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SHORT COMMUNICATION

Zinc and Manganese Imbalances in BALB/c Mice Experimentally Infected with *Leishmania* (*Leishmania*) *amazonensis*

Caroline Sobotyk[1,6] · Fernanda Giesel Baldissera[2] · Luiz Carlos Rodrigues Junior[2] · Pedro Roosevelt Torres Romão[2] · Juliana Sorraila de Oliveira[3] · Guilherme Lopes Dornelles[3] · Cinthia Melazzo de Andrade[3] · Roberto Marinho Maciel[4] · Cristiane Cademartori Danesi[4] · Rafael Vicente de Padua Ferreira[5] · Maria Helena Bellini[5] · Sônia de Avila Botton[6] · Fernanda Silveira Flores Vogel[6] · Luis Antonio Sangioni[6]

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Abstract

Purpose The clinical progression of *Leishmania (Leishmania) amazonensis* infection depends on multiple factors, including immunological status of the host and their genotypic interaction. Several immunological processes depend directly on minerals for an efficient performance. Therefore, this study used an experimental model to investigate the alterations of trace metals in *L. amazonensis* infection associate with clinical outcome, parasite load, and histopathological lesions, and the effect of CD4 + *T* cells depletion on these parameters.

Methods A total of 28 BALB/c mice were divided into 4 groups: 1—non-infected; 2—treated with anti-CD4 antibody; 3—infected with *L. amazonensis*; and 4—treated with anti-CD4 antibody and infected with *L. amazonensis*. After 24 weeks post-infection, levels of calcium (Ca), iron (Fe), magnesium (Mg), manganese (Mn), Cu, and Zn were determined by inductively coupled plasma optical emission spectroscopy using tissue samples of the spleen, liver, and kidneys. Additionally, parasite burdens were determined in the infected footpad (inoculation site) and samples of inguinal lymph node, spleen, liver, and kidneys were submitted to histopathological analysis.

Results Despite no significant difference was observed between groups 3 and 4, *L. amazonensis*-infected mice had a significant reduction of Zn (65.68–68.32%) and Mn (65.98 to 82.17%) levels. Presence of *L. amazonensis* amastigotes was also detected in the inguinal lymph node, spleen, and liver samples in all infected animals.

Conclusion The results showed that significant alterations in micro-elements levels occur in BALB/c mice experimentally infected with *L. amazonensis* and may increase the susceptibility of individuals to the infection.

Keywords *Leishmania* sp. · Cutaneous leishmaniasis · Microelements · Trace elements · CD4 + T cells

Leishmania (*Leishmania*) *amazonensis* has been associated with cutaneous, diffuse cutaneous, and mucocutaneous clinical forms of the disease. This protozoan is distributed in numerous countries, including Brazil, Bolivia, Colombia, French Guiana, Paraguay, and Peru [1–6]. Rodents are considered the natural host for *L. amazonensis* but

✉ Caroline Sobotyk
csobotyk@vet.upenn.edu

[1] Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA

[2] Departamento de Ciências Básicas da Saúde, Universidade Federal de Ciências da Saúde de Porto Alegre, Rua Sarmento Leite, 245, Porto Alegre, RS 90050-170, Brazil

[3] Programa de Pós-graduação em Medicina Veterinária (PPGMV), Departamento de Clínica de Pequenos Animais, Universidade Federal de Santa (UFSM), Av. Roraima, 1000, Camobi, Santa Maria, RS 97105900, Brazil

[4] Departamento de Patologia, UFSM, Av. Roraima, 1000, Camobi, Santa Maria, RS 97105900, Brazil

[5] Laboratório de Biologia Celular e Molecular do Câncer, Centro de Biotecnologia, Instituto de Pesquisas Energéticas e Nucleares, Av. Professor Lineu Prestes, 2242, Cidade Universitária, Butantã, São Paulo, SP 05508-000, Brazil

[6] Departamento de Medicina Veterinária Preventiva, PPGMV, UFSM, Av. Roraima, 1000, Camobi, Santa Maria, RS 97105900, Brazil

autochthonous cases in marsupials, forest foxes, bats, dogs, cats, and humans have been also reported [2, 7–11].

