Experimental Parasitology 127 (2011) 719–723

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Research Brief

Leishmania chagasi: Effect of the iron deficiency on the infection in BALB/c mice

Guilherme Malafaia [a,*], Letícia de Nadai Marcon [b], Liliane de Fátima Pereira [c], Maria Lúcia Pedrosa [d], Simone Aparecida Rezende [e]

[a] *Laboratório de Ciências Ambientais, Departamento de Ciências Biológicas, Instituto Federal de Educação, Ciência e Tecnologia Goiano – Campus Urutaí (IF Goiano), Núcleo de Pesquisa em Ciências Ambientais e Biológicas (NPCAB). Rodovia Geraldo Nascimento Silva, 2.5 km, Zona Rural, CEP 75790-000, Urutaí, GO, Brazil*

[b] *Laboratório de Imunoparasitologia, Programa de Pós-Graduação em Ciências Biológicas, Universidade Federal de Ouro Preto (UFOP). Campus Universitário Morro do Cruzeiro, Bauxita, CEP 35400-000, Ouro Preto, MG, Brazil*

[c] *Graduanda em Farmácia, Universidade Federal de Ouro Preto (UFOP). Campus Universitário Morro do Cruzeiro, Bauxita, CEP 35400-000, Ouro Preto, MG, Brazil*

[d] *Laboratório de Bioquímica e Biologia Molecular, Departamento de Ciências Biológicas, Programa de Pós-Graduação em Ciências Biológicas, Núcleo de Pesquisa em Ciências Biológicas (NUPEB), Universidade Federal de Ouro Preto (UFOP). Campus Universitário Morro do Cruzeiro, Bauxita, CEP 35400-000, Ouro Preto, MG, Brazil*

[e] *Laboratório de Imunoparasitologia, Departamento de Análises Clínicas, Escola de Farmácia, Programa de Pós-Graduação em Ciências Biológicas, Núcleo de Pesquisa em Ciências Biológicas (NUPEB), Universidade Federal de Ouro Preto (UFOP). Campus Universitário Morro do Cruzeiro, Bauxita, CEP 35400-000, Ouro Preto, MG, Brazil*

ARTICLE INFO

Article history:
Received 27 January 2010
Received in revised form 4 November 2010
Accepted 17 November 2010
Available online 24 November 2010

Keywords:
Leishmania chagasi
Iron deficiency
BALB/c
Visceral leishmaniasis
Hemoglobin
IFN-gamma
IL-4

ABSTRACT

Iron deficiency and visceral leishmaniasis are serious problems of public health. The aim of this study was to evaluate the effect of iron deficiency, induced by the iron chelator desferrioxamine, on the course of the infection by *Leishmania chagasi* in BALB/c mice. Our data show that the iron chelator caused significant reduction in hemoglobin concentration of treated mice and reduction in parasite load in spleen and liver. Significant differences were not observed in the production of IFN-gamma and IL-4 among the experimental groups. In conclusion, the data reported in this paper suggest that iron deficiency may favor the host. If there is not enough iron available to the parasite, its multiplication may be reduced and infection attenuated.

1. Introduction

Currently, both iron deficiency and infection caused by *Leishmania* parasites are considered serious problems of public health which affect millions of people. Visceral leishmaniasis (VL) has an annual incidence of 500,000 new cases (WHO, 2005), and recently, an increase in the number of cases has been observed in many areas where there is a high prevalence of iron deficiency.

Iron deficiency, according to the World Health Organization (WHO), is the most prevalent nutritional disorder in the world affects 1.62 billion people, (24.8% of the world population) (WHO, 2008) and affects mainly children under four years old, breast-feeding and pregnant women and women of fertile age. In relation to the genus *Leishmania* (Protozoa: Kinetoplastida), only a few studies have evaluated the effect of iron deficiency on the infection (Huynh et al., 2006; Huynh and Andrews, 2008; Das et al., 2009; Carvalho et al., 2009 and Jacques et al., 2010). These authors have demonstrated the importance of elemental iron for the replication of pathogens in the host.

