

## **Leishmaniasis: effect of enalaprilat on the production of (ON) and cytokines *in vitro***

Leishmanioses: efeito de enalaprilato na produção de óxido nítrico (ON) e citocinas *in vitro*

Leishmaniasis: Efecto de enalaprilat en la producción de óxido nítrico (NO) y citoquinas *in vitro*

Received: 02/14/2021 | Reviewed: 03/22/2021 | Accept: 03/06/2021 | Published: 03/14/2021

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### **Abstract**

Leishmaniasis is neglected diseases; the drugs used have significant toxic effects that compromise patient compliance, and increased loss of efficacy due to increased resistant infectious strains. Assays on Swiss mice infected with *Leishmania brasiliensis* and treated with Angiotensin-Suppressing Enzyme (ACE) inhibitors showed that, in addition to the classical effects on the cardiovascular system, some of these inhibitors had effects on nonhemodynamic, immunomediated functions such as cytokine production. In this study, the effect of enalaprilate on nitric oxide (NO) production and cytokines were evaluated, i.e., interleukins-10 and 12 and IFN- $\gamma$  in J774 A.1 macrophages infected by *Leishmania brasiliensis*. Enalaprilate demonstrated in vitro immunomodulatory capacity, increasing the release of NO to induce IL-12 and IFN- $\gamma$  also decreasing il-10 release.

**Keywords:** Leishmaniasis; Enalaprilate; Immunomodulation; Cytokines.

### **Resumo**

As Leishmanioses são doenças negligenciadas, os medicamentos usados têm efeitos tóxicos significativos que comprometem a conformidade do paciente, e aumento da perda de eficácia devido ao aumento de cepas infecciosas resistentes. Ensaios em camundongos *swiss* infectados com *Leishmania brasiliensis* e tratados com inibidores de Enzima Conversora de Angiotensina (ACE) mostraram que, além dos efeitos clássicos sobre o sistema cardiovascular, alguns desses inibidores, apresentaram efeitos sobre funções não hemodinâmicas, imunomediadas, como a produção de citocinas. Neste estudo, foram avaliados o efeito do enalaprilato na produção de óxido nítrico (NO), e nas citocinas, ou seja, interleucinas-10 e 12 e IFN- $\gamma$  nos macrófagos J774 A.1 infectados por *Leishmania brasiliensis*. O enalaprilato demonstrou capacidade imunomodulatória *in vitro*, aumentando a liberação de NO para induzir IL-12 e IFN- $\gamma$  também diminuindo a liberação do IL-10.

**Palavras-chave:** Leishmanioses; Enalaprilato; Imunomodulação; Citocinas.

### **Resumen**

La leishmaniasis son enfermedades desatendidas, los fármacos utilizados tienen efectos tóxicos significativos que comprometen el cumplimiento del paciente, y una mayor pérdida de eficacia debido al aumento de las cepas infecciosas resistentes. Los ensayos en ratones *Swiss* infectados con *Leishmania brasiliensis* y tratados con inhibidores de la enzima supresora de angiotensina (ECA) mostraron que, además de los efectos clásicos sobre el sistema cardiovascular, algunos de estos inhibidores tenían efectos sobre funciones no biodinámicas e inmunomediadas, como la producción de citoquinas. En este estudio, se evaluó el efecto del enalaprilato en la producción de óxido nítrico (NO) y las citoquinas, es decir, interleukins-10 y 12 y IFN- $\gamma$  en macrófagos J774 a.1 infectados por *Leishmania brasiliensis*. Enalaprilate demostró capacidad inmunomoduladora *in vitro*, aumentando la liberación de NO para inducir IL-12 e IFN- $\gamma$  también disminuyendo la liberación de il-10.

**Palabras clave:** Leishmaniasis; Enalaprilato; Inmunomodulación; Citoquinas.

## 1. Introduction

Leishmaniasis represent a complex of infectious and parasitic diseases transmitted to humans by the *Phlebotomines* carried out by the mosquitoes of the genus *Phlebotomus* caused by protozoa of the genus *Leishmania* (Brasil 2010a; Araújo et al 2020). Leishmaniasis affects thousands of people around the world and no pharmacological treatment is fully effective or safe. An estimated 1.3 million new cases and 20,000 to 30,000 deaths occur annually (Who 2014). However, despite its epidemiological importance, there are few drugs available commercially, and these, when available, have significant toxicity, which favors the therapeutic intervention abandonment (Brasil 2010a). In spite of meglumine antimoniate being the drug of first choice in the treatment of cutaneous leishmaniasis, long-term use is associated with severe cardiotoxic effects (Rodrigues, Hueb, Nery & Fontes 2007). The low number of therapeutic agents is due in part to the affected public, who mostly reside in developing countries and of low income. Thus, research into new drugs is of low potential profit (Brasil 2010b).

