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Immunomodulatory effect of Canova medication on experimental *Leishmania amazonensis* infection

W.K.V. Pereira[a], M.V.C. Lonardoni[b], R. Grespan[a], S.M. Caparroz-Assef[a], R.K.N. Cuman[a], C.A. Bersani-Amado[a,*]

[a]*Laboratory of Inflammation, Department of Pharmacy and Pharmacology, University of Maringá, Avenida Colombo, 5790, 87020-900 Maringá-PR, Brazil*
[b]*Laboratory of Immunology, Department of Pharmacy and Pharmacology, University of Maringá, Avenida Colombo, 5790, 87020-900 Maringá-PR, Brazil*

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Abstract This study investigates the action of Canova medication (CM) on experimental infection by *Leishmania* (*Leishmania*) *amazonensis*, utilizing in vitro and in vivo assays. For the in vitro tests, Balb/c mouse peritoneal macrophages (5×10^5 cells in 500 μl of culture medium, supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (0.1 mg/ml) (were distributed in 24-well plates and CM was added at concentrations of 20 or 40%. Twenty-four hours later, the macrophages were infected with *Leishmania* amastigotes in culture medium. The effect of CM on macrophages leishmanicidal activity in 24 and 48 h cultures was evaluated by determining infection index and measuring nitric oxide (NO) production. The in vivo tests were performed in mice infected with 10^7 *L.* (*L.*) *amazonensis* promastigotes injected in to the right hind footpad (25 μl in phosphate buffered saline). The progression of the lesions was examined over a 9-week period by measuring footpad swelling, and the parasite load in regional lymph nodes and spleen. The in vitro results showed that at 40% CM reduced the infection index, and induced NO production in the elicited macrophages, which suggests that the inhibitory effect on infection index may be mediated by NO. In the in vivo infection, when administered, orally or subcutaneously in mice, CM reduced infection by *L.* (*L.*) *amazonensis* in the paws, resulting in smaller lesions. CM treatment also decreased parasite load in the regional popliteal lymph nodes and in the spleen. These results suggest that CM modulates experimental infection by *L.* (*L.*) *amazonensis*, controlling infection progression and limiting dissemination.

Introduction

Leishmaniosis results from infection by protozoa with a worldwide distribution. The disease can be manifest as visceral, cutaneous and mucocutaneous

* Corresponding author.
E-mail address: cabamado@uem.br (C.A. Bersani-Amado).

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forms, depending on clinical symptoms. Characteristically, *Leishmania* parasites multiply exclusively in the cells of the mononuclear phagocyte system.[1] In murine resident macrophages, *Leishmania* parasites can survive within the phagolysosome and multiply extensively.[2]

In infection by *Leishmania*, the macrophages constitute both the host cells and the effector cells against the parasites.[3] The immunological mechanisms conferring resistance and susceptibility to infection by *Leishmania* have been widely studied; however, the consequences of the initial interactions between the macrophages and the parasites as regards the capability of *Leishmania* to live and multiply within these cells are not well known.[4]

It has been well established in murine models that, in activated macrophages, increased leishmanicidal activity is correlated with increased NO (nitric oxide) production.[5-7]

Several studies have reported that certain products of natural origin are effective in curing diseases, and maintaining an organism's resistance to infection.[8] The mechanisms by which these agents may cause these effects seem to be activation of immunity.[8-12] The newest forms of immunomodulatory therapy can be directed to specific cells, or to their products which contribute to the immune response. These forms of immunotherapy are known as 'biological response modifiers'.[13]

Today, in Brazil and Argentina, at homeopathic drugstores, a product indicated to patients with depressed immunologic system is sold. This product, named Canova medication, contains as fundamental substances: *Aconitum napellus*, *Arsenicum album*, *Bryonia alba*, *Lachesis muta* and *Thuya occidentalis* (information obtained from homeopathic manipulator).

Sasaki et al.[14] performed the first clinical study showing the effectiveness of Canova medication in patients carrying the acquired immune deficiency syndrome virus (HIV). This study showed that after treatment with Canova medication, patients exhibited decrease in both viral load and in opportunistic infections, in addition to a reduction in damage caused by the disease itself.