However, very little is known about the mechanisms involved in the relationship between infection by *L. chagasi* and iron deficiency. As discussed by Malafaia (2008a), studies concerning iron and infection have presented results which are frequently contradictory, some showing that iron shortage increases susceptibility to infectious processes whereas others show that iron excess is much more harmful to the human host and that the iron shortage could even play a protective role in certain infections. In the case of infection by *Leishmania*, the capture of iron ions required for anti-oxidizing functions and other metabolic reactions seems to be crucial for their survival and multiplication (Marquis and Gros, 2007; Huynh and Andrews, 2008; Malafaia, 2008b; Das et al., 2009).

Thus, the present work aimed to evaluate the effect of iron deficiency induced by the iron chelator desferrioxamine on *L. chagasi* infection in BALB/c mice. Since few works have dealt with the existing association between iron deficiency and VL caused specifically by *L. chagasi*, this study may help to elucidate the complex

* Corresponding author. Address: Departamento de Ciências Biológicas, Rodovia Geraldo Silva, 2.5 km, Zona Rural, CEP 75790-000, Urutaí, GO, Brazil.
E-mail addresses: guilherme@nupeb.ufop.br, guilhermebioufop@yahoo.com.br (G. Malafaia).

0014-4894
doi:10.1016/j.exppara.2010.11.010

relationships that govern this micronutrient deficiency and the course of VL.

2. Materials and methods

2.1. Animals, division of groups and experimental design

Female BALB/c mice (3–5 weeks old) were used and were randomly divided into three experimental groups: uninfected and not treated with desferrioxamine (DFO) (UNT) group, infected with *L. chagasi*, but not treated (INT) group and infected and treated (IT) group. The data presented in this study are from two experiments performed independently (n = 8 mice/per group/per experiment).

Initially, all animals of the treated groups received an intraperitoneal (ip) injection of 10 mg of DFO (Desferal®, Novartis, Basel, Switzerland) in 100 μL of PBS (3 doses per week). The treatment protocol was based on Arantes et al. (2007), regarding the route of inoculation and concentration of the drug administered in each animal (double the concentration used by Arantes et al. (2007) was used in this study). The animals of the groups not treated with DFO received the same amount of PBS over the same period.

After two weeks of treatment, animals in the infection groups were infected with *L. chagasi* promastigotes, given intravenously by lateral tail vein. In order to do this, promastigote forms of *L. chagasi* strain (MHOM/BR/1974/M2682) were used. *L. chagasi* was cultured at 26 °C in Grace's Insect Medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS; Cripion, Andradina, SP, Brazil), 2 mM-glutamine (Gibco BRL) and 100 U/mL penicillin G potassium (USB Corporation, Cleveland, OH, USA), pH 6.5 at 26 °C. Infectivity was maintained by serial passage in BALB/c mice. For the inoculation, promastigotes of *L. chagasi* were harvested from late-log-phase cultures by centrifugation and washed three times in PBS. For the experimental infection, 1×10^7 promastigotes were suspended in 200 μL RPMI, pH 7.2.

Two, four and six weeks after the infection with *L. chagasi*, the infected mice were sacrificed and spleen and liver parasite loads were determined. In this case, the parasite load was determined by quantitative limiting dilution culture as described by Titus et al. (1985) and modified by Marques-da-Silva et al. (2005), with some modifications. Fragments of spleen and liver were obtained and weighed separately for parasite quantification. In addition we decided to assess some immunological parameters including production of cytokines IFN-gamma and IL-4, and the production of NO by splenocytes. Single-cell suspensions of spleen were obtained by tissue grinder homogenization and processed as described previously (Marques-da-Silva et al., 2005) and the production of IFN-gamma and IL-4 was determined in these supernatants by ELISA (Afonso and Scott, 1993). The production of nitric oxide (NO) was determined by the Greiss method (Green et al., 1982). To prepare the *L. chagasi* antigen, parasites were disrupted by three rounds of freezing and thawing (freeze–thawed antigen), protein content was estimated by the Lowry method (Lowry et al., 1951) and the preparation was frozen at −20 °C until use.

2.2. Levels of hemoglobin and physical parameters

To determine if the treatment with DFO was capable of reducing the blood levels of hemoglobin (Hb) (which indicates a reduction in the levels of iron), its concentration was determined in blood samples, collected from the ocular plexus, using a commercial assay procedure (Labtest Kit catalogue No. 43). Evaluations were performed in the second, fourth and sixth week after infection, on the day of sacrifice.

