# MARBOFLOXACIN INDUCES LEISHMANICIDAL ACTIVITY AND LESS INFLAMMATORY RESPONSE IN *Leishmania chagasi* INFECTED MACROPHAGES

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## ABSTRACT

Marbofloxacin has promising leishmanicidal activity due to the direct action on the *Leishmania chagasi* amastigotes. It was developed only for veterinary medicine uses, and it could be used as an drug for the treatment of Canine Visceral Leishmaniasis (CVL). In the present study, we evaluated the leishmanicidal effect and macrophage modulation of marbofloxacin in macrophages infected with amastigotes of *L. chagasi*. Macrophages were collected from peritoneum of BALB/c mice and infected with promastigotes of *L. chagasi*. After internalization and transformation into amastigotes forms, cells were treated with marbofloxacin in concentration of 100, 500 and 750 µg/mL for 18 hours. The leishmanicidal effect was evaluated by morphological spects of amastigotes inside of macrophages by phagocytosis assay and detection of death amastigotes and macrophages by TUNEL assay. Macrophage modulation was evaluated by release of cytotoxic metabolites and cytokine production. The results showed that *L. chagasi*-infected macrophages treated with the highest concentration of the drug showed lower amount of amastigotes into the macrophages ($p<0,0632$) than untreated infected cells. There was pronounced presence of cellular vacuoles in the treated infected-cells, and more apoptotic amastigotes in alive macrophages. It was observed decreased levels of $H_2O_2$, IL-1β, IL-6, and TNF-α dose-dependent of marbofloxacin in infected macrophages. The results indicate the leishmanicidal effect of marbofloxacin in infected macrophages and immunomodulation actions in these cells.

**Keywords**: *Leishmania chagasi*, marbofloxacin, infected macrophages.

# MARBOFLOXACINA INDUZ ATIVIDADE LEISHMANICIDA E MENOS RESPOSTA INFLAMATÓRIA EM MACRÓFAGOS INFECTADOS POR *Leishmania chagasi*

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## RESUMO

A marbofloxacina tem promissora atividade leishmanicida devido à ação direta sobre as amastigotas de *Leishmania chagasi.* Foi desenvolvido apenas para uso em medicina veterinária, podendo ser utilizado no tratamento da Leishmaniose Visceral Canina (LVC). No presente estudo, avaliamos o efeito leishmanicida e a modulação macrofágica da marbofloxacina em macrófagos infectados com amastigotas de *L. chagasi*. Os macrófagos foram coletados do peritônio de camundongos BALB/c infectados com promastigotas de *L. chagasi*. Após internalização e transformação em formas amastigotas, as células foram tratadas com marbofloxacina nas concentrações 100, 500 e 750µg / mL por 18 horas. O efeito leishmanicida foi avaliado pelos aspectos morfológicos de amastigotas dentro de macrófagos, por ensaio de fagocitose e detecção de amastigotas e macrófagos mortos por ensaio TUNEL. A modulação dos macrófagos foi avaliada pela liberação de metabólitos citotóxicos e produção de citocinas. Os resultados mostraram que macrófagos infectados com *L. chagasi* tratados com a maior concentração da droga apresentaram menor quantidade de amastigotas nos macrófagos ($p < 0,0632$) do que células infectadas não tratadas. Houve a presença pronunciada de vacúolos celulares nas células infectadas tratadas, e mais amastigotas apoptóticas em macrófagos vivos. Observou-se diminuição dos níveis de $H_2O_2$, IL-1β, IL-6 e TNF-α dependente da dose de marbofloxacina em macrófagos infectados. Os resultados indicam o efeito leishmanicida da marbofloxacina em macrófagos infectados e ações imunomoduladoras nessas células.

Palavras-chave: *Leishmania chagasi*, marbofloxacina, macrófagos infectados.