Patients with neoplasms who undergo Canova medication therapy also show an improvement in their infectious and inflammatory conditions, with a decrease in tumor mass and, in some cases, regression of the neoplasic process.[15] It has been assumed that the effect of the medication is not a direct anti-tumoral action, but via activation of the immunological system, which has led some researchers to perform studies on the immunomodulatory effects of this compound.

In vitro studies by Piemonte and Buchi[16] suggested that Canova medication may exert an effect on macrophages, which somehow would stimulate the lymphocytes, increasing their cytotoxic effects. Experiments using tumor infiltrating lymphocytes allowed us to infer that the Canova medication may act as a cytokine inducer.[17]

In experimental animals, the median lethal dose (LD 50) of Canova medication is 100 times greater than the usual dose; further, the medication presents neither genotoxicity nor identifiable mutagenicity at the chromosome level.[18]

Other experimental investigations have demonstrated that Canova medication induces activation of murine peritoneal macrophages incubated with parasites such as *Toxoplasma gondii*,[19] suggesting that it may stimulate the immune response in infectious diseases.

Although Canova medication has been used clinically, few experimental studies have been carried out to examine the medication's biological activity and mechanisms of action. Thus, the aim of this research was to examine the effect of Canova medication on (1) macrophage leishmanicidal activity; and (2) the evolution of infection induced by *Leishmania* (*Leishmania*) *amazonensis*.

Materials and methods

Canova medication

Canova medication was obtained from pharmacies in Brazil. The Hahenemannian Homeopathic method used to prepare the Canova medication is described in the Farmacopéia Homeopática Brasileira.[20] Starting from the original mother tincture (in the case of a plant this is an alcoholic extract) several dinamizations are performed. The final product, Canova, contains 19dH *Thuya occidentalis* (Cupresaceae) made from the bark; 18dH *Bryonia alba* (Curcubitaceae) made from fresh roots; 11dH *Aconitum napellus* (Ranunculaceae) made from fresh preparations of the whole plant, including the roots, at the beginning of flowering; 19dH *Arsenicum album* (arsenic trioxide) and 18dH *Lachesis muta* (Viperidae) venom. The active ingredients were extracted or diluted in equal parts 70% alcohol. dH units were used to describe the doses employed; one dH unit is a one-tenth dilution. The number given before (dH) is the number of times the decimal dilution was made; thus, 10dH represents 1×10^{-10}.

Mice and parasites

Male Balb/c mice, weighing between 20 and 25 g were used. The *Leishmania* (*Leishmania*) *amazonensis* strain (MHOM/BR/73/M2269), was kindly provided by J. J. Shaw, Instituto Evandro Chagas, Belém, Pará, Brazil, and maintained as amastigotes by inoculation into the footpads of golden hamsters every 4–6 weeks. Amastigote suspensions were prepared as previously described.[21] Briefly, the excised lesions were homogenized using a Potter glass homogenizer. The resulting supernatant was centrifuged at 1400×*g* for 10 min, and the pellet was resuspended in RPMI 1640. Promastigotes were isolated from the lymph nodes of infected mice and were cultured in Medium 199 containing 20% fetal calf serum (FCS), 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (0.1 mg/ml) at 26 °C. They were then used in the stationary phase of growth (day 6 of culture). The protocol for these experiments was previously approved by the Animal Ethics Committee of Maringá State University.

Macrophage leishmanicidal activity

Macrophages were harvested from the peritoneal cavities of Balb/c mice by lavage with phosphate-buffered saline (PBS) 4 days after injection of 1 ml 3% TG (TG-elicited macrophages). Macrophages (5×10^5 cells in 500 μl of RPMI medium), supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (0.1 mg/ml) were distributed in sterile, 24-well plates, containing sterile, 13-mm diameter, glass coverslips. The plates were maintained at 37 °C in a humid atmosphere containing 5% CO_2 during 60 min. The coverslips were then washed three times with PBS to remove the non-adherent cells. The Canova medication was added to the plates, at 20 or 40% concentrations in relation to the culture medium. The culture medium was the only addition made to the control groups. Twenty-four hours later, the supernatant was removed and the macrophages were infected with amastigote forms of *Leishmania* at a ratio of 3:1 in culture medium supplemented with RPMI. The plates were incubated for 24 or 48 h at 37 °C in an atmosphere containing 5% CO_2. The coverslips were washed with PBS, stained with a HEMA 3-Stain set, dried, mounted on glass slides and examined microscopically. The number of infected macrophages and the average number of parasites per macrophage was estimated in 200 cells. The results were expressed as an infection index, which is the percentage of infected macrophages multiplied by the average number of amastigotes per macrophage.