The pathogenesis of leishmaniasis, its clinical manifestations and course of the disease, depend on complex interactions between the virulence characteristic of each species of protozoan parasite and the immune response mediated by the host cells (Gollob, Viana & Dutra 2014). However, despite the wide range of clinical forms, the location of amastigotes inside macrophages make infection control dependent on a cell-mediated immune response, the severity of the infection is intrinsically related to the development of the response pattern. In fact, when the specific cellular response is well modulated with a predominance of type 1 cytokines, there is a tendency to spontaneous healing and good response to treatment (Brazil 2010). Experimentally, it has been demonstrated in mice that the resistance or susceptibility to leishmaniasis are linked to two populations of CD4 + T cells, those that produce type 1 cytokines and those that secrete type-2 cytokines, respectively (Dutra et al 2011).

Experimentally, some authors have demonstrated that inhibitors of angiotensin converting enzyme (ACE) inhibitors can modulate the immune response by altering the pattern of cytokines in murine models (Schieffer, et al., 2000; De Albuquerque, et al., 2004). In fact, human monocytes stimulated with tumor necrosis factor (TNF)  $-\alpha$  and GM-CSF secrete angiotensin II (Kim, Zhou & Wahl 2005) suggesting that there is a link between the renin-angiotensin system and production of cytokines that can be independent of hemodynamic factors. In addition, ACE inhibitors can, by inhibiting angiotensin II formation in cells of the immune system, regulate apoptosis of T cells (Albuquerque et al 2004).

In this context, it can be postulated that ACE I may alter the pattern of lymphocyte polarization, stimulating NO synthesis and IL-12 cytokines, IFN- $\gamma$  and TNF (Th1), and decrease the release of IL-10 (Th2 pattern), as this may improve the treatment of leishmaniasis. In humans, cutaneous and mucosa leishmaniasis are associated with strong local inflammatory reaction, and increased expression of TNF, IFN- $\gamma$  and few parasites in the lesion (Gollob et al 2014).

Thus, because of the central role of macrophages as well as the aforementioned cytokines and nitric oxide, the ability of enalaprilat in modulating the immune response to a pattern of cytokine protection against cutaneous leishmaniasis was evaluated in this study.

## 2. Methodology

### 2.1 Material

#### 2.1.1 Reagents and Drugs

The following reagents and drugs were used: kanamycin, trypsin (Gibco, NY), fetal bovine serum (FBS) (Cultilab, SP, Brazil). Alkaline phosphatase-conjugated streptavidin, recombinant IFN- $\gamma$  mouse monoclonal antibody of mouse IL-10 and standard mouse recombinant IL10, IL-12 monoclonal antibody of mouse and standard mouse recombinant IL12 were purchased from BD Bioscience (PharMingen™, CA, USA) and alamar Blue® (Invitrogen, CA, USA). All other reagents were of analytical grades (Sigma Aldrich, MO, USA).

### 2.1.2 Cell lines

Murine macrophages line J774 A.1 (ATCC CR-107), macrophage/monocyte cellular type, derived from a sarcoma of an adult female of the Balb/c were used and grown in RPMI-1640 cell culture medium (Sigma, St. Louis, MO, USA), supplemented with the antibiotics: streptomycin (10mg/mL), penicillin (6mg/mL), kanamycin (2mg/mL; Gibco, Grand Island, NY, USA) and 10% FBS, maintained at 37°C and 5% of CO<sub>2</sub>. Weekly subculture for the establishment of cell culture were performed. J774 A.1 were obtained from the cell bank of Rio de Janeiro (BCRJ), Rio de Janeiro, Brazil.

### 2.1.3 Parasites

The stationary phase promastigotes forms of *L. braziliensis* MHOM / BR / 1975 / M2903 were used and were maintained in Schneider culture media supplemented with streptomycin (10mg/mL; Sigma, St. Louis, MO, USA) and penicillin (6mg/mL; Sigma, St. Louis, MO, USA), 20% FBS (SBF; Cultilab, Campinas, SP, BR), maintained at 26°C in B.O.D incubator. The parasites used were obtained from cell culture that were 6 to 7 days of growth, when the promastigote forms are in the stationary phase. *Leishmania* were purchased from *Leishmania* collections of Instituto Oswaldo Cruz- CLIOC- Rio de Janeiro, RJ, Brazil.