The pathogenicity and virulence of *Leishmania* sp. are influenced by several factors such as the genetic and immunological aspects of the host and *Leishmania* species, as well as the host–parasite interaction [12]. Previous studies demonstrated that *L. amazonensis* can evade the host protective mechanisms of the innate and adaptive immune systems, even in immunocompetent hosts [13]. Several trace elements are directly linked with immunological functions and cellular actions such as cell membrane stability, apoptosis, host metabolism, and enzymatic activities [14–18]. Few reports have focused on the alteration of trace elements and cutaneous leishmaniasis [19–21]. However, the association between trace element, clinical outcome, and pathogenicity of *L. amazonensis* is poorly understood. Therefore, this study aims to investigate the changes in macro- and microelements in BALB/c experimentally infected with *L. amazonensis* and the potential association with the clinical outcome, including parasite load and histopathological lesions, and determine the effect of depletion of CD4 + T cells during *L. amazonensis* infection on these parameters.

A total of 28 6-week-old BALB/c mice were randomly divided into 4 groups (n = seven mice/group): 1—control (non-infected animals); 2—treated with anti-CD4 antibody 3—infected with *L. amazonensis*; 4—treated with anti-CD4 antibody and infected with *L. amazonensis.* Groups 2 and 4 were treated intraperitoneally with three 50 µg doses of anti-CD4 antibody purified from the hybridome GK 1.5 (ATCC TIB 207) on days 1, 5, and 8 of the experiment. The efficiency of immunosuppression was evaluated by the quantification of peripheral CD4 + T cells using flow cytometry. After $CD4^+$ T cells depletion, animals from groups 3 and 4 were inoculated (sc) in the left footpad with 2×10^6 stationary-phase *L. amazonensis* promastigotes at day 9. *Leishmania* (*Leishmania*) a*mazonensis* strain IFLA/BR/67/PH8 was used in this study, and its promastigote forms were cultured *in vitro* as previously described [22]. The kinetics of the cutaneous lesion was evaluated weekly and was expressed as the difference between the infected and uninfected contralateral footpad. After 24 weeks post-infection, tissue samples from the footpad (inoculation site), lymph nodes, liver, spleen, and kidneys were collected. Parasite burdens were analyzed in inoculation site by microtiter culture technique according to Buffet *et al*. [23]. Samples of the inguinal lymph node, spleen, liver, and kidneys were used for the histopathological analysis according to the method of Klüver and Barrera [24].

Levels of calcium (Ca), iron (Fe), magnesium (Mg), manganese (Mn), Cu, and Zn were determined by inductively coupled plasma optical emission spectroscopy (ICP-OES) (Optima 7000 DV, Perkin Elmer Co. USA) using tissue samples of the infected footpad, spleen, liver, and kidneys. For the digestion step, 2 mL of 65% nitric acid (14.44 mol/L HNO_3) (ACS reagent grade, Merck, Rio de Janeiro, Brazil) was added to each sample. The solution was digested with 2.0 mL of 30% hydrogen peroxide (ACS reagent grade, Merck, Rio de Janeiro, Brazil) on a hot plate at 54 °C (Quimis, model Q313A, Sao Paulo, Brazil). After the digestion, diluted solutions were prepared for each sample using 5% HNO_3 solution (Suprapur® Merck, Darmstadt, Germany). A reagent blank was prepared under the same conditions. Metal determination by ICP-OES (Optima 7000 DV) was performed with external calibration using analytical solutions prepared in 5% HNO_3 (Suprapur®) by appropriate dilution of the stock solution (ICP phosphorus and 21 multi-element standard solutions Inorganic Ventures, Christiansburg, USA). The measurement conditions for analyses are described in Tables S1 and S2.

The statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA). The results obtained were compared among groups using one-way ANOVA and Tukey post hoc test. The analysis was followed by residual analyses to check for the error distribution and suitability of the normal model. Differences were considered significant when $p \leq 0.05$.

The efficiency of CD4 + T cells depletion was evaluated using a peripheral blood sample taken from animals treated with anti-CD4. As shown in Fig. S1, after the depletion scheme, the circulating CD4 + T cells reduced 50%. In our study, the depletion of CD4 + T cells right before the infection did not change the course of *L. amazonensis* infection. All infected animals presented a chronic cutaneous disease over 24 weeks, including presence of non-ulcerative nodular lesion and footpad swelling. Groups 3 and 4 had similar lesion sizes at all points of the kinetic curve and there were no statistical differences between the groups (Fig. S2). Groups 3 and 4 also presented similar parasite load, with median of 3.6×10^5 and 3.9×10^5 parasites/g, respectively ($p > 0.05$).

The Zn concentration was significantly lower in the spleen tissues in both groups infected with *L. amazonensis* (3 and 4) compared to control (group 1) ($p < 0.05$; Fig. 1). The tissue Zn level in both groups 3 and 4 ranged from 0.0212 to 0.0413 and 0.0211 to 0.0387 mg per mg of tissue, respectively. The average reduction of Zn levels observed in the group 3 was 68.32% (0.0279 ± 0.0091) and 65.68% (0.0302 ± 0.0080) in the group 4 compared to non-infected animals (group 1) (0.0881 ± 0.0297). In contrast, Zn level in the spleen tissue was 44.42% (0.1436 ± 0.0315) higher in mice with CD4 + T cells depletion (group 2) compared to group 1 ($p < 0.05$).

