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Trypanosoma cruzi: Treatment with the iron chelator desferrioxamine reduces parasitemia and mortality in experimentally infected mice

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Abstract

The effects of prolonged treatment with iron chelator (desferrioxamine) on the development of infection in mice inoculated with Y *Trypanosoma cruzi* were determined. Infected/treated mice presented lower levels of parasitemia and reduced mortality rate compared with infected/non-treated animals. The five out of twenty infected/treated mice that survived the acute phase of infection showed negative hemoculture and positive ELISA in the acute and chronic phases and positive PCR in the acute phase: in the chronic phase, three of the animals presented negative PCR. The single surviving infected/non-treated animal exhibited positive hemoculture, PCR and ELISA in both phases of infection. Infected groups presented lower levels of iron in the liver compared with treated/non-infected or non-treated/non-infected animals. The serum iron levels of the infected/non-treated group were higher on the 21st day post-infection in comparison with control and infected/treated groups. These results suggest that decrease of iron in the host leads to *T. cruzi* infection attenuation.

Index Descriptors and Abbreviations: *Trypanosoma cruzi*; ANOVA, analysis of variance; DNA, deoxyribonucleic acid; DFO, desferrioxamine; d.p.i., day post-infection; ELISA, enzyme-linked immunosorbent assay; EDTA, ethylenediamine tetraacetic acid; i.p., intraperitoneal; LIT, live infusion tryptose medium; PCR, polymerase chain reaction; IT, treatment groups—infected with *T. cruzi* and treated with DFO; INT, infected with *T. cruzi* but not treated with DFO; NIT, DFO-treated/non-infected control; NINT, non-treated/non-infected control; Mice; Iron chelator

1. Introduction

Iron is the most abundant of the heavy metal micronutrients in body fluids and tissues (Crichton and Ward, 1992), and is essential for the continued growth and proliferation of almost all living cells. Moreover, iron homeostasis is fundamental in the regulation of the human immune system (Weinberg, 1984; Kent et al., 1990), affecting both humoral and cellular immunity (Blakley and Hamilton, 1988; Galan et al., 1988). Modulation of the availability of iron represents, therefore, a potential strategy for augmenting host defence levels and restricting the development of disease.

In relation to the immune system, it has been demonstrated that the iron chelator desferrioxamine (DFO) blocks expression of the IL-12 receptor in human T cells, inhibits DNA synthesis through the inactivation of ribonucleotide reductase (Carotenuto et al., 1986), and

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up-regulates the expression of cyclooxygenase-2 and prostaglandin in human macrophages (Tanjii et al., 2001). *In vitro* and *in vivo* studies have demonstrated that various malignant human and experimental animals tumours are sensitive to therapies that include DFO (Wolfe et al., 1988; Hann et al., 1992; DonFrancesco et al., 1996; Richardson, 1997; Wang et al., 1999). DFO also exhibits a significant *in vitro* antiviral effect against cytomegalovirus (Cinatl et al., 1994, 1995; Martelius et al., 1999) and is effective in reducing trauma injuries mediated by free radicals (Shadid et al., 1998).

Parasitic protozoa also depend on iron for their survival, and iron chelators have been employed in various studies aimed to evaluating the relationship between the iron status of the host and the development of infection (Dhur et al., 1989). Studies in human malaria have shown that treatment with DFO, either alone or in combination with standard therapies, enhanced parasite clearance in asymptomatic and in severe malaria (Traore et al., 1991; Gordeuk et al., 1992; Mabeza et al., 1996). DFO has also been shown to inhibit the growth of *Plasmodium falciparum* (Hershko and Peto, 1988) and of bloodstream forms of *Trypanosoma brucei* (Breidbach et al., 2002) and this chelator was considered to be a promising drug against *Toxoplasma gondii* (Mahmoud, 1999).

The first reports of a correlation between iron levels and the development of infection by *Trypanosoma cruzi* were published by Lalonde and Holbein (1984) and Loo and Lalonde (1984). These authors observed that the depletion of iron stores in mice that had been treated with DFO or maintained on an iron-deficient diet, reduced the parasitemia and mortality of the infection. Subsequently, Pedrosa et al. (1990) evaluated the effects of iron deficiency on the evolution of experimental *T. cruzi* infection in mice and observed a strain-dependency. Compared with the control group, mice infected with the YuYu strain developed a less severe form of the disease when treated with DFO at a dose of 10 mg/mouse in the 5th d.p.i., but no differences were observed in mice infected with Y and CL strains. Lima and Villalta (1989) showed that amastigote forms of *T. cruzi* possess receptors for human transferrin, the major iron transport protein in mammalian plasma. It would thus appear that the iron essential for amastigote growth is delivered by receptor-mediated transferrin endocytosis.