To evaluate the effect of infection *by L. chagasi* and of DFO on physical parameters, we measured the body weight and liver and spleen mass of the mice. The assessment of body weight was performed weekly until the sacrifice of animals. The evaluations of organ weight were performed on the day of animals' sacrifice (2, 4 and 6 weeks after infection).

2.3. Statistical analyses and ethics issues

All data were analyzed by Kolmogorov–Smirnov normality test. Data with a normal distribution were analyzed by Student's t test (data of body weight, organ weight and blood hemoglobin levels). Data whose distributions were not considered normal were submitted to non-parametric Mann–Whitney's test (data of parasite load, cytokines and NO). Differences with a p value <0.05 were considered statistically significant.

All animal procedures were approved by the Committee on Ethics in Research of the Universidade Federal de Ouro Preto-MG, Brazil, and followed the guidelines for the use and care of animals for research published by the Canadian Council on Animal Care (1980, 1984).

3. Results

3.1. Effect of treatment with DFO on total body weight and liver and spleen mass

No difference in the total body weight between the experimental groups was observed (data not shown). In addition, no significant difference between the liver mass of the INT and IT groups measured in the second, fourth and sixth weeks after infection were observed. A significant difference was observed between UNT and INT groups and UNT and IT groups only in the sixth week of evaluation (Fig. 1A).

There was no difference in spleen mass between the INT and IT groups in the second, fourth and sixth weeks after infection. However, a significant difference was observed between UNT and INT groups and UNT and IT groups also only in sixth week of evaluation (Fig. 1B).

3.2. Levels of hemoglobin

We did not observe significant differences in hemoglobin between UNT and INT groups measured in the second, fourth and sixth experimental weeks. However, significant differences between INT and IT groups and UNT and IT groups were observed 4 and 6 weeks after infection (Fig. 2).

3.3. Parasite load in spleen and liver

In order to study the influence of iron deficiency on *L. chagasi* infection in BALB/c mice, this study evaluated the parasite load in spleen and liver. Our data show that the IT group had a significantly smaller splenic parasitic load compared to INT group 6 weeks after infection (Fig. 3A). Regarding hepatic parasitic load, a significant difference in number of parasites between the INT and IT groups was observed when the evaluations were carried out after 4 and 6 weeks of infection (Fig. 3B).

3.4. Determination of production of cytokine and NO

After mice were killed, spleen cells were harvested and incubated in the presence or absence of freeze–thawed *L. chagasi* antigen (50 μg/mL), in order to determine the cytokine levels (IFN-gamma and IL-4) in culture supernatants. It was observed that the treatment with DFO did not influence the production of IFN-gamma and IL-4. Although the *Leishmania* antigen was able