## MARBOFLOXACINO INDUCE ACTIVIDAD LEISHMANICIDA Y MENOR RESPUESTA INFLAMATORIA EN MACRÓFAGOS INFECTADOS CON *Leishmania chagasi*

## RESUMÉN

El marbofloxacino tiene una actividad leishmanicida prometedora debido a la acción directa sobre amastigotes de *Leishmania chagasi*. Fue desarrollado solo para uso en medicina veterinaria y puede usarse en el tratamiento de la leishmaniasis visceral canina (LVC). En el presente estudio, evaluamos el efecto leishmanicida y la modulación de macrófagos de marbofloxacino en macrófagos infectados con amastigotes de *L. chagasi*. Se recogieron macrófagos del peritoneo de ratones BALB/c infectados con promastigotes de *L. chagasi.* Después de la internalizacion y transformacion en formas amastigotes, las células se trataron con marbofloxacina a concentraciones de 100, 500 y 750 µg / ml durante 18 horas. El efecto leishmanicida se evaluó mediante los aspectos morfológicos de amastigotes dentro de macrófagos, mediante ensayo de fagocitosis y detección de amastigotes y macrófagos muertos mediante ensayo TUNEL. La modulación de los macrófagos se evaluó mediante la liberación de metabolitos citotóxicos y la producción de citocinas. Los resultados mostraron que los macrófagos infectados con *L. chagasi* tratados con la concentración más altas del fármaco tenían una menor cantidad de amastigotes en los macrófagos ($p < 0,0632$) que las células infectadas no tratadas. Hubo una presencia pronunciada de vacuolas celulares en las células infectadas tratadas y más amastigotes apoptóticas en macrófagos vivos. Se observó una disminuicón en los niveles de $H_2O_2$, IL-1β, IL-6 y TNF-α dependiendo de la dosis de marbofloxacino en macrófagos infectados. Los resultados indican el efecto leishmanicida de el marbofloxacino en macrófagos infectados y acciones inmunomoduladoras en estas células.

**Palabras clave**: *Leishmania chagasi*, marbofloxacino, macrófagos infectados

## INTRODUCTION

Canine Visceral Leishmaniasis is a zoonosis of worldwide distribution and a serious public health problem in Brazil (1). It is considered the second most important protozoonosis and one of the six main infectious-parasitary diseases in the world (2). It is caused by a mandatory intracellular protozoan parasite *Leishmania chagasi* (*infantum*) species and the vectors are phlebotomines (*Lutzomyia longipalpis* in Americas) (3).

Dogs are the main domestic parasite reservoir, which are infected by phlebotomine females biting during its blood feeding through the skin or the peripheral blood of the reservoirs inoculating metacyclic promastigotes, known as the infective stage of *Leishmania* (4). In the vertebrate host, the promastigote forms are fagocitated by macrophages and differentiated into amastigote forms (5).

Macrophages are cells frequently observed in lesions during infection caused by *Leishmania* spp. and the parasites usually multiply inside of these cells and also contributes for the immunopathology of the disease by intensify an unprotective and intense inflammatory response (6). The parasite-macrophage interplay begins when macrophages detect parasite molecules through membrane receptors, which then stimulate the production of proinflammatory cytokines, such as TNF-α, IFN-γ, IL-12, IL-1β and of hydrogen peroxide, triggering the Th-1 type cellular response, which is related to the efficiency in the elimination of the intracellular parasites (7). However, in the amastigote form, the parasite is able to inhibit hydrolytic enzymes and deplete reactive species of nitrogen and oxygen while remaining protected and is capable of multiplying in the phagolysosome (8).

The clinical signs in the dog are usually systemic and of chronic evolution. It can be observed apathy, weight loss, abdominal distension due to hepatosplenomegaly, palpable spleen, lymphadenomegaly, skin lesions and/or alopecia, emaciation, ocular lesions, onicogryphosis, haemorrhages, diarrhea, vomiting, pneumonia and locomotors and neurological alterations (9). The clinical presentations are dependent on the animal's immune response, being able to reach severe cases of debilitation (10).

It is a disease whose control depends on the vector control and on the success of the treatment, which aims to control clinical signs, improve the animal's immunity and reduce parasitic burden at non-transmissible levels (11). Parasitological cure with anti-leishmania drug is extremely difficult, in this way it is of great importance the study of alternative drugs for the treatment of leishmaniasis.

Marbofloxacin is a third generation fluorquinolone developed for veterinary use with leishmanicide action in macrophages amastigotes inside the host. Its molecule shows a very high volume of distribution, which is widely diffused throughout the organism (12).

Duo to the ability of amastigotes survive inside of macrophages, it is important the discovery of new drugs that are able to penetrate in macrophages and kill the amastigotes forms and also modulate the immune response. In the present study, we evaluated the marbofloxacin effect in BALB/c mouse peritoneal macrophages that were infected with amastigotes forms of *L. chagasi* by determination of phagocytosis index, production of inflammatory cytokines and cytotoxic metabolites production, and quantification of death amastigotes inside of alive macrophages.