The Mn concentration in the spleen was significantly lower in *L. amazonensis*-infected animals (group 3 and 4) compared to non-infected (group 1) ($p < 0.05$; Fig. 2). The tissue Mn concentration in both non-infected groups (1 and

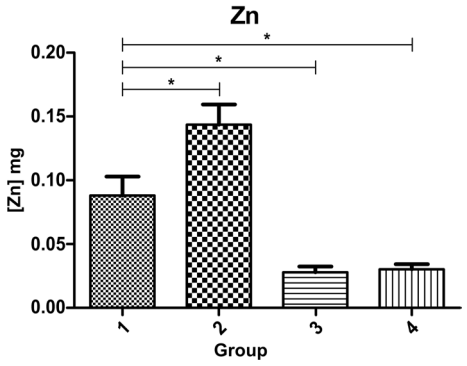

Fig. 1 Zinc concentrations in tissue of the spleen in BALB/c mice treated with anti-CD4 antibody (group 2), infected mice with *Leishmania (Leishmania) amazonensis* (group 3) and mice treated with anti-CD4 antibody and infected with *L. amazonensis* (group 4) compared to uninfected control mice (group 1). Significant differences between sample means are indicated: *, $p < 0.05$)

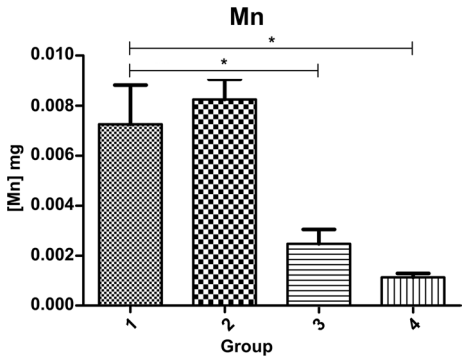

Fig. 2 Manganese concentrations in tissue of the spleen in BALB/c mice treated with anti-CD4 antibody (group 2), infected mice with *Leishmania (Leishmania) amazonensis* (group 3), and mice treated with anti-CD4 antibody and infected with *L. amazonensis* (group 4) compared to uninfected control mice (group 1). Significant differences between sample means are indicated: *, $p < 0.05$)

2) ranged from 0.0046 to 0.0117 and 0.0070 to 0.0105 mg/mg, respectively, while the *L. amazonensis*-infected groups 3 and 4 ranged from 0.0012 to 0.0035 and 0.0010 to 0.0015 mg/mg, respectively. No significant difference was noted between animals infected with *L. amazonensis* (group 3) and animals both treated with anti-CD4 antibody and infected with *L. amazonensis* (group 4) ($p > 0.05$). However, the group 4 presented lower reduction value of Mn levels (82.17%; 0.0012 ± 0.0002) compared to group 3 (65.98%; 0.0024 ± 0.0011). No significant differences in Cu, Ca, Fe, and Mg concentration were observed in spleen tissue. Also, no significant difference in the macro- and micro-element concentrations was observed in the liver and kidney tissue.

We observed the presence of *L. amazonensis* amastigotes in the inguinal lymph node, spleen, and liver samples in the infected groups, but no significant difference was observed between infected mice with *L amazonensis* (group 3) and treated with anti-CD4 antibody and infected with *L. amazonensis* (group 4) ($p > 0.05$). In both infected groups (groups 3 and 4), we observed tissue damage in (i) liver: lymphoplasmacytic and granulomatous periportal hepatitis; (ii) spleen: pulp hyperplasia; and (iii) inguinal lymph node: chronic granulomatous lymphadenitis. No histological alterations were observed in the kidneys.