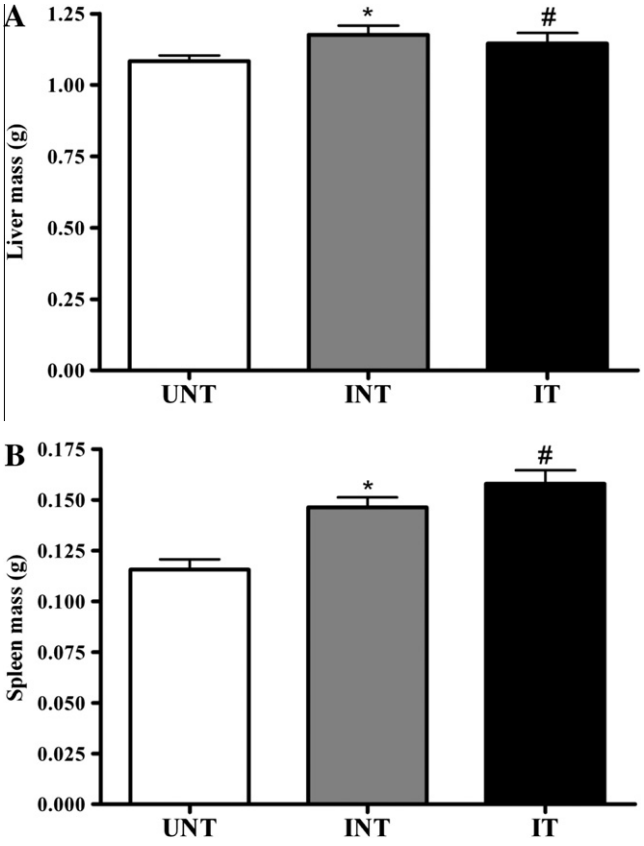

Fig. 1. Liver (A) and spleen (B) mass of mice. The bars represent the means + standard deviation of data obtained in the sixth experimental week of two independent experiments ($n = 8$/for group in each experiment). Statistical differences were determined by Student's t test. Statistical differences between UNT and INT groups and UNT and IT groups are represented by $*$ and #, respectively ($p < 0.05$). Animals were randomly divided into three experimental groups: uninfected and not treated with desferrioxamine (DFO) (UNT) group, infected with *L. chagasi*, but not treated (INT) group and infected and treated (IT) group.

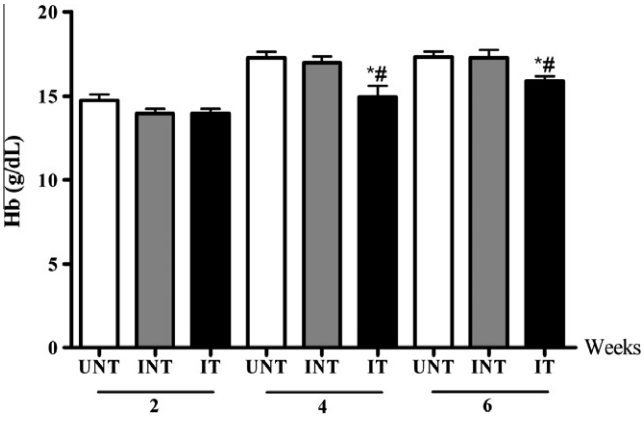

Fig. 2. Levels of hemoglobin in mice in control and treated groups. Hemoglobin concentration was determined, immediately after collection, in blood samples via ocular plexus. The bars represent the means + standard deviation of data obtained in the sixth experimental week of two independent experiments ($n = 8$/for group in each experiment. Statistical differences were determined by Student's t test. Statistical differences between UNT and INT groups and UNT and IT groups are represented by $*$ and #, respectively ($p < 0.05$). Animals were randomly divided into three experimental groups: uninfected and not treated with desferrioxamine (DFO) (UNT) group, infected with *L. chagasi*, but not treated (INT) group and infected and treated (IT) group.

