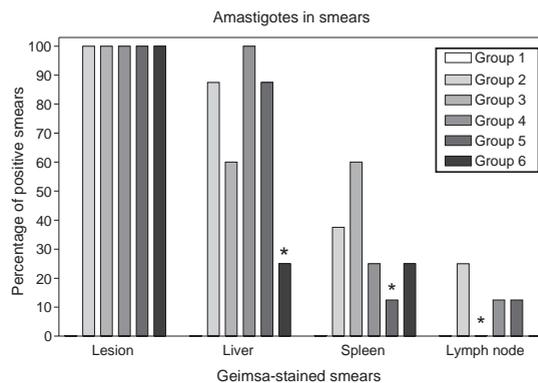


Fig 4—Percentages of positive smears from target tissues of Balb/c mice infected with *L. major*. Positive Geimsa-stained smears were counted from lesion, the liver, the spleen and lymph nodes of control and test groups of mice infected with *L. major* at the end of the experimental period. Lesions were cleaned with methanol and punctured at the margins with a sterile lancet and exudation material was smeared. Impression smears were prepared from the liver, the spleen and lymph nodes by placing a small piece of tissue between two glass slides and pushing them in different directions. Smears were dried in air, fixed by methanol and stained with Giemsa for examination by light microscopy ( $n=8$  mice/group).

Pathophysiological signs including hepatomegaly, splenomegaly, survival rate and body weight all were evaluated in experimental animals. Leishmania parasite increased hepatomegaly as a pathological consequence of disease in infected mice. INDO decreased sharply splenomegaly ( $p < 0.001$ ) (Group 3) as compared with control (Group 2). Combination therapy by L-Arg + INDO reduced survival rate ( $p < 0.01$ ) and body weight ( $p < 0.001$ ) as a toxicity point of view (Fig 6).

PG production was measured in sera of all groups once at final stage. This may ex-

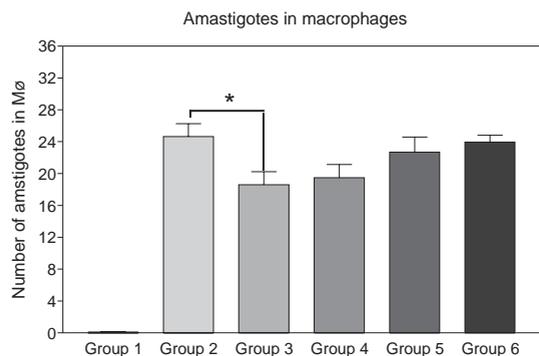


Fig 5—Comparative proliferation of amastigotes inside Mφ from lesions of Balb/c mice infected with *L. major*. Proliferation of amastigotes inside Mφ was made by observation of Geimsa-stained smears of lesions in leishmania groups at the end of the experimental period. The proliferation of parasite was evaluated by counting and calculation of mean amastigotes inside five random Mφ on Geimsa stained lesion smears. Analysis of differences was determined by an unpaired Student's *t*-test using Graph Pad Prism ( $n=8$  mice/group).

plain no alterations of PG values at this time. Leishmania probably had some antagonistic effects on INDO therapy (Fig 7).

## DISCUSSION

Experimental *L. major* infection is one of the best elucidated models for studying defense mechanisms in murine model (Iniesta *et al*, 2001) and exploring the immune responses underlying susceptibility/resistance to intracellular infection (Sacks and Noben-Trauth, 2002). The relationship between the immune system and the evolution of leishmaniasis is well studied in the experimental murine models and substantial progress in the immunopathogenesis of CL has been achieved (Sacks and Noben-Trauth, 2002). Although a majority of inbred

mice develop small, self-healing lesions following infection with the *L. major*, Balb/c mice, develop large, nonhealing lesions with visceralized parasites, systemic disease and death (Li *et al*, 2002; Nahrevanian *et al*, 2007; Radwanska *et al*, 2007). This is associated with a Th2 response characterized by secretion of cytokines and PG (Arendse *et al*, 2005). In contrast, resistance to *L. major* infection is mediated by Th1 response, cytokines and NO (Iniesta *et al*, 2005; Holscher *et al*, 2006). PG inhibits Th1 responses and NO production, which contributes to the predominance of a Th2 response in Balb/c mice infected with *L. major* (Freitas *et al*, 1999).