Previous studies concerning the effect of DFO on the development of *T. cruzi* infection have typically involved short-term treatments, i.e. up to the 5th (Pedrosa et al., 1990) or 5th and 6th (Lalonde and Holbein, 1984) d.p.i. Considering that DFO is rapidly cleared from the circulation (Brittenham, 1988), it is suggested that a longer period of treatment with this chelator could give rise to a more pronounced effect on the development of *T. cruzi* infection. In order to test this hypothesis, mice were experimentally infected with *T. cruzi* Y strain that not affect the course of infection when used a single dose of DFO (Pedrosa et al., 1990), herein we used a daily treatment with a dose (5 mg/mouse) of DFO from 14 days before infection up until the 14th or 21st d.p.i. The effect of the iron chelator on parasite virulence and host survival was determined during the acute phase of the infection. Our results clarify that this DFO prolonged treatment resulting in powerful protection of infection with *T. cruzi* Y leading attenuation of parasitemia and mortality in infected mice.

2. Materials and methods

2.1. Animals and experimental design

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).

Eighty Swiss male mice, each *ca.* 30 days old, were fed throughout the 45 days of the study on a commercial non-purified diet consisting of Purina Rodent Chow (Purina, São Paulo, Brazil) provided in pellet form. Forty mice received a daily dose (5 mg; 0.05 ml) of DFO (Desferal®, Novartis, Basel, Switzerland) by i.p. injection during a period commencing 14 days prior to infection and continuing up to 14 or 21 d.p.i. The second set of 40 animals received a daily i.p. injection of 0.05 ml of sterile water. On day 14 of the study period, 20 animals that had been receiving the DFO treatment were infected with *T. cruzi* Y strain (Silva and Nussenzweig, 1953) by i.p. injection of 500 bloodstream forms, thus forming the infected/treated (IT) group. Twenty animals that had not received DFO treatment were similarly infected with *T. cruzi* Y strain forming the infected/non-treated (INT) group. The remaining 40 mice were not infected with *T. cruzi* and formed the non-infected/treated (NIT) and the non-infected/non-treated (NINT) groups, respectively.

2.2. Parameters evaluated

Levels of hemoglobin and of iron in the liver and serum were evaluated in mice from all four groups on the 14th and 21st d.p.i. as appropriate. In all infected animals (i.e. those in groups IT and INT), parasitemia was measured on the 4th d.p.i. and daily thereafter according to the method of Brener (1962): the prepatent period, the patent period, the maximum parasitemia, and the day of maximum parasitemia were thus determined. Mortalities were recorded on a daily basis and expressed as a cumulative percentage up to the 32nd d.p.i. In the case of animals that survived the acute phase of the infection, blood samples were collected in the acute (60th d.p.i.) and chronic (240th d.p.i.) phases and submitted to parasitological (hemoculture and PCR) and serological (ELISA) tests.

2.3. Hemoculture

Hemocultures were carried out according to the method of Filardi and Brener (1987). Blood collected from the orbi-

tal sinus vein was inoculated into tubes containing 3 ml of LIT medium (Camargo, 1964). Tubes were maintained at 28 °C and examined for the presence of parasites after 30, 60, 90, and 180 days of incubation.

2.4. PCR amplifications

Blood samples were mixed in a proportion of 1:2 with 6 M guanidine in 0.2 M EDTA (pH 8.0) and stored at room temperature (Ávila et al., 1991). DNA was extracted according to the method of Wincker et al. (1994) as modified by Gomes et al. (1998). PCR amplifications were performed according to Gomes et al. (1998) in 9 µl of reaction mixture containing 10 mM Tris–HCl (pH 9.0), 0.1% Triton X-100, 75 mM KCl, 3.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotide (Sigma, St. Louis, MO, USA), 0.5 U of Platinum Taq DNA polymerase (Invitrogen, São Paulo, Brazil), and 10 pmoles of each oligonucleotide primer (S35 and S36 described by Ávila et al., 1991; Invitrogen). The reaction mixture was added to 2 µl of the sample and overlaid with 30 µl of mineral oil in order to avoid evaporation. Amplifications were performed in an MJ Research (Ramsey, MN, USA) model PTC-150 thermal cycler using the following protocol: initial denaturation step of 5 min at 94 °C, followed by 35 cycles consisting of 1 min denaturation at 95 °C, 1 min annealing at 65 °C and 1 min extension at 72 °C, and a final extension step of 10 min at 72 °C. Amplicons were separated by electrophoresis on 6.0% polyacrylamide gels and revealed by silver staining (Santos et al., 1993). Positive, negative, and reagent controls were processed in parallel with each assay.