### 2.1.4 Enalaprilat acquisition

Enalapril maleate (CAS No. 76095-16-4) was purchased from Sigma Aldrich (purity  $\geq$ 98%). All chemicals used were of highest degree of purity commercially available. Water used in preparing solutions was distilled twice and purified by MilliQ System.

## 2.2 Methods

### 2.2.1 Enalapril Effect on promastigotes forms

Enalaprilat was evaluated for its ability to inhibit the proliferation of *Leishmania* promastigotes (Fournet et al 1993). Promastigote forms of *Leishmania braziliensis* in stationary phase of growth, at a concentration of  $1 \times 10^5$  parasites/ml, were cultivated for 8 days, at the temperature of 26°C, in Schneider culture medium, in 96 well plates (Kasvi) with standard drug Amphotericin B with 10 mg/ml (Afotérica B sigma Aldrich), enalaprilat was diluted and tested at different doses to establish the potential anti-proliferative effect on the parasite. As a negative control, *Leishmania* was grown in Schneider medium containing diluent. After 72 hours, counting was performed in Neubauer chamber to determine the number of live parasites. The results were expressed as IC<sub>50</sub> and percentage inhibition (PI) relative to the control, considered as 100% viable promastigotes.

### 2.2.2 Cytotoxicity and Selectivity Index (SI) of Enalaprilat on J774 A.1 cells.

Murine macrophages from J774 A.1 cell line were grown in cell culture flasks of 25 cm<sup>2</sup> in RPMI-1640 (Sigma) supplemented with streptomycin (10 mg / ml), penicillin (6 mg / ml) and kanamycin (2mg / ml) and 10% FBS maintained at 37°C and 5% of CO<sub>2</sub>. The cell culture after reaching semi-confluence was washed once with Hanks buffer, trypsinized and counted in a Neubauer chamber, adjusting the number of cells to  $2 \times 10^5$  cells/mL in complete RPMI medium. This suspension cells were seeded in 96-well plates and incubated at 37 and 5% CO<sub>2</sub> for 24 hours. Thereafter, enalaprilat at different concentrations were prepared and added to the cells after removal of the medium, and then incubated at 37°C and 5% CO<sub>2</sub> for 24 hours. As a negative control the cells were cultivated with the diluent. Doxorubicin (10 mg / mL, Eurofarma, São Paulo, SP, BR) was used as a positive control in this bioassay. The cytotoxicity index (IC) and cell proliferation of enalaprilat were assessed by the redox indicator (redox) Alamar blue (AB; Biosource, Camarillo, CA) according to Al-Nasiry et al (2007). After the incubation period, the culture medium was removed and then 20  $\mu$ L of alamar blue and 180  $\mu$ L of RPMI complete was added. After 6 hours of incubation, the absorbance was read in filters of 570 nm (oxidised) and 595 nm (reduced status) in the ELISA reader (BIORAD 640). The data were analyzed by linear regression curve (GraphPad Prism version 5.02 for Windows, GraphPad Software, California, USA) and the results were expressed

as CC<sub>50</sub>, with CC<sub>50</sub> <50 µg/ml regarded as cytotoxicity according to Froelich et al (2007). The experiments were performed in triplicate. The selectivity index was calculated taking into consideration the ratio of CC<sub>50</sub> and IC<sub>50</sub>.

### 2.2.3 Evaluation of enalaprilat effects on cells infected with species of *Leishmania* genus.

J774 A.1 cells ( $2.5 \times 10^5$  / ml) were seeded on round glass coverslips in 24-well plates in RPMI 1640 medium (Sigma) supplemented with 10% FBS for 24 hours at 37 ° C in the presence 5% CO<sub>2</sub> for cell adhesion. After 24 hours, non-adhering cells did were removed and the adherent washed in incomplete RPMI and infected with infective promastigote forms of *Leishmania braziliensis* in a proportion of 5: 1 (parasite: cell) for 6 hours in CO<sub>2</sub> incubator at 37 ° C. After 6 hours of incubation, the infected cells were cultured in the presence of the drug, amphotericin B (10 mg / ml) or untreated (medium). Subsequently, the coverslips were washed in RPMI 1640 to remove nonadherent cells, immediately dried with an air jet and then stained with Giemsa (Sigma, St. Louis, MO, USA) for thirty minutes. The infection rate was determined through random analysis of 100 cells, by evaluating the percentage of infected cells and the average number of intracellular parasites, as described by Chang (1980). The results were expressed as IC<sub>50</sub> and percentage inhibition (PI) of intracellular amastigotes was determined as described by Lakshmi, et al., (2007), using the formula:  $PI = 100 - (T/C * 100)$ .