Infection by *Leishmania* in vivo

Mice were infected subcutaneously with 10^7 promastigotes of *L.* (*L.*) *amazonensis* in 25 μl PBS in the right hind footpad, and with the same volume of PBS alone in the left paw. Footpad swelling was monitored weekly, during 9 weeks, with the help of a digital plethysmograph (Ugo Basile), and the result was expressed as an increase in footpad volume compared to initial volume. The final result was obtained by subtracting the value of the paw inoculated with the parasites from the value of the control footpad.

Treatment with Canova medication

Mice received a single daily dose of 0.2 ml Canova medication, either intragastrically or subcutaneously. The treatment was carried out for 9 weeks. Animals in the control group received the same volume of vehicle (grain alcohol).

Preparation of macrophage cultures for nitric oxide measurement

To evaluate nitric oxide (NO) production, total peritoneal cell suspensions (200 μl) containing 2×10^5 macrophages were spread on 96-well plates. Canova medication, at concentrations of 20 or 40%, and the *Leishmania* suspension in a 3:1 ratio in RPMI medium containing 10% fetal calf serum (FCS) and antibiotics were added, providing a final volume of 200 μl. In some experiments, the same procedure was carried out in the absence of parasites. In addition, other cultures were previously treated with lipopolysaccharide (LPS) at a concentration of 1 mg/ml. The plates were maintained at 37 °C in an atmosphere containing 5% CO_2 during 24 or 48 h. The experiments were carried out in triplicate.

Nitrites measurement

Nitric oxide (NO) production was assayed by measuring the concentration of nitrites present in the supernatant of the macrophage cultures, according to the protocol described by.[22] Fifty microlitre of supernatant from the cell cultures were incubated with an equal volume of Griess reagent (1% sulfonilamide, 0.1% naphthylenediamine dihydrochloride and 2.5% orthophosphoric acid) during 10 min at room temperature.

Absorbance was measured using a

Spectrophotometer (Elx 800 Universal Microplate Reader) with a 550-ηm filter, against a blank constituted by medium plus Griess reagent (v/v). The results were expressed as μM of NO_2, based on a standard curve, from known concentrations of sodium nitrite ($NaNO_2$) dissolved in medium.

Parasite load in the lymph nodes and spleen

Balb/c mice were infected in one of the hind paws with *L.* (*L.*) *amazonensis* and treated daily with Canova medication (0.2 ml) by intragastrically. Treatment was continued for 9 weeks at which time the animals were killed and the popliteal lymph nodes and spleen were aseptically removed, weighed, and then homogenized with a Potter glass homogenizer in Medium 199 supplemented with 20% FCS 2 mM L-glutamine penicillin (100 U/ml), and streptomycin (0.1 mg/ml) as previously described.[23] Briefly, under sterile conditions, serial, four-fold dilutions were prepared and distributed in 96-well microtiter plates in triplicate. After 7 or 14 days of incubation at 26 °C, the wells were examined in an inverted microscope. The final titer was the last dilution at which the well contained at least one parasite. The parasite load (number of parasites/gram of tissue) was calculated by dividing the geometric mean of the reciprocal of the positive titers from each duplicate by the weight of the lymph node or spleen. The value obtained was multiplied by the reciprocal fraction of the homogenized organ inoculated into the first well.

Statistical analysis

The data were subjected to the Kolmogorov-Smirnov test: those showing normal distribuition were submitted to Student's *t*-test when comparing two groups or analysis of variance (ANOVA) followed by the Tukey test, for more than two groups. All analyses were made using the GraphPad Prism Program. *P* values of less than 0.05 were consideres significant.