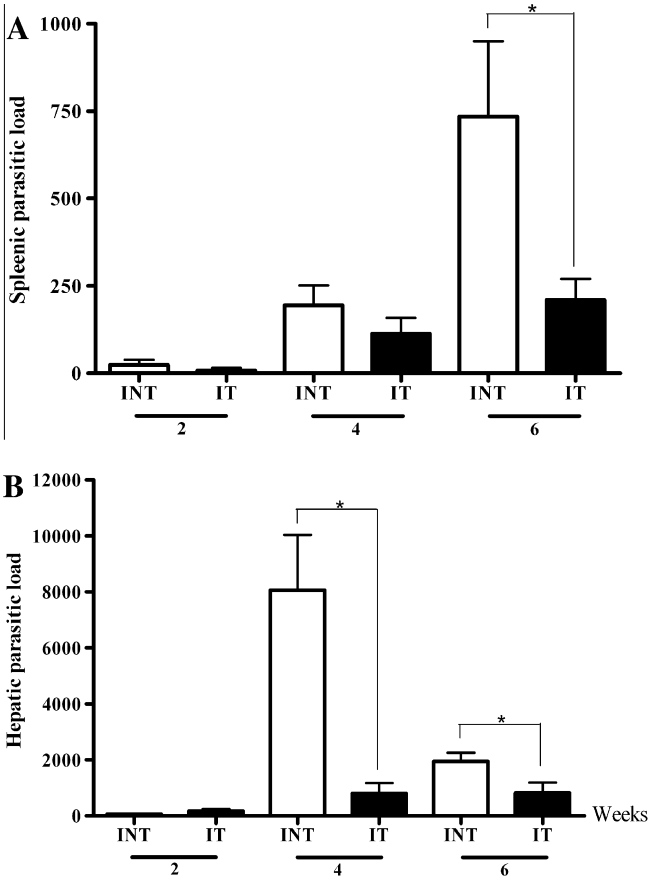

Fig. 3. Splenic (A) and hepatic (B) parasitic load of mice. Two, four and six weeks after inoculation of parasites, mice were sacrificed and their spleen and liver were harvested for parasite quantification by limiting-dilution quantitative culture. The bars represent the means + standard deviation of data obtained in the second, fourth and sixth experimental weeks of two independent experiments (eight animals for group were sacrificed in each period of the evaluation). Statistical differences were determined by non-parametric Mann–Whitney's test. Statistical differences between groups are represented by one asterisk ($p < 344$ 0.05). Animals were randomly divided into three experimental groups: uninfected and not treated with desferrioxamine (DFO) (UNT) group, infected with *L. chagasi*, but not treated (INT) group and infected and treated (IT) group.

to induce production of IFN-gamma, no statistically significant difference was observed between the INT and IT groups, when the evaluations were performed in the second, fourth and sixth weeks after infection (Fig. 4A). Furthermore, no difference in the production of IL-4 between INT and IT groups (Fig. 4B) was observed. No significant levels of NO were detected in the supernatants by the method used (data not shown).

4. Discussion

Iron is the most studied and best described micronutrients in the literature, and plays important roles in human metabolism, such as transport and storage of oxygen, energy release reactions in the electron transport chain, conversion of ribose into deoxyribose, and is a co-factor for some enzymatic reactions and other essential metabolic processes (Cook et al., 1992). The greatest amount of iron is found in hemoglobin; the remainder being distributed in other proteins, enzymes and in iron deposits (ferritin and hemosiderin) (Yip and Dallman, 1997). As discussed by Marquis and Gros (2007), Malafaia (2008b) and Das et al. (2009), considering infections, findings available in literature show that either iron deficiency may favor the proliferation of parasites, once such

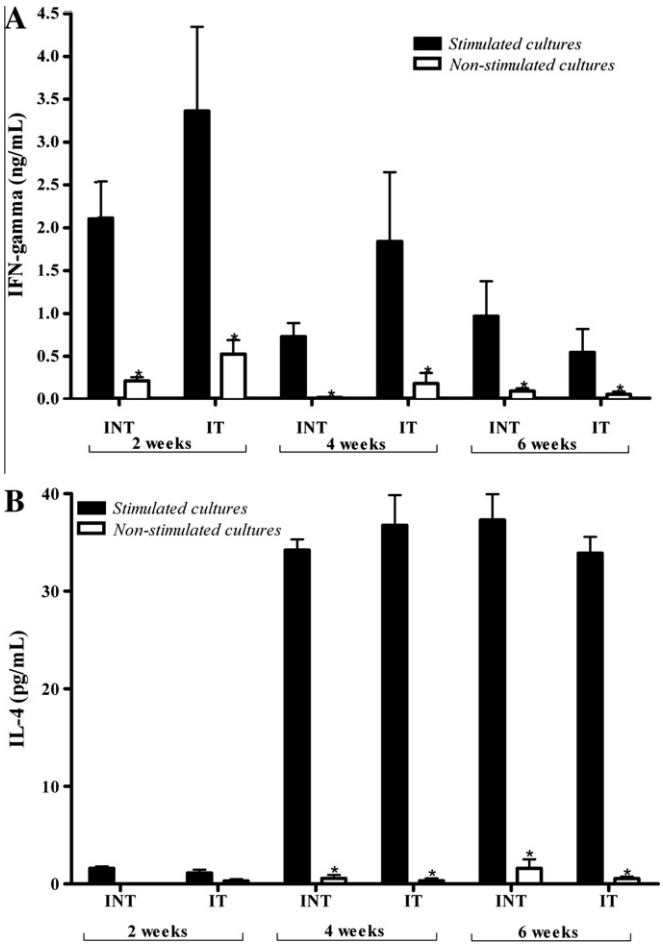

Fig. 4. Production of IFN-gamma (A) and IL- 4 (B) by spleen cells of mice studied. The bars represent the means + standard deviation of data obtained in the second, fourth and sixth experimental weeks of two independent experiments (eight animals for group were sacrificed in each period of the evaluation). Statistical differences were determined by non-parametric Mann–Whitney's test. Statistical differences between non-stimulated cultures and stimulated cultures are represented by one asterisk ($p < 0.05$). Animals were randomly divided into three experimental groups: uninfected and not treated with desferrioxamine (DFO) (UNT) group, infected with *L. chagasi*, but not treated (INT) group and infected and treated (IT) group.

deficiency leads to damage to the immune system, or alternatively, that iron deficiency may favor the control of infection, as it limits the amount of iron available to the parasite. There have been no studies of infection caused by *L. chagasi*.