## MATERIAL AND METHODS

### *Leishmania* strain

*L. chagasi* promastigotes (strain MHOM/BR/2002/LPC-RPV) were maintained in LIT (***L****iver* ***I****nfusion* ***T****riptose*) culture medium at 28° C, supplemented with 10% fetal calf serum (Gibco, EUA) maintained at the concentration of 1 x  promastigotes/ml.

### Mice

BALB/c female mice with 45 days-old were obtained from Lauro de Souza Lima Institute (ILSL), Bauru, SP, Brazil and maintained at the Laboratory of Experimental Immunopathology Laboratory (LIPE) of the Faculty of Sciences, UNESP, Bauru, receiving *ad-libitum* balanced feed and sterile water. The study was approved by the Ethics Committee on the Use of Animals (CEUA) of the Faculty of Veterinary Medicine and Animal Science (FMVZ), UNESP-Botucatu / SP (Protocol # 57/2015).

### Drug

Marbofloxacin was purchased from Vétoquinol (France).

### Determination of cellular viability to monitor drug toxicity

The possible toxic effects of different concentrations of marbofloxacin, we used spleen cells4 culture by consider that lymphocytes are more sensitive to external danger and thus, these cells is a gold standard to evaluate citotoxicity. For this protocol, we performed the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromidetetrazolium) (13). Fragments of the spleen were collected and homogenized in ice-cold sterile PBS. The red blood cells were lysed with 0.15 M ammonium nitrate. After washing, the cellular suspension was centrifuged, and the cells were resuspended in 1.0 ml of RPMI-1640 (Nutricell, Campinas, SP, Brazil) supplemented with 10% heat-inactivated fetal calf serum (Nutricell), penicillin (100 UI $ml^{-1}$), streptomycin (100 mg $ml^{-1}$) (Sigma-Aldrich, St. Louis, MO, USA). The cell concentration was adjusted to 2.0 x $10^6$ cells/mL, as determined by 0.1% trypan blue staining. Spleen cells ($2.10^6$ cells / ml) were stimulated with three different concentrations of marbofloxacin (100, 500 and 750 µg / ml) incubated for 96 hours at 37 ºC in 5% CO2. Thereafter, the culture plate was centrifuged for 5 minutes at 1500 rpm. The supernatant was discarded, the cells resuspended in complete RPMI medium containing MTT (Sigma-Aldrich, 5 mg / ml). The plate was incubated again for two hours at 37ºC in 5% $CO_2$ and then centrifuged for 5 minutes at 1500 rpm. The supernatant was removed and the cells from each well were resuspended in 100 ml dimethylsulfoxide (DMSO). After 5 minutes, the samples were submitted to determination of absorbance in a ELISA reader set up at 540 nm.

### Ex vivo peritoneal macrophage cell culture

Mice were euthanized via $CO_2$ asphyxiation. Peritoneal lavage (PL) was performed using cold and sterile phosphate buffered saline (PBS). The PL suspensions were centrifuged, and the cells were resuspended in 1.0 ml of supplemented RPMI-1640. Cell concentration was adjusted to one cell for five parasites (5:1) mononuclear phagocytes $ml^{-1}$ as judged by the uptake of 0.02% neutral red (Sigma) and expression of F4/80 by Fluorescence-activated cell sorting (FACS). Cells were placed in plates (Greiner BioOne, Frickenhausen, Germany) and incubated for two hours at 37°C and 5% $CO_2$ in a humidified chamber to allow cells to adhere

and spread. Non-adherent cells were removed by washing the wells three times with RPMI, and the remaining adherent cells (>95% mononuclear phagocytes as assessed by morphological examination and by FACS) were co-cultured for 6 hours with promastigotes of *L. chagasi* (5 parasites:1 cell*)* for infection and transformation into amastigotes in macrophages. After this period, the supernatant with the non-internalized promastigotes was removed, and the cultured were incubated in the presence or absence of 100, 500 and 750 µg / ml of marbofloxacin for 18 hours.

### Evaluation of anti-amastigote activity by phagocytosis

Peritoneal macrophages (5x macrophages/well) and *L. chagasi (*15 x parasites/well) treated, or not, with marbofloxacin were cultivated in 24-well plates with slides. After 18 hours, the samples were fixed with absolute methanol, submitted to Giemsa staining and examined microscopically with a 40x magnification. The number of intracellular amastigotes was determined by counting 100 infected macrophages, and the results expressed as percent inhibition relative to the negative control.