Results

Effect of Canova medication on infection index

To evaluate the effect of Canova medication on infection by *Leishmania* in vitro, peritoneal murine macrophages were treated with the medication 24 h before infection with *L.* (*L.*) *amazonensis* amastigotes. Treatment with Canova Medication, at concentration of 40%, significantly reduced the infection index. The 20% concentration had no effect on the index. This effect was observed both in the 24 and 48-h periods after infection, giving decreases of 16.8 and 11.4%, respectively, compared to the control group (Fig. 1(A) and (B)), wich suggests that Canova medication can stimulate macrophage leishmanicidal activity. These results also demonstrate that Canova medication at concentrations of 10, 20 on 40%, does not affect the promastigote forms of parasite growth, thus suggesting that it have no direct toxic action on *Leishmania* (data not shown).

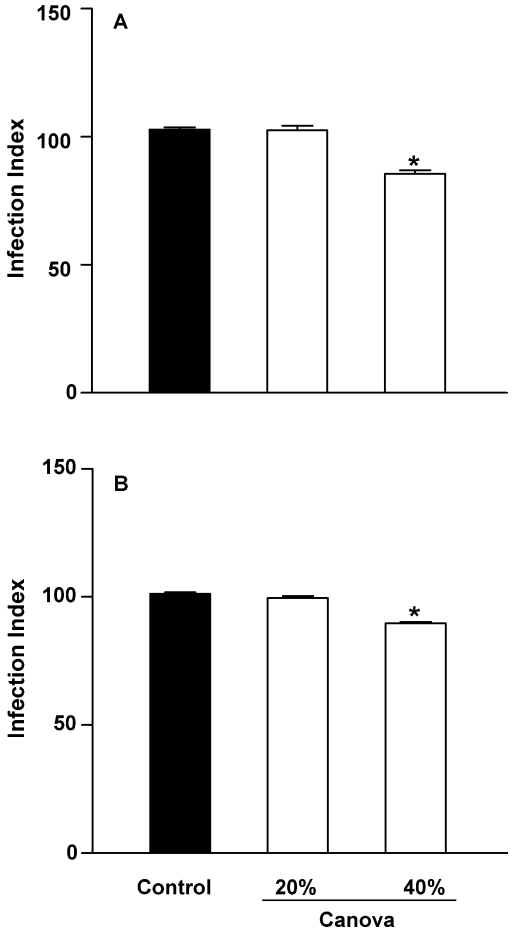

Figure 1 Effect of Canova medication on macrophages infection index. Balb/c mouse peritoneal macrophages, elicited with thioglycate, and adhered to glass slides (5×10^5 cells) were treated with Canova medication at 20 or 40% concentration, 24 h before infection with *L.* (*L.*) *amazonensis* amastigotes. After 24 h (A) and 48 h (B), infection index was estimated. Data are the mean ± SEM of four experiments. *$P<0.01$ (compared to control group, Tukey test).

Effect of Canova medication on nitric oxide production

Important among several mechanisms protection of an organism against a wide variety of agents involves the nitric oxide (NO) production by many cell types, including activated macrophages.[24,25] Thus, we evaluated whether Canova medication might increase NO production in elicited macrophages, in the absence or presence of *Leishmania*. Canova medication, at 20 and 40% concentrations, significantly increased NO levels in the macrophage cultures, more than two-fold both in the absence (Fig. 2) or in presence of *Leishmania* (Fig. 3), compared to the untreated control group. Another experimental series evaluated whether Canova medication modified nitric oxide (NO) production in macrophage cultures previously stimulated by lipopolysaccharide (LPS). Figs. 4 and 5 show that the LPS caused a significant increase in NO production in macrophage cultures both in the presence or absence of *Leishmania*. Treatment with Canova medication did not boost this effect (Figs. 4 and 5). This increase in NO production in response to treatment, suggests that Canova medication may act as a macrophage activator.