### 2.2.4 Assessing nitrite concentration by Griess method

The release of NO was indirectly assessed by determining the levels of nitrite (Ding et al, 1988). For quantification of nitrite, 0.1 ml aliquot of the culture supernatants was used, in the presence or absence of enalaprilat (at concentrations between 100 µg/mL and 6.25 µg/mL) at different times. The supernatants were incubated with an equal volume of Griess reagent (1% sulfanilamide, 0.1% dihydrochloride N- (1-naphtil) ethylenediamine, 2.5% H<sub>3</sub>PO<sub>4</sub>) at room temperature for 10 minutes. Absorbance was read in ELISA reader at 540 nm (Titertek Multiskan Reader). As positive controls concentrations of 1 µg / ml IFN-γ (IFN-γ Recombinant Mouse / BD Pharmingen, San Diego, CA, USA) and 50 µg/ml of LPS (Lipopolysaccharide from *Escherichia coli* Serotype 0111:B4 – Sigma, St. Louis, MO, USA) were used. Nitrite levels were determined by comparing the absorbance obtained with a predetermined standard curve. The Limulus ameocyte lysate assay (Sigma) was employed. All reagents and materials besides enalaprilat were LPS-free (<0.2 ng / ml of endotoxin).

### 2.2.5 Infection of macrophages with *Leishmania* and treatment with enalapril.

J774 A.1 Macrophage ( $10^5$  cells per 0.1 ml) were seeded on round glass coverslips in the interior of 24-well plates in RPMI 1640 medium (Sigma) supplemented with 10% FBS for 24 hours at 37 ° C in the presence of 5 % CO<sub>2</sub> for cell adhesion. After this period the non-adherent cells were removed, while the adherent cells were washed in incomplete RPMI and then infected with  $2 \times 10^6$  infective form of *Leishmania braziliensis* promastigotes at ratio 20:1, after 12 hours the promastigote extracellular forms were removed by washing the wells with PBS shortly after the cells were treated with 2.3 ug / ml enalaprilat, LPS (50ng / ml), IFN-γ (100 U / ml), LPS and IFN-γ and incubated for 24, 48 and 72 hours at 37 in an atmosphere of 5% CO<sub>2</sub>.

Macrophages were fixed with methanol and stained with hematoxylin / eosin (HE). intracellular amastigotes were counted and the rate of infection was determined by multiplying the percentage of macrophages infected with the average number of amastigotes per macrophage. Macrophages count in each coverslip was standardized to two hundred per coverslip (De Souza Carmo et al 2010).

### 2.2.6 Quantification of IFN-γ, IL-12 and IL-10 by immunoassay

IFN-γ, IL-12 and IL-10 were determined by ELISA employing antibodies and standards obtained from Pharmingen™ (San Diego, CA, USA). Polystyrene 96-wells plates (Dynatech, Alexandria, VA, USA) were maintained overnight at 4°C with

50 µl of monoclonal antibodies (anti-mouse) to IFN-γ, IL-12 and IL10, thereafter the plates were washed with PBS and 0.05% of Tween-20, blocked for 60 min at 37°C with FBS diluted with PBS. 100 µL of the supernatant of the established cultures, subjected to treatments at various time periods (24, 48 ad 72h) with enalaprilat and then 100 µl of the standard added (IFN-γ, IL-12 and IL-10 recombinants) in triplicates to the well plates and incubated at 37°C for 24 h. After washing the plates, they were added 100 l of anti-IFN-γ mouse monoclonal antibodies, IL-12 and IL-10 in each well for 45 min at 37 ° C. Streptavidin conjugated to alkaline phosphatase (Pharmingen) was added and incubated for 30 min at 37 ° C after incubation 100 µl of p-nitrophenyl-phosphate (Sigma, up to 1mg/ml) diluted in 0.1M glycine buffer was also added. The plates were incubated for 30 min at 37 ° C in the dark, they were then immediately read at a wavelength of 405 nm in an ELISA reader (Titertek Multiskan Microplate Reader).