2.5. ELISA assay

Serum samples were assayed at 1:80 dilution in phosphate-buffered saline using the technique described by Voller et al. (1976). ELISA assay plates were sensitised with 4.5 µg/ml of *T. cruzi* antigen prepared by alkaline extraction of Y strain parasites in exponential growth phase in LIT medium. Antibody binding was determined with peroxidase-labelled anti-mouse immunoglobulin G (Sigma) at 1:5000 dilution, and absorbance was measured on a Bio-Rad (Hercules, CA, USA) model 3550 spectrophotometer equipped with a 490 nm filter. Positive and negative controls were processed in parallel with each assay. Cut-off values were calculated for each plate as $\overline{X} \pm 2\sigma$, where $\overline{X}$ is the mean absorbance ($n = 10$) of negative control serum and σ is the standard deviation.

2.6. Iron levels in the liver

Liver samples were digested in HNO_3 at 100 °C, evaporated to dryness, and the iron quantified colorimetrically using the orthophenanthroline method of the Association of Official Analytical Chemists (1980). An external iron standard solution of concentration 89.5 µmol/l was employed.

2.7. Iron levels in sera

Serum iron concentrations were determined spectrophotometrically in non-hemolysed serum samples using the Ferrozine® dye-binding method (Labtest Kit catalogue no. 38; Labtest Diagnostica and Bioclin Química Básica, Belo Horizonte, MG, Brazil). An external iron standard solution of concentration 89.5 µmol/l was employed.

2.8. Levels of hemoglobin

Hemoglobin concentrations were determined in blood samples collected from the tails of mice (Henry et al., 1974) immediately prior to infection and on the 14th and 21st d.p.i using Labtest Kit catalogue no. 43: a solution containing 10 g/l of cyanmethemoglobin (Labtest Standard catalogue no. 47) was used as standard.

2.9. Statistical analysis

Statistical analyses of the data were carried out using MINITAB 13 Statistical Software (Minitab, State College, PA, USA). Data were initially assessed by two-way ANOVA: when interactions were significant, the Tukey test was used to determine the specific differences between mean values. The Kolmogorov–Smirnov test was employed to compare parasitemia between treated and not-treated/infected groups. One-way variance analysis or Mann–Whitney U tests were used to compare values of the prepatent period, the patent period and the maximum parasitemia, and the day of maximum parasitemia, between different groups. Values are expressed as mean ± standard deviation: a difference in mean values was considered significant at the $p < 0.05$ level.

3. Results and discussion

3.1. Effect of treatment with DFO on parasitemia and mortality

The parasitemia curves of *T. cruzi* infected mice that had either been treated with DFO (the IT group) or had received no such treatment (the INT group) are shown in Fig. 1. Whilst significant differences were detected in the patent period between the IT and the NIT groups (21 and 11 days, respectively), no differences were observed in the prepatent period (7 days for both groups) or in the day of the peak of parasitemia (10th and 11th d.p.i., respectively). The average parasitemia value in IT mice (274,666 trypomastigotes/0.1 ml of blood) was five times lower than in INT animals (1,397,000 trypomastigotes/0.1 ml of blood) ($p < 0.05$). The cumulative mortality rates for DFO-treated and non-treated mice that had been infected with *T. cruzi* are presented in Fig. 2. It was observed that 67.75% of the animals

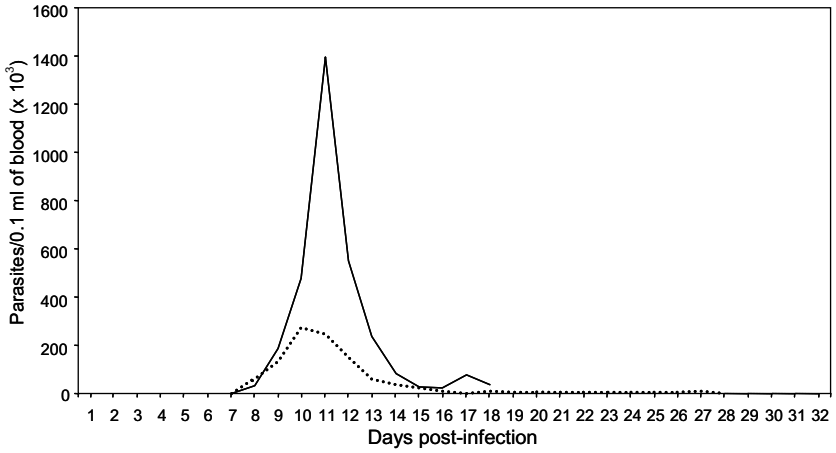

Fig. 1. Parasitaemia curves of Swiss mice that had been inoculated with 500 blood trypomastigotes of *Trypanosoma cruzi* Y strain and had received no treatment (INT —) or had been treated with desferrioxamine (IT ···).