### Evaluation of anti-amastigote activity by TUNEL

In order to determine the amount of dead amastigotes of *L. chagasi* inside of alive macrophages, the cell cultures were submitted to terminal deoxynucleotidyl transferase-mediated dUTP-Fluorescein nickend-labeling (TUNEL) technique, using the Apoptosis Detection System, Fluorescein kit (Roche, Germany). according to the manufacturer's protocol. The slides were mounting in VECTASHIELD®. Mountingwith DAPI (Vector Labs). Cells were analyzed using a fluorescence microscope (Nikon Eclipse 80i) and images were captured using NIS elements software (version Ar 3.10). Green color means death of cell and blue nucleus.

### Determination of release of cytotoxic metabolites and cytokines

Peritoneal macrophages (1.0x macrophages/well) and *L. chagasi* (5 x parasites/well) treated, or not, with marbofloxacin were cultivated in 96-well plates and after the incubation period were submitted to determination of $H_2O_2$, NO, IL-1β, TNF-α, IL-10 and IL-6 levels. The production of H2O2 was performed according to (14) and modified by (15). NO concentration and measuring nitrites in the supernatant of the macrophage culture according to (16). Quantification of IL-1β, TNF-α, IL-10 and IL-6 cytokines in cell-free supernatant was performed using the Duo-Set Kit (R&D Systems, Minneapolis, MI, USA), as instructed by the manufacturer.

### Statistical analyses

Statistical tests were performed using GraphPad InStat software version 5.1 for Windows (GraphPad Software, San Diego, CA, USA) and the significance level established to verify the null hypothesis was 5.0%. The comparison of two paired samples was done by the Student t test, whereas the one of more than two paired samples was done by the ANOVA test.

## RESULTS

**The highest evaluated concentration of marbofloxacin induced death of amastigotes and the macrophages remained alive**.

Before to perform the protocol in *L. chagasi* infected-macrophages, we evaluated the cytotoxicity effect of the marbofloxacin in non-infected splenic cells. The lower concentration (100 µg/ml) of marbofloxacin exhibited no changes in its cellular viability (Figure 1). The concentration of 500 mg/ml induced 22% of cell cytotoxicity and the highest concentration (750 µg / ml), 33% of cytotoxicity. Independently of the concentration of marbofloxacin, the phagocytosis index was similar among the groups (Fig. 2A). On the other hand, the number of amastigotes per cell decreased according to the higher concentrations of marbofloxacin (Figure 2B). In addition, it was observed pronounced presence of cytoplasmic vacuoles in the infected macrophages treated with the highest concentration of the drug (Figure 3), suggesting dead of parasites.

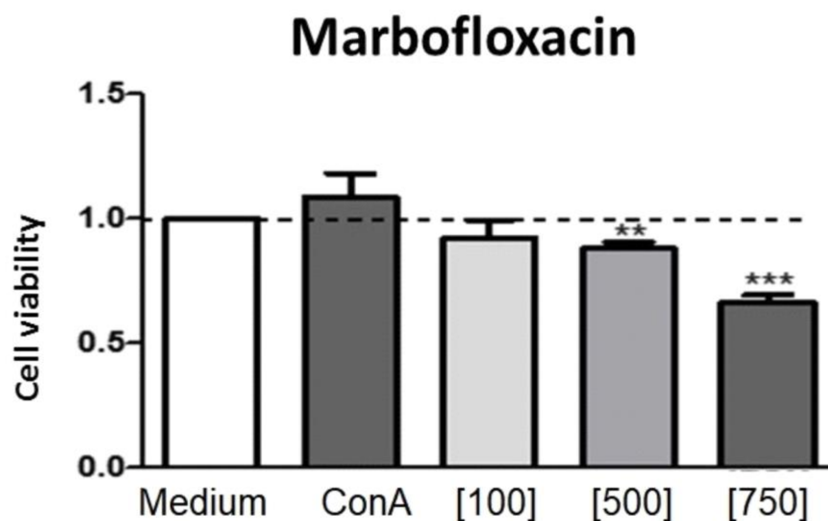


**Figure 1**.Evaluation of cytotoxic effect of marbofloxacin in normal cells. Spleen cells of female BALB/c mice were cultivated in the presence of marbofloxacin at three concentrations (100, 500 and 750 µg / ml) for 96 hours and the cellular viability was determined by the MTT assay. Cell viability index represents the ratio of tested culture and control culture. The results are expressed as mean ± SD (paired t-test; n=6; *p <0.05, ** p<0.01, *** p<0.01 in comparison with control, represented by the dashed line).