In authors' previous studies, applications of NO modulators, NO inhibitors, time courses, NO variations and its pathophysiological consequences during infection with intracellular parasites were described (Nahrevanian and Dascombe 2001, 2006; Nahrevanian, 2006; Nahrevanian *et al*, 2007). This experiment investigated the role of L-Arg as NO-inducer and INDO as PG-inhibitor in Balb/c mice infected with *L. major*.

The modulation of NO was able to modify clinical signs and could affect the proliferation of amastigotes inside Mφ, lesion sizes, survival rates, degrees of hepatosplenomegaly and presence of amastigotes in smears of the liver, spleen and lymph node. Analysis of data revealed an association between NO with the evolution of disease in the spleen, which had an effect on pathological signs. A partial role for NO is highlighted here, which is in accordance with published reports, describing that intracellular control of *Leishmania* infection is partly NO dependent, and has been confirmed by several laboratories (Ajadary *et al*, 2000; Qadoumi *et al*, 2002; Nahrevanian *et al*, 2007). Activated Mφs kill the amastigotes of *L. major in vitro* (Cillari *et al*, 1994), which may point to involvement of NO in the cy-

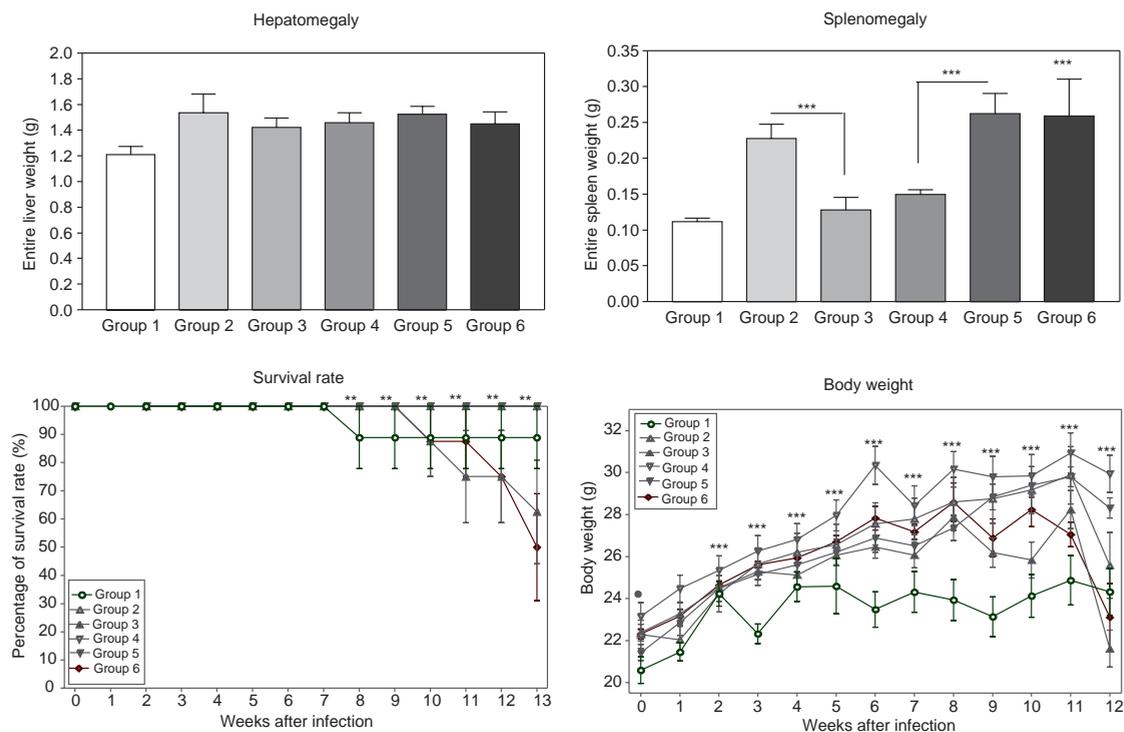


Fig 6–Pathophysiological evaluation on six experimental groups of Balb/c mice. Pathophysiological signs including hepatomegaly, splenomegaly (at the end of the experimental period), survival rate and body weight (at every week after inoculation), all were evaluated in all six groups of Balb/c mice. For measurement of hepato/splenomegaly, entire livers and spleens were removed *post mortem* from mice after terminal general anesthesia. Organ wet weights were measured as indices of possible hepatomegaly and splenomegaly. Survival rate was presented as the percentage of survived experimental mice and body weight was measured using a top pan balance. Significance of differences ( $p < 0.001$ ) was determined by an unpaired Student's *t*-test and One-Way ANOVA test using Graph Pad Prism ( $n=8$  mice/group).