Treatment with DFO did not have any influence on the physical parameters evaluated (total body weight and liver and spleen mass). However, an increase in liver and spleen mass was observed in mice of the infected groups (INT and/or IT) if compared to UNT group only in the evaluation at the sixth week of infection (Fig. 1). These data suggest a developing of hepatosplenomegaly in mice, a typical sign of VL. However, this was not observed at the second and fourth weeks after infection, which is not surprising as it is progressive process.

Our data indicate that treatment with DFO was able to reduce hemoglobin blood levels in the fourth and sixth weeks after infection (Fig. 2). In experimental models and humans, three stages of iron deficiency have been described. The initial stage, iron depletion, occurs when stored iron in the bone marrow diminishes due to an insufficient supply of iron. Generally this stage is asymptomatic, creates no overt effect on erythropoiesis, and escapes detection by hemoglobin or hematocrit screening. Continued iron store depletion leads to the second stage, iron deficiency, during which storage levels become substantially reduced and hemoglobin synthesis begins to be affected. The final stage, iron deficiency anemia, develops when iron stores are insufficient to maintain hemoglobin production (Cook, 1999; Wu et al., 2002). Thus, the results obtained in our study allow us to infer possible iron deficiency in the treated mice.

Our data show that the DFO was able to provoke a significant reduction of hepatic parasitic load when the evaluations were performed 4 and 6 weeks after infection (Fig. 3B). This was especially apparent in the fourth week after infection (when the reduction was greater), which coincides with a peak of hepatic parasitism in mice infected by *L. chagasi* (Marques-da-Silva et al., 2005). In the spleen, the decrease in parasite load was observed at the sixth week after infection (Fig. 3A), which also coincides with the peak of parasitism (Marques-da-Silva et al., 2005).

Similar data were observed in the recent works of Arantes et al. (2007) and Francisco et al. (2008), involving murine infection by *Trypanosoma cruzi* (Y strain) and use of DFO. These studies are important because they also evaluated another representative of the Trypanosomatidae family. In the first case, the authors observed a reduction in the parasite levels and a longer patent period in the infected group treated with DFO. Francisco et al. (2008), evaluated the effect of the treatment in Swiss mice infected with Y strain of *T. cruzi* with the drug benznidazol (BZ) associated with DFO. The results of this study showed that the treatment with DFO, associated with BZ, was able to enhance BZ efficacy and, therefore, that modification in iron stores increases BZ efficacy.

The reduction of splenic and hepatic parasite burden observed could be related to the effects of DFO on the production of IFN-gamma and NO (cytokine and substance, respectively, directly involved in the control of infection by *L. chagasi* in BALB/c mice). However, the results in the present study do not support this, since no significant differences were observed between the production of IFN-gamma (Fig. 4A) and the production of NO by the splenocytes of treated mice. In addition, no changes were observed in IL-4 (Th2 profile) production in the animals of the treated group (Fig. 4B).

Thus, the results presented in this paper provide clues that iron deficiency may contribute to control the murine infection with *L. chagasi* and that, therefore, may be related to lower parasite levels. However, there must be a critical point at which iron depletion is not so great as to negatively interfere in the functioning of the immune system while at the same time is enough to damage the parasite.

These results are a first step in understanding the relationship between iron deficiency and VL. It is clear that further work will be needed to elucidate other aspects such as the mechanism by which iron deficiency reduces the parasite load in mice infected with *L. chagasi*. Further investigation is also needed into of the influence of iron deficiency on the immune response and the production of other cytokines, such as IL-10 and TNF, which may be altered in iron deficiency conditions and VL?

References

Afonso, L.C.C., Scott, P., 1993. Immune responses associated with the susceptibility of C57BL/10 to *Leishmania amazonensis*. Infection and Immunity 61, 2952–2959.