### 3. Results

#### 3.1 Effect of Enalaprilat on Promastigotes forms of different *Leishmania* species and murine J774 A.1. Macrophages

The leishmanicide potential of enalaprilat against *Leishmania braziliensis*, *L. major*, *L. amazonensis* and *L. chagasi*, was evaluated at concentrations ranging from 0.1µg / ml to 50 µg / ml analyzed at different time periods (24, 48 and 72 hours) to determine the inhibitory concentration of these compounds across the tested strains of *Leishmania*. Our results showed greater efficacy of enalaprilat against promastigotes of *L. braziliensis* and *L.chagasi* exhibiting IC<sub>50</sub> of 2.36µg/ml and 9.10 µg/ml respectively. The alamarBlue (AB) was used as an effective tool to evaluate the metabolic activity and proliferation of cell lines. In this bioassay, reduction of resazurin to resorufin was quantified and which demonstrated low toxicity against the J774 A.1. cell line at the tested concentrations. The CC50 results of enalaprilat of 61.05 (Table 1), demonstrate that enalaprilat could be tested at concentrations below 50 µg/ml. The selectivity indexes (SI) of the drug against parasites were 25.86 and 6.7 (Table 1) against *L. braziliensis* and *L. chagasi* respectively, indicate a greater efficacy and safety of these compounds.

**Table 1.** Effect of enalaprilat on different promastigote forms of *Leishmania* species and murine J774 A.1 macrophage cell line

Treatment	CC50 (µg/mL) J774 Cell	<i>L. pifanoi</i>		<i>L. chagasi</i>		<i>L. brasiliensis</i>		<i>L. lansoni</i>	
		IC50 (µg/mL)	SI	IC50 (µg/mL)	SI	IC50 (µg/mL)	SI	IC50 (µg/mL)	SI
Enalaprilat	61.05 (53.01-70.05)	128.03 (104.1-141.2)	0.47	9,.10 (12.2-37.8)	6,70	2.36 (48.67-87.34)	25,86	204.7 (198.3-210.60)	0.29

CC50 = cytotoxic concentration 50%, CI50= inhibitory concentration 50%, SI = selectivity index determined by CC50/CI50. Experimental data were obtained from three independent experiments. Data in parentheses represent confidence intervals (minimum and maximum) of IC50 values obtained by linear regression curve. Source: Authors.

#### 3.2 The effect of Enalaprilat on parasite load in J774 A.1. Macrophages

Macrophages infected and treated with 2.3 µg/ml of enalaprilat exhibited a significant reduction ( $p<0.001$ ) the number of intracellular amastigote observed at 24, 48 and 72 h. The cultures of macrophages treated with LPS and/or IFN-γ also had higher reduced rate of intracellular infection when compared to the control cultures (Figure 1A).