toxic activity of M $\phi$  against this parasite (Vouldoukis *et al*, 1994). M $\phi$  when exposed to microorganisms, exhibit a burst in oxygen consumption and generation of ROI and NO with H<sub>2</sub>O<sub>2</sub>, shows more potent leishmaniacidal effect by being converted into more toxic derivatives (Nahrevanian *et al*, 2007). In addition, NO is unlikely to be the only effectors mechanism in CL, killing of *L. major* parasites is reported to be dependent on cytokines, suggesting a correlation between NO production and disease outcome (Qadoumi *et al*, 2002). Consequently, it is conceivable that the protection induced

by NO inducer against *L. major* may be attributable partly or entirely to effects other than the concurrent increase in NO (Dascombe and Nahrevanian, 2003).

When Balb/c mice infected with *L. major*, they fail to develop a Th1 response, but instead mount a Th2 response and die of the disease. PG production is elevated and that *in vivo* treatment with the cyclooxygenase inhibitor, INDO can significantly slow the growth of parasitized lesions (Farrell and Kirkpatrick, 1987) and production large amounts of PGE<sub>2</sub> could be reversed with this medication (Li *et al*, 2002). In addition, PG

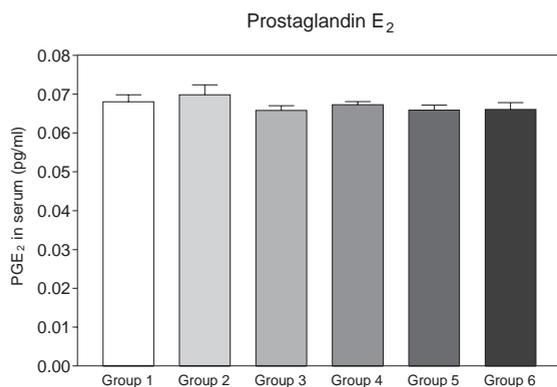


Fig 7-PG production in sera of six groups of Balb/c mice. PGE<sub>2</sub> quantification was determined in sera by ELISA kit (DRG Instruments, Germany) and the optical density read at 405 nm using a microplate reader.

inhibits the development of Th1 responses; therefore, application of INDO inhibits PG production and enhance the production of Th1-type cytokines in *L. major* infected mice. INDO promoted a Th1 response, presented anti-leishmanial activity, lessened disease severity (parasite burden and pathology), lesion size and reduced pathology in association with raised serum NO. This could emphasize the role of PG inhibition to act against experimental *L. major* infection in murine model.

In this study, NO production by hepatocytes in the liver was decreased after treatment with INDO. It is possible that NO synthesis by Mø was able to decrease NO production by hepatocytes via PGE<sub>2</sub> (Griffon *et al*, 1998). It is indicated that in cells, INDO inhibits cyclooxygenase to promote NOS activity (Hrabak *et al*, 2001). Both L-Arg and INDO showed their ability to elevate spleen NO in leishmanial mice. Spleen cells are reported to be more susceptible to INDO, suggesting that the drug acts on NO production via the inhibition of PG synthesis (Hrabak *et al*, 2001).

In this study, the liver, spleen and lymph nodes were studied as target organs to detect amastigotes and to evaluate visceralization of this CL form of parasite. The data obtained in the present study indicated that activated Mø released NO, which might be involved in the leishmanicidal activity of these cells against *L. major*. The resistance of amastigotes inside the Mø was affected by INDO. It had anti-leishmanial activity by reduction positive smears from liver and lymph nodes.

Consequently, L-Arg increased spleen NO; declined proliferation of amastigotes in the liver and lymph node but it had no effects on host survival. INDO, although had some beneficial activity including elevation of spleen NO, decline of amastigote proliferation inside Møs, effects on lesion size and help to healing of CL lesion; however it decreased survival rate, and caused hepato / splenomegaly. Moreover, combination therapy with L-Arg + INDO increased spleen NO, declined proliferation of amastigotes in the liver and lymph node and reduced survival rate.

It is concluded that there is a need for compounds with a pharmacological profile *in vivo* in leishmania with longer biological action and greater selectivity. These results demonstrated that INDO had leishmanicidal activity as a potential therapeutic agent. Perhaps, NO and PG come from several cellular sources, which can contribute towards the immune responses against *Leishmania*. Further investigation in defining these sources will be important for the understanding of cell-mediated defense mechanism(s) in leishmania.

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