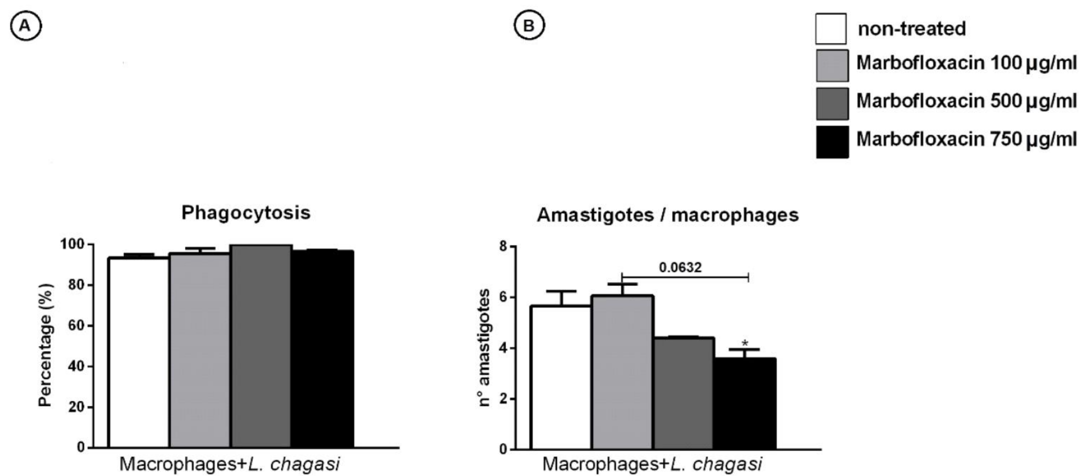


**Figure 2**. Effect of marbofloxacin in L. chagasi-infected macrophages. Murine peritoneal macrophages were infected with *L.chagasi* and after six hours, the infected cells were treated with marbofloxacin (100, 500 and 750 µg/ml) for 18 hours. **A**. The phagocytosis index maintained the same despite the drug concentration. **B**. The number of amastigotes inside the macrophages decreased as the drug concentration increased

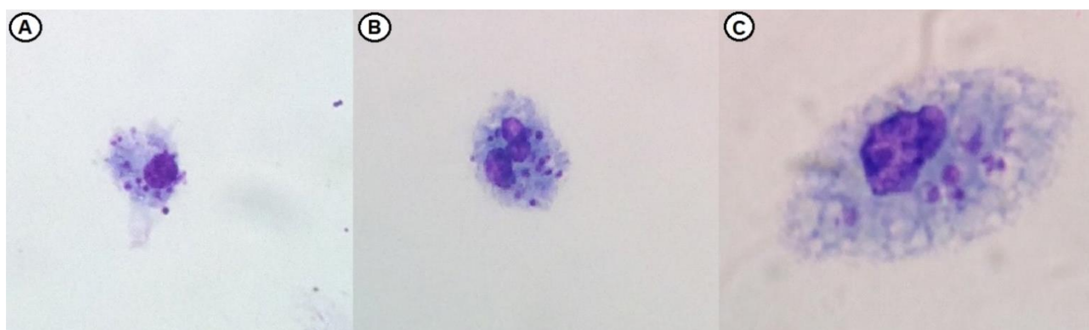


**Figure 3.** Photomicrographs of *L. chagasi* infected macrophages treated with marbofloxacin in three concentrations.**A:** Marbofloxacin at the concentration of 100µg/ml. **B**: 500µg/ml and **C**: 750µg/ml. Arrows are showing formation of vacuoles and parasite fragmentation (Objectives of40x and 100x).

Even the highest concentration (750 µg/ml) showing 33% of cytotoxicity, it is less than 50% of index of citotoxicity (50%), a safe concentration. In order to verified the viability of macrophages in this concentration, we performed the TUNEL assay and the findings confirmed the leishmanicidal effect of marbofloxacin (750 µg/ml) without trigger death of macrophages (Fig, 4).