#### 3.3 Effect of Enalaprilat on the production Nitric Oxide (NO).

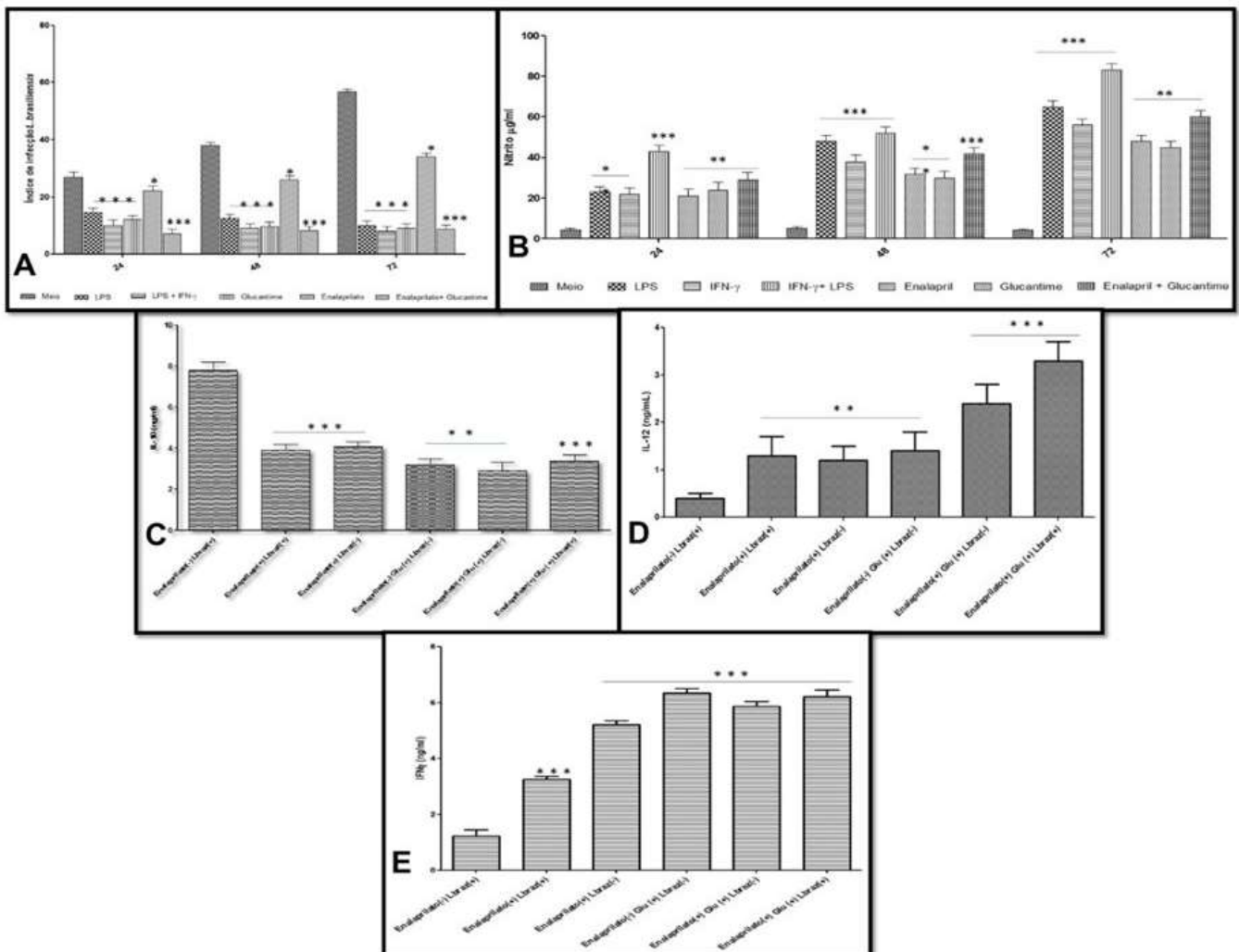
The production of nitrite (NO<sub>2</sub><sup>-</sup>) was measured in the supernatants of macrophages at 24, 48 and 72 hours in the presence or absence of LPS (50ng / ml) and IFN-γ (100 U / ml). The results presented in (Figure 1B) showed a significant

increase ( $p < 0.001$ ) in nitric oxide production in macrophages stimulated with enalaprilat at all analyzed time periods when compared to non-stimulated macrophages. Cells stimulated with LPS and/or IFN- $\gamma$  also exhibited significant increases in NO production.

### 3.4 Determination of cytokines typical of TH1 and TH2 in cultures of J774 A.1. Macrophages treated with Enalaprilat

The synthesis of IFN- $\gamma$ , IL-12 and IL-10 cytokines was measured in supernatants from cultures of cells, in this sense, we evaluated the synthesis of Th1 and Th2 cytokines in J774 A.1. macrophages infected with forms of *L. braziliensis* promastigote treated with 2.3 $\mu$ g/ml of enalaprilat. Enalaprilat was able to significantly attenuate ( $p < 0.001$ ) IL-10 production, suggesting that the antileishmanial activity of this compound may be related to its modulation of IL-10 (Figure 1C). In the present study significant increase was observed ( $p < 0.001$ ) in IL-12 levels (Figure 1D) and IFN- $\gamma$  (Figure 1E) in the supernatants of macrophages infected with *L. braziliensis* in the presence of enalaprilat. This result reveals the cellular pattern of cytokines modulation crucial to the control of leishmaniasis.

**Figure 1: Effect of Enalaprilate on parasitic load, nitric oxide production, IL-10, IL-12, IFN- $\gamma$ , Macrophages J774 A.1 infected and uninfected were incubated for 24, 48 72 hours with enalaprilate to 2,3  $\mu$ /mg/mL LPS (50 ng/mL), IFN-  $\gamma$  (100U/mL). LPS. The controls were incubated only with full RPMII 1640 medium. The sobrenaants were collected independently, in triplicate. \*\*  $p < 0,01$  and \*  $p < 0,05$  when compared to the control macrophages infectade the cytokines were quantified by ELISA on 405NM. The data were expressed as  $\pm$  SD of three experiments. Figure 1A parasitic cargo, Figure – 1B nitric oxide production, Figure – 1C Interleukin 10, Figure – 1D Interleukin 12, Figura 1E Interferon gama.**



Source: Authors.