Effect of Canova medication on the progression of *Leishmania* infection

Based on the above results, we investigated whether Canova medication affected in vivo infection development, when induced by intradermal inoculation of the promastigote form of *L.* (*L.*) *amazonensis* into mouse paws. Progression of the lesion was monitored until week 9 by plethysmographic measurement of footpad swelling. The control animals developed larger lesions with progression during the course of infection. The

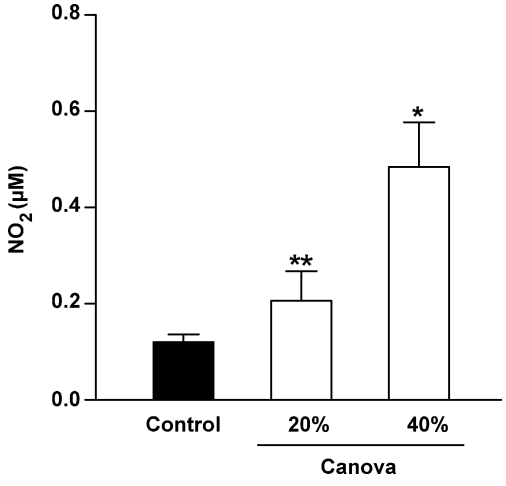

Figure 2 Nitric oxide (NO) production in macrophages treated with Canova medication. Balb/c mouse macrophages elicited with thioglycate, were treated with Canova medication at 20 or 40% concentration. After 24 h, nitric oxide levels were measured. Data are the mean ± SEM of four experiments. *$P<0.001$, **$P<0.05$ (compared to control group, Tukey test).

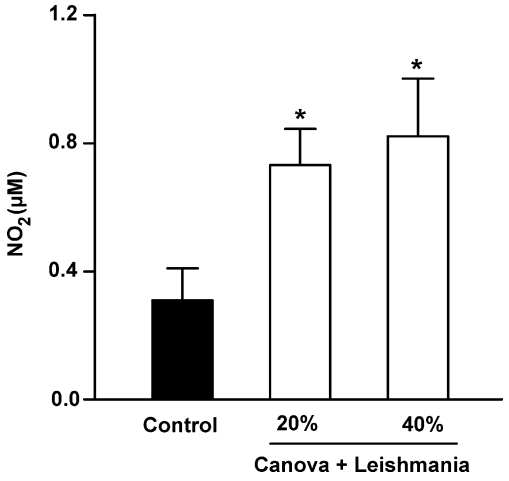

Figure 3 Nitric oxide (NO) production in infected macrophages treated with Canova medication. Balb/c mouse peritoneal macrophages, elicited with thioglycate, were treated with Canova medication at 20 or 40% concentration, and infected with *L.* (*L.*) *amazonensis* amastigotes. Nitric oxide levels were measured 24 h later. Data are the mean ± SEM of four experiments. *$P<0.001$ (compared to control group, Tukey test).

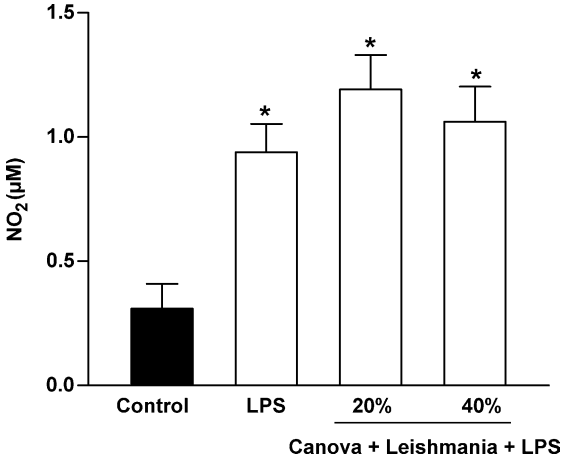

Figure 4 Nitric oxide (NO) production in infected macrophages treated with Canova medication. Balb/c mouse peritoneal macrophages, elicited with thioglycate and stimulated with LPS, were treated with Canova medication at 20 or 40 concentration and infected with *L.* (*L.*) *amazonensis* amastigotes. Nitric oxide levels were measured 24 h later. Data are the mean ± SEM of five experiments. *$P<0.001$ (compared to control group, Tukey test).