Arantes, J.M., Pedrosa, M.L., Martins, H.R., Veloso, V.M., Lana, M., Bahia, T., Tafuri, W.L., Carneiro, C.M., 2007. *Trypanosoma cruzi*: treatment with the iron chelator desferrioxamine reduces parasitemia and mortality in experimentally infected mice. Experimental Parasitology 117, 43–50.

Canadian Council on Animal Care, 1980. Guide to the care and use of experimental animals. CCAC, Ottawa.

Canadian Council on Animal Care, 1984. Guide to the care and use of experimental animals. CCAC, Ottawa.

Carvalho, S., Cruz, T., Santarém, N., Castro, H., Costa, V., Tomás, A.M., 2009. Heme as a source of iron to *Leishmania infantum* amastigotes. Acta Tropica 109, 131–135.

Cook, J.D., Baynes, R.D., Skikne, B.S., 1992. Iron deficiency and the measurement of iron status. Nutrition Research Reviews 5, 189–202.
Cook, J., 1999. The nutritional assessment of iron status. Achivos Latinoamericanos de Nutrición 49, 11S–14S.
Das, N.K., Biswas, S., Solanki, S., Mukhopadhyay, C.K., 2009. *Leishmania donovani* depletes labile iron pool to exploit iron uptake capacity of macrophage for its intracellular growth. Cellular Microbiology 11, 83–94.
Francisco, A.F., Vieira, P.M.A., Arantes, J.M., Pedrosa, M.L., Martins, H.R., Silva, M., Veloso, V.M., Lana, M., Bahia, T., Tafuri, W.L., 2008. *Trypanosoma cruzi*: effect of benznidazole therapy combined with the iron chelator desferrioxamine in infected mice. Experimental Parasitology 120, 314–319.
Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S., Tannenbaum, S.R., 1982. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. Analytical Biochemistry 126, 131–138.
Huynh, C., Sacks, D.L., Andrews, N.W., 2006. *Leishmania amazonensis* ZIP family iron transporter is essential for parasite replication within macrophage phagolysosomes. The Journal of Experimental Medicine 203, 2263–2375.
Huynh, C., Andrews, N.W., 2008. Iron acquisition within host cells and the pathogenicity of *Leishmania*. Cellular Microbiology 10, 293–300.
Jacques, I., Andrews, N.W., Huynh, C., 2010. Functional characterization of LIT1, the *Leishmania amazonensis* ferrous iron transporter. Molecular and Biochemical Parasitology 170, 28–36.
Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the folin phenol reagent. Journal of Biological Chemistry 193, 265–275.
Malafaia, G., 2008a. Iron deficiency: resistance or susceptibility against infections? Revista Médica de Minas Gerais 18, 191–196.
Malafaia, G., 2008b. Iron uptake by *Leishmania* parasites. Revista Biociência 4, 41–50.
Marques-Da-Silva, E.A., Coelho, E.A.F., Gomes, D.C.O., Vilela, M.C., Masioli, C.Z., Tavares, C.A.P., Fernandes, A.P., Afonso, L.C.C., Rezende, S.A., 2005. Intramuscular immunization with p36(LACK) DNA vaccine induces IFN-gamma production but does not protect BALB/c mice against *Leishmania chagasi* intravenous challenge. Parasitology Research 9, 67–74.
Marquis, F.F., Gros, P., 2007. Intracellular Leishmania: your iron or mine? Trends in Microbiology 15, 93–95.
Titus, R.G., Marchand, M., Boon, T., Louis, J.A., 1985. A limiting dilution assay for quantifying *Leishmania major* in tissues of infected mice. Parasite Immunology 7, 545–555.
World Health Organization (WHO), 2005. Program for the surveillance and control of leishmaniasis. World Health Organization, Geneva.
World Health Organization (WHO), 2008. Worldwide prevalence of anemia 1993–2005:WHO global data base on anemia. World Health Organization, Geneva.
Wu, A.C., Lesperance, L., Bernstein, H., 2002. Screening for iron deficiency. Pediatrics in Review 23, 171–178.
Yip, R., Dallman, P.H., 1997. Organización Panamericana de la Salud. International Life Sciences Institute. Conocimientos Actuales Sobre Nutrición. OPAS – Publicación Científica, Washington.