#### 4. Discussion

Pentavalent antimony are drugs of choice used in the treatment of leishmaniasis. However, treatment with these drugs causes serious side effects. McGwire and Satoskar (2014) demonstrated some side effects presented by pentavalent antimony in the treatment of leishmaniasis such as high toxicity, very long treatment regimens and which are not always effective.

Potential cardioprotective effects of ACE inhibitors have been observed in patients with cutaneous leishmaniasis treated with antimony (Rodrigues et al 2007). The clinical manifestations, as well as the severity of the disease depend on the species and intrinsic factors of the parasite in developing resistance to antimony (Hadighi et al 2006; Rojas et al 2006) as well as the profile of the host immune response (Malla & Mahajan 2006). In this context, recent data obtained from experimental and clinical studies have emphasized an important role of ACE inhibitors on non-hemodynamic functions, immune mediated, such as cytokine production (Schieffer et al 2004; De Albuquerque et al 2004; Penitente et al 2015). In addition to the discovery of novel compounds, introduction of drugs that can potentiate the immune response is an important strategy in advancing the treatment of leishmaniasis. In this sense, the immunomodulatory evidence from the use of ACE inhibitors make their study pivotal, since these agents are low cost and have a high level of toxicological safety.

The cytotoxicity of enalaprilat to J774A.1 macrophages was compared to that of *Leishmania brasiliensis* using the selectivity index (SI). The results shown in (Table 1) demonstrate that enalaprilat showed low toxicity against J774A.1 cell line, corroborating literature findings. This result indicates that this drug can be tested with security.

In the present study murine macrophages infected with *Leishmania brasiliensis* treated with enalaprilat increased release of NO, and induce IL-12 cytokines, IFN- $\gamma$  and TNF, and reduce the release of IL-10 by the J774 A.1 (ATCC CR-107) cell line.

Well established mechanisms explain antileishmanial activity against forms of promastigotes and amastigotes such as inhibition of topoisomerase, interference in the parasite's metabolism and production of nitric oxide by macrophages (Das, et al., 2006). In this study, the probable enalaprilat mechanism of action involved in the killing of intracellular amastigotes of *L. brasiliensis* in J774 cells 1. A. was the production of nitric oxide. The increase of NO in this experimental model was directly correlated with the parasite load, the likely leishmanicidal effect was due to increased production of NO and corresponding decrease in the number of intracellular parasites (Figures 1A and 1 B).

Enalaprilat demonstrated immunomodulatory capacity in cutaneous leishmaniasis with consistent indicating the involvement of NO pathway in its mechanism of action. The production of cytokines capable of inducing nitric oxide production suggests that enalaprilat is a potential immunomodulator, which may also be useful not only in the treatment of leishmaniasis, but also of other diseases whose healing and control depends on Th1. The immunomodulatory capacities in leishmaniasis of some compounds have consistently demonstrate the participation of NO in their mechanisms of action (Bhattacharjee, et al., 2009). Our results are in accordance with these investigations, since enalaprilat was able to induce NO production in the treated macrophages (Figure 1B).

Cellular immunity is the major defense mechanism of the host against *Leishmania*. Susceptible strains such as Balb/c show an increased mRNA expression for IL-4 as well as the production of IL-5, IL-10 and IL-13 (Himmelrich et al 2000; Dudeck, Suender, Kostka, Von Stebut & Maurer 2011). In humans the immune response to leishmaniasis is more complex than in mice, in different clinical forms the immune response is T cell-dependent, these cells have a central role in resistance or susceptibility according to based on the expansion of Th1 and Th2 cells (Dutra et al 2014).

As part of strategy in stemming the menace of leishmaniasis, research has been focused on identifying new compounds that can modulate the immune response to a set of cytokines (Siegers et al 1999; Amoa-Bosompem et al 2016). In this context, it is known that IL-10, an anti-inflammatory cytokine, is partly responsible for the parasite's virulence in the macrophages. In the present study, the results seen in macrophages infected with *L. brasiliensis* and treated with enalaprilat showed reduced IL-

IL-10 signaling a positive immunomodulatory action to control the parasite load of the species studied (Figure 1C). Grutz (2005) demonstrated that IL-10 reduces the expression of MHC Class I and II interfering with the antigenic action. It was also shown that IL-10 reduces transcription and transduction of pro-inflammatory cytokines such as TNF, IL-12 and IL-18 (Conti et al 2003; Sharma et al 2011).