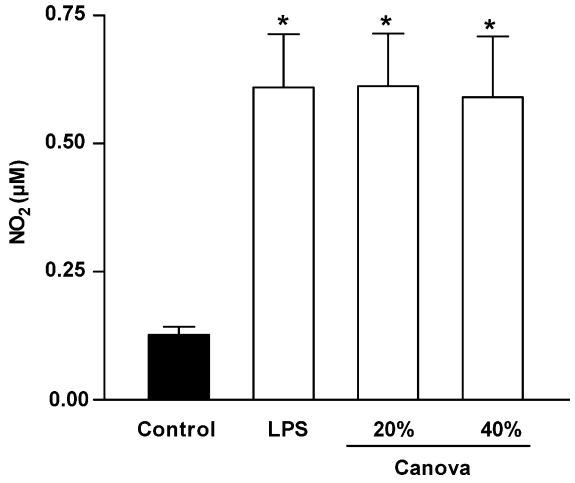

Figure 5 Nitric oxide (NO) production in macrophages treated with Canova medication. Balb/c mouse peritoneal macrophages, elicited with thioglycate and stimulated with LPS, were treated with Canova medication at 20 or 40% concentration. Nitric oxide levels were measured 24 h later. Data are the mean ± SEM of four experiments. *$P<0.001$ (compared to control group, Tukey test).

lesions development was initially slow increasing intensively after day 40 of infection (Fig. 6). Treatment with a single, daily 0.2 ml dose of Canova medication during the experimental period, either intragastrically or subcutaneously, significanly decreased footpad swelling after day 50 of infection by 35 and 21% on day 50, and 53 and 48% on day 65, respectively, when compared to the untreated control group (Fig. 6). These results suggest that Canova medication acts during the later phase of infection, i.e. after a specific immune response has been established.

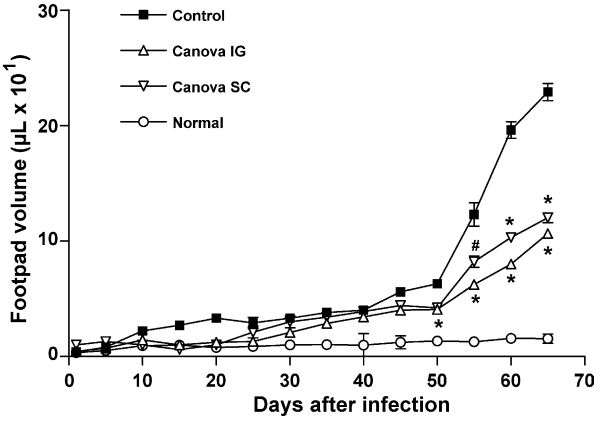

Figure 6 Effect OF Canova medication on infection progression. Balb/c mice were inoculated with 1×10^7 *L.* (*L.*) *amazonensis* promastigotes in one of the hind paws. Treatment with Canova medication (a daily 0.2 ml dose) was provided by intragastric (IG) or subcutaneous (SC) route during the 9-week experimental period. Lesion evolution was evaluated weekly and was expressed as the difference between the infected paw and the uninfected, contralateral paw. Data are the mean ± SEM of 10 animals/group. *$P<0.001$, # $P<0.018$ (compared to control group, Student's *t*-test).

Effect of Canova medication on lymph node and spleen parasite load

Figs. 7 and 8 show that the parasite load in both the popliteal lymph nodes and spleen of animals treated with Canova medication was significantly reduced, compared to non-treated, control animals. These results confirm the data obtained in vitro, suggesting that Canova medication modulates infection by *L.* (*L.*) *amazonensis* in vivo, activating mechanisms that positively affect the host capacity to eliminate the parasites from the infected cells, thus controling parasite dissemination.

Discussion

Macrophages constitute both the host and effector cells against infection by *Leishmania* parasites and by other intracellular micro-organisms.[3] Maintenance and continuity of infection require parasites present in the lesion, and the ability to invade new host cells and multiply. Thus, macrophage activation is fundamental for infection control.[26]

Canova medication can stimulate immunological

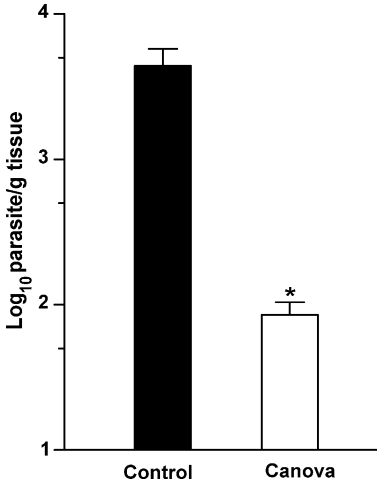

Figure 7 Effect of treatment with the Canova medication on spleen parasite number. Balb/c mice were inoculated with *L.* (*L.*) *amazonensis* promastigotes, and treated with the Canova medication (0.2 ml/day, intragastrically) for 9 weeks. The spleens of six animals were then removed and parasite number was estimated using the limiting dilution technique. *$P<0.001$ (compared to control group, Student's *t*-test).