The present study showed that *L. amazonensis* infection can decrease the Zn and Mn levels in spleen tissue even after $CD4^+$ T cells depletion. Alterations in trace mineral levels have been described in cutaneous and visceral *Leishmania* infections in different hosts [20, 25–28]. [20] observed lower levels of Zn in serum from susceptible BALB/c mice infected with *Leishmania* (*Leishmania*) *major*. Similar results were also reported by Pasa *et al*. [25] and Souza *et al*. [27] in symptomatic dogs naturally infected with *Leishmania* (*Leishmania*) *infantum.* In protozoan infections, this mineral has been implicated as a factor for the inability of the host to eliminate the parasite and occurrence of the inflammation reaction with deficient production of several cytokines and enzymes [20, 29, 30]. Our findings associated with the literature suggest that decreased Zn levels could contribute to survival and persistence of visceral and cutaneous *Leishmania* infections and play an important role in the development of *L. amazonensis* chronic status. The occurrence of higher levels of Zn in non-infected mice treated with anti-CD4 antibody reinforces the association between *Leishmania* positive hosts and lower concentrations of trace elements. It is essential to understand that the concentration of micro- and macro-elements change under distinct situations of infections and/or inflammations. Therefore, excess or deficiency of trace elements could damage the functioning of the immune system cells and increase the risk of development and progression of infectious diseases, including leishmaniasis. In our study, the concentration of trace elements in serum was not evaluated due to the limited serum volume obtained from the murine models. However, previous study has demonstrated that the distribution of minerals in experimentally infected animals presents similar and comparable pattern in both serum and tissue samples [15].

There are no reports of Mn imbalance in spleen tissue in both cutaneous and visceral leishmaniasis, which suggests that Mn deficiency could be restricted to *L. amazonensis* infections. In contrast with our results, previous studies have observed alteration of Cu and Fe serum concentrations in *L. major* and *L. infantum* infections in dogs, humans, and animal models [20, 25, 26, 28, 31]. This variability in trace elements could be explained by the host susceptibility or resistance to the infection, and different virulent factors and pathogenic behavior of *Leishmania* species. The Mn imbalance could be induced by immunoregulatory cytokines in response to a systemic inflammatory reaction to the infection

as a result of the host immune system strategy [32]. However, the trace element deficiency decreases the possibility of control and elimination of pathogenic agents and increases the susceptibility to several diseases [33]. In this way, Mn concentrations could be altered by *L. amazonensis* during the infection as a survival strategy, and thus should be considered for the prevention, control, and treatment. While this study provides insights on how trace elements concentrations might influence the clinical outcome of cutaneous leishmaniosis, several limitations should be considered in the present study. First, the anti-*Leishmania* humoral and cellular immune responses were not assessed in the present study. As such, it is not possible to determine the correlation between trace element concentrations, the quality of the immune response, and infection outcome, and thus results should be interpreted carefully. Another important limitation is the CD4 T cell depletion protocol which was performed during the infection period. This could explain similar trace elements concentrations between groups 3 and 4 and limited result interpretations in some infection parameters.

Despite the evidence of the role of nutrients in the immune system and cutaneous leishmaniosis, the correlation between pathogenesis, host immune response, and trace elements has not been fully elucidated. The pattern of Zn and Mn observed in spleen tissue samples may indicate a defense activity and a higher parasite load in this tissue compared to liver and kidneys, and aggravation of the disease. Additionally, our study demonstrated the ability of *L. amazonensis* to migrate to other organs. Although *L. amazonensis* is considered the agent of American cutaneous leishmaniasis, there are few reports of visceral infections in dogs and humans [11, 34–37]. The mechanism of infection, pathogenesis, and the wide spectrum of clinical features is still poorly understood. A meta-analytical study showed that cutaneous *Leishmania* species can migrate to different organs, including skin, lymph nodes, spleen, liver, and cause canine visceral disease [38]. Due to the wide range of clinical signs, *L. amazonensis* infections are often misdiagnosed as and treated for canine visceral leishmaniasis (caused by *L. infantum*). This lack of epidemiological information on the prevalence of *L. amazonensis* could be due to the low specificity of serological tests and the similarity of clinical presentation between canine *L. infantum* infection and *L. amazonensis* visceral form. Therefore, *L. amazonensis* should be included in the differential diagnosis of visceral leishmaniasis in both dogs and humans especially in co-endemics areas.

In summary, this study demonstrated that chronic *L. amazonensis* infections could induce Zn and Mn imbalance even in immunocompetent hosts. The lower concentration of Zn and Mn in spleen tissue suggests a relationship between the trace elements imbalance and the persistence of *L. amazonensis* infection with the development of visceral disease. Future studies should be performed to mitigate the effects of mineral imbalance in the immunological response and treatment of *L. amazonensis* infections.

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Author Contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by CS, FGB, LCRJ, PRTR, JSdO, GLD, CMdA, RMM, CCD, RVdPF, MHB. Supervision was performed by Sd AB, FSFV, and LAS. The first draft of the manuscript was written by CS and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability All data generated or analysed during this study are included in this published article and its supplementary information files.

Declarations

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. All procedures performed were in accordance with the ethical standards of the institution at which the studies were conducted (Committee on Ethics in the Use of Animals, Universidade Federal de Ciências da Saúde de Porto Alegre, number 505/17).

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