Among the cytokines secreted by macrophages and other antigen-presenting cells is IL-12, which is essential for the development of the Th1 cytokines, through the induction of IFN- $\gamma$  which activates macrophages. IL-12 is critical at onset of infection by *Leishmania* and decisive for the disease control (Okwor & Uzonna, 2016). Enalaprilat significantly induced production of IL-12 (Figure 1D). This result coupled with the decreased IL-10 and increase production of NO by J774 A.1 cells an effective signals a modulation effective against *L. braziliensis*.

Many studies have reported increased production of IFN $\gamma$ , decreased tumor necrosis factor (TNF) and regulatory cytokines like IL-10 and IL-4 as being fundamental to clinical cure of leishmaniasis (Castellano et al 2009; Muniz et al 2016). The enalaprilat was able to induce IFN- $\gamma$  reinforcing the hypothesis in modulating capability of this compound an effective cellular response correlates with the prior induction of IL-12 with consequent production of high levels of nitric oxide (Figure 1E).

In leishmaniasis the phenotype of phagocytic cells is altered by reducing the production of reactive oxygen species, and pro-inflammatory cytokines, such as IFN- $\gamma$ , IL-12 and TNF- $\alpha$ . These initial events limit the activation of Th1 effector cells, promoting an initial control of innate immunity on the part of the pathogen by reducing the proliferation of CD4 T cells (+) and CD8 (+) which results in the production of anti-inflammatory cytokines, such as TGF- $\beta$ , and IL-10 (Gannavaram, Bhattacharya, Ismail, Kaul, Singh & Nakhasi 2016).

The susceptibility of host to developing *Leishmania* infection may be correlated to the presence of immature macrophages. Moreover, IFN- $\gamma$ , TNF- $\alpha$  and LPS activate macrophages and render them capable of killing intracellular parasites, through the induction of NOS-II enzyme expression leading to increase production of nitric oxide. Mice deficient in NOS-II were not capable of controlling the infection caused by *L. major*, thus further confirming the activity of nitric oxide against intracellular parasites (Liew & O'donnell 1993).

An exacerbated production of cytokines may be related to suppression of cellular immune responses and the absence of IFN- $\gamma$ , an activator of macrophages, is of great interest. Successful treatment of leishmaniasis requires the development of an effective immune response mediated by cells, in addition to the death of the parasite, the immunomodulation is fundamental to control and development of these pathologies, as well as its clinical manifestations. The inability to activate macrophages is a limiting factor in the control of the parasite and the involvement of multiple organs. In the present study the Enalaprilato demonstrated in vitro the ability to increase the release of NO, induce IL-12 and IFN- $\gamma$  in addition to reducing the release of IL-10 in macrophages infected with *Leishmania braziliensis*. This result is relevant because, this drug has already demonstrated cardioprotective effects in hypertensive patients with leishmaniasis, treated with antimonial (Rodrigues et al 2007).

The methylglucamine antimonate is especially effective in the treatment of cutaneous, mucocutaneous and visceral leishmaniasis, however its pronounced toxicity linked to excessive generation of reactive species, both of oxygen (ROS) and nitrogen (RNA), affect its therapeutic benefit. Furthermore, failures in the therapy owing to low doses and discontinuous treatments, increase the number of resistant strains of parasites, decreasing the therapeutic efficacy of this drug, which frequently occur in the case of co-infection with HIV or other deficiencies due to immunological and or physiological nature. In contrast enalapril own antioxidant effect can help alleviates the toxic effects of meglumine antimoniate.



## 5. Conclusion

The drug combination for the treatment of leishmaniasis is an option to combat drug resistance developed by monotherapy, which can broaden the spectrum, due to increase in the activity of the drug by additive or synergistic action, and may thus decrease treatment duration and dosage, may consequently lead to fewer side effects and reduction in the cost of treatment. This study showed immunomodulation of enalaprilat by increasing soluble factors and effective molecules in controlling the parasite load, indicating a potential favorable association with glucantime as a strategy in therapy to combat cutaneous leishmaniasis, but safety studies on the use of this association remains to be evaluated *in vitro* and *in vivo*.

The number of drugs for the treatment of leishmaniasis is still very small, and expensive and difficult to administer.

## Acknowledgments

To the Universidade Federal do Mato Grosso (UFMT), the Universidade Federal da Paraíba (UFPB) and the Universidade do Estado de Mato Grosso (UNEMAT) partners in the execution of the experiments, the Fundação de Amparo a Pesquisa do Mato Grosso (FAPEMAT) by promotion, à Universidade Federal da Paraíba (UFPB) for funding the study.

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