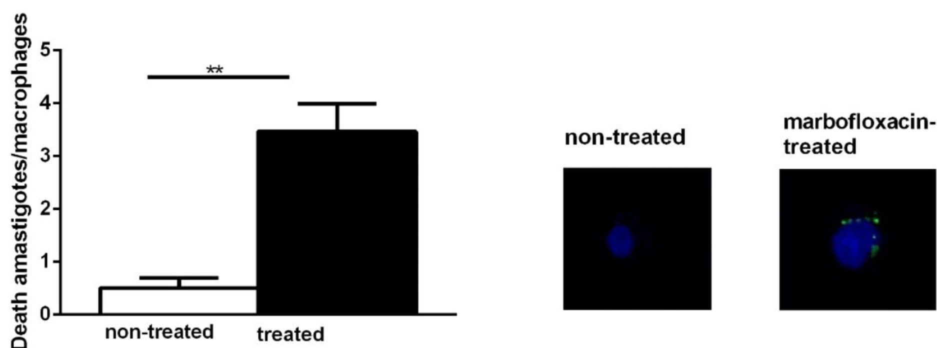

**Figure 4**.TUNEL apoptosis assay. Determination of dead amastigote forms by macrophage treated with 750 µg/ml of marbofloxacin and representative photomicrography of infected macrophages treated or not with marbofloxacin (750 µg/ml). Blue staining (DAPI) corresponds to the cell nucleus and green staining (fluorescein) corresponds to the incorporation of d-UTP, marker of apoptosis (dead amastigotes) (Objective of 100x). Paired t-test, n=6, * $p<0.05$ and ** $p <0.01$ in comparison to control.

**Marbofloxacin (750 µg/ml) treatment down-modulates the immune response of infected macrophages**

In the group without treatment is possible note that infected macrophages increased the production of cytotoxic metabolites ($H_2O_2$, NO) and pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) compared with no-infected macrophages. With the addition of marbofloxacin (750 µg / ml), the infected macrophages decreased the production of $H_2O_2$, TNF-α, IL-1β showing an immunomodulatory effect of this drug that down modulated the activated macrophages (Fig. 4). No statistical differences were observed in the production of IL-10 and IL-6 by the different groups of treated or no-infected macrophages.

## DISCUSSION

Currently, the association of meglumine antimoniate with allopurinol (17) has been used for the treatment of LVC in some countries. However, it is not allowed in Brazil, because the complete elimination of the parasite does not occur. With the results obtained in the present study, analyzed as a whole (reduction of amastigotes associated with the presence of vacuoles, more death-amastigotes inside of treated-macrophages and decrease of inflammatory metabolites and cytokines), it is suggested that the action of marbofloxacin should be considered to treat this disease.

After 6 hours of co-culture with *L. chagasi*, we observed an increase of inflammatory mediators (Fig. 5) produced by macrophages in response of infection by parasites. In this period, we started our experiments by adding the marbofloxacin in the infected macrophage culture.