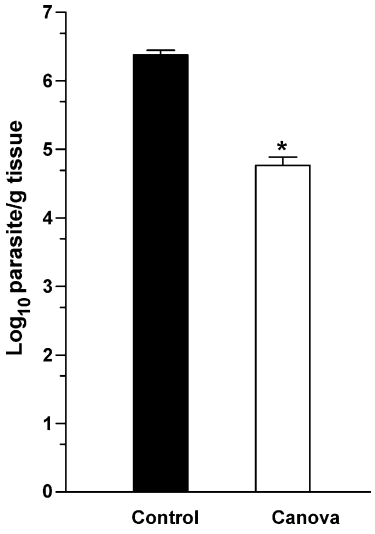

Figure 8 Effect of treatment with Canova medication on lymph node parasite number. Balb/c mice were inoculated with *L. (L.) amazonensis* promastigotes and treated with the Canova medication (0.2 ml/day, intragastrically) for 9 weeks. The popliteal lymph nodes of 6 animals were then removed and parasite number was estimated using the limiting dilution technique. $^{*}P < 0.001$ (compared to control group, Student's *t*-test).

responses, activating macrophages. Oliveira et al.[27] recently showed that macrophages infected with different microorganisms, such as *Saccharomyces cerevisiae* and *Trypanossoma cruzi* exhibit an increase in phagocytotic capacity when submitted to Canova medication treatment. Piemonte and Buchi[16] also demonstrated that when Canova medication is added to macrophage cultures infected by *T. cruzi* and *Leishmania amazonensis* tripomastigotes, cell increases, and morphological alterations such as cell elongation occur.

In this study, we demonstrate that treatment of elicited, peritoneal, murine macrophages with Canova medication causes a significant decrease in the macrophage infection index by *L.* (*L.*) *amazonensis*. This effect was not due to a direct, toxic action on *Leishmania*, since the treatment did not affect promastigote growth.

In murine macrophages, the main effector molecules of anti-parasite defense include nitric oxide (NO), the production of reactive oxygen species and generation of peroxinitrite. Such metabolites can cause protein, lipid and nucleic acid oxidation.[28–30] The function of NO in the leishmanicidal activity of activated macrophages has been demonstrated both in vitro and in vivo.[7,28,31–33]

Our data demonstrate that Canova medication treatment stimulates NO production in macrophages, suggesting that the inhibitory effect of the medication on infection index is mediated by nitric oxide. Although NO levels increased, these were not expressive. This may be relevant since some studies show that the release of high NO levels may be toxic to the cells themselves.[34]

The in vitro experiments clearly revealed that Canova medication can modulate macrophage infection by *Leishmania*. For this reason, we performed experiments to examine whether the medication might affect infection by *L.* (*L.*) *amazonensis* in vivo.

Canova medication, when administered orally or subcutaneously in mice for a 9 week period, controlled the infection induced by *L.* (*L.*) *amazonensis* in the paws, resulting in smaller lesions when compared to those in the control animals. The study also demonstrates that Canova medication treatment decreased parasite load, both in the regional popliteal lymph nodes and in the spleen.

On the whole, our data show that Canova medication affects not only the regulatory mechanisms that control *L.* (*L.*) *amazonensis* infection, but also seems to limit parasite dissemination.

NO production by macrophages alone does not fully explain the notable inhibitory effect of Canova medication on lesions induced by *Leishmania* in vivo. Thus, while macrophages are one of the main sources of NO, this radical may also be realese by other cells involved in the infectious process.[35–37] Other substances such as platelet-activating factor (PAF) and prostaglandin (PGE), for example, may also, modulate the host response in this experimental model.

The great number of cytocines and other mediators released from activated cells, that modify macrophage functions, underscore the complexity of the process.[1,38]

Given these considerations, many further studies will be necessary to elucidate the mechanisms of action of Canova medication in the defense of the organism against infection, creating new perspectives for investigation of other mediators and/or cytocines.

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