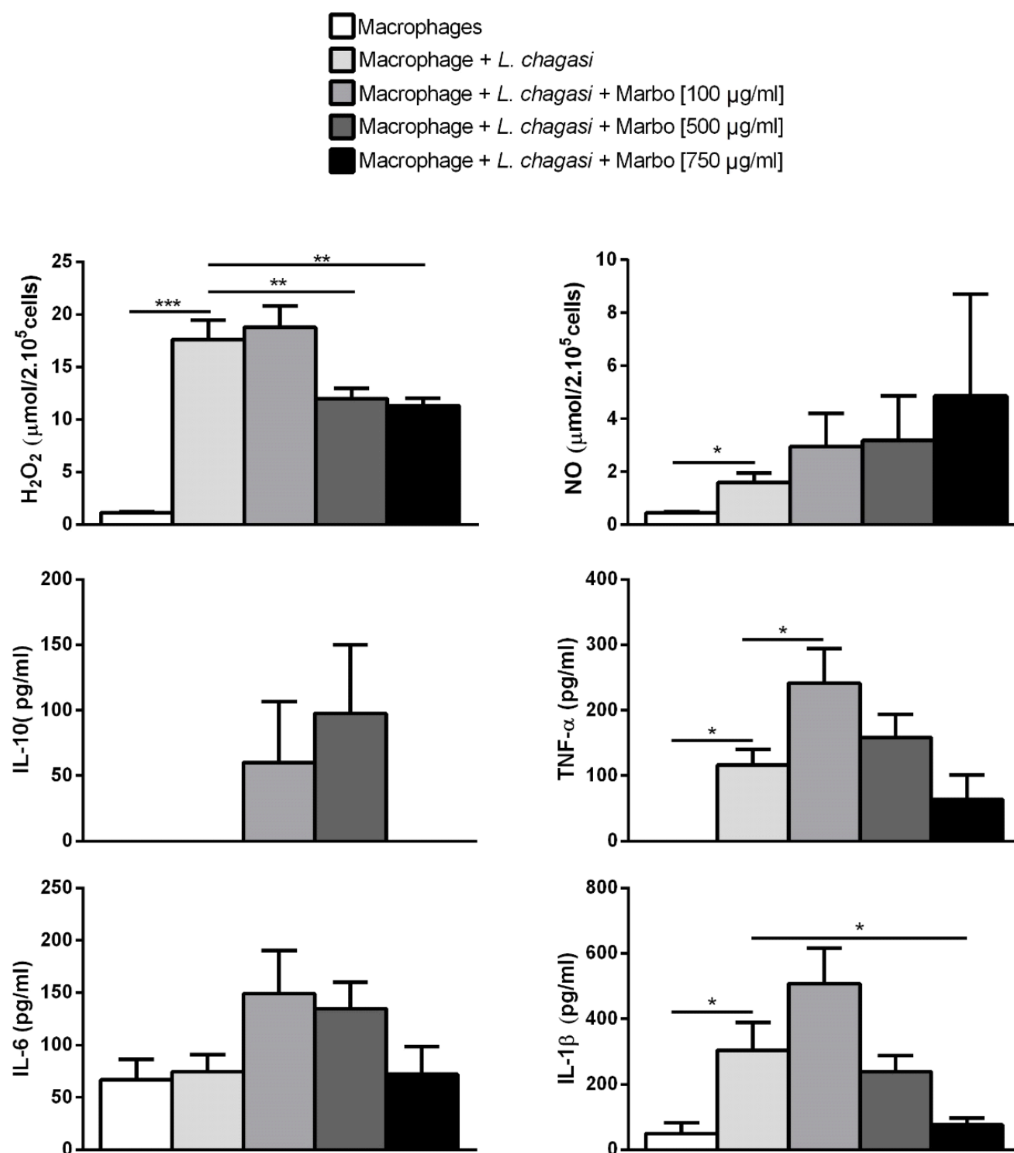


**Figure 5**. Effect of marbofloxacin in the production of cytotoxic metabolites (NO and H2O2) and cytokines (IL-10, TNF-α, IL-6 and IL-1β) by *L. chagasi*-infected macrophages. After 6 hours of infection, macrophages were treated, or not, with marbofloxacin (100, 500 and 750 µg/ml) for 24 hours. Paired t-test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

After 18 hours of treatment, we observed decrease in the number of amastigotes per cell, more cytoplasmic vacuoles and more number of dead *L. chagasi* inside of macrophages. In addition, treated macrophages decreased the production of pro- inflammatory mediators showing that the marbofloxacin induces death of amastigotes inside of alive macrophages and also modulated the inflammatory response by the drug. LVC is characterized as a disease that induces an exacerbated inflammatory response, worsening the disease (chronicity) since these cytokines lead to tissue damage (18). With marbofloxacin treatment, we observed down modulation of inflammatory response by infected macrophages, what is a good point because this response cause less tissue damage beyond to kill the parasite, what can help in the resolution of the symptoms, what is a problem in this disease.

The results show strong evidence of the leishmanicidal action of marbofloxacin, and it can be considered as an alternative for the treatment of LVC, due to the impossibility of treatment in the national territory with drugs recommended for the human treatment of

visceral leishmaniasis (19). Further studies are suggested in this line of research, comparing the action of marbofloxacin with that of other drugs, as well as studies in experimental animals such as hamsters, for better evaluation of the drug.

## CONCLUSION

The present study demonstrates that mabofloxacin are potential leishmanicidal drug in non-cytotoxic concentration and immunomodulatory effect in infected macrophages, with the *in vitro* capacity against amastigotes of *L. chagasi* inside of macrophages and consequent down-regulation of this cells. Its necessary future experimental studies to confirm this capacity *in vivo*.

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