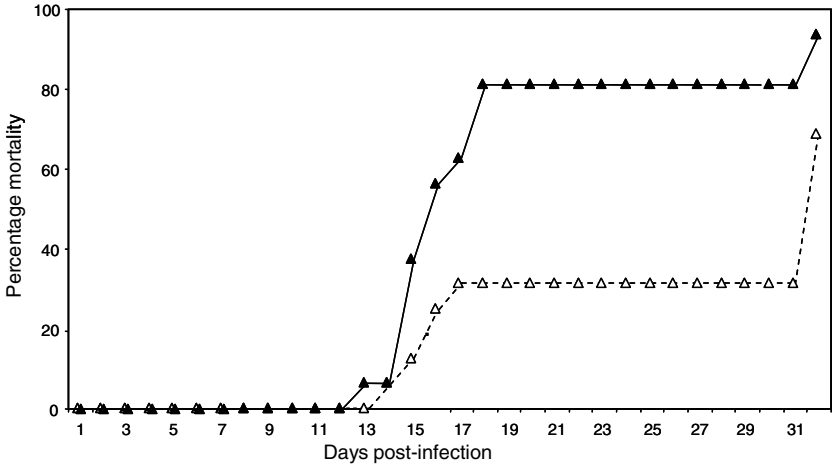

Fig. 2. Percentage mortality of Swiss mice that had been inoculated with 500 blood trypomastigotes of *Trypanosoma cruzi* Y strain and had received no treatment (INT —▲—) or had been treated with desferrioxamine (IT ···△···).

in the IT group died, with mortality commencing on the 14th d.p.i., whilst the death rate in the NIT group was 93.75%, with mortality commencing on the 13th d.p.i.

If the hypothesis that iron is important for the proliferation of *T. cruzi* is valid, then removal of the heavy metal through the use of a chelator could moderate the proliferation of the parasite and hence reduce its levels in the blood. In the present study, a reduction in the parasitemia level, accompanied by an increase in the patent period, of animals in the IT group was observed, indicating that the severity of infection by the Y strain was attenuated by treatment with the iron chelator. This finding is in accord with that of Lalonde and Holbein (1984) who demonstrated that depletion of iron supplies, either by treatment with DFO or by administration of a diet deficient in iron, promoted the reduction of mortality in mice infected with *T. cruzi.* The demonstration that parasite reproduction is negatively correlated with the level of iron in the plasma would appear to support the original hypothesis. In contrast to the results outlined above, Pedrosa et al. (1990) reported that *T. cruzi* Y strain was not sensitive to DFO treatment, and that an effect on parasite replication could only be observed when mice were fed on an iron-free diet. The authors explained this result in terms of the more rapid multiplication rate of the Y strain compared with the YuYu strain, which permitted earlier attainment of the parasitemia peak. However, the inoculum used in this study (i.e. 1400 trypomastigotes forms/animal) and principal the DFO treatment administered (one dose of DFO on the 5th d.p.i.) were very different from those presently employed and could explain the divergence in the results obtained.

The beneficial effects of DFO treatment have not only been observed with respect to parasite infections. Weinberg (1994) demonstrated the *in vivo* use of the chelator against *Pneumocystis carinii* by establishing that daily injections of the drug reduced the intensity of infection in rats and mice and produced alterations in the morphology. These authors also demonstrated that daily administration of DFO led to a more pronounced reduction in the replication of trophozoites compared with weekly i.p. injections.

3.2. Effect of treatment with DFO on hemoculture, PCR, and ELISA assays

The results of the parasitological and serological tests are displayed in Table 1. All of the *T. cruzi* infected mice that had been treated with DFO (IT group) presented negative hemoculture, while the infected/non-treated (INT) group exhibited positive hemoculture in the acute and chronic phases of infection. This result was surprising since Filardi and Brener (1987) had previously found that 50% of animals infected with *T. cruzi* Y strain and treated with benznidazole presented positive hemoculture, while the untreated group exhibited 100% positive hemoculture.

In order to determine if infection was still present in an animal, both PCR and ELISA assays were also performed in parallel. Only one mouse from the INT group survived the infection and this animal presented, PCR- and ELISA-positive in both the acute and chronic phases. All of the mice in the IT group that survived the acute infection ($n = 5$) were PCR- and ELISA-positive in the acute phase, whilst in the chronic phase, three animals were PCR-negative although all 5 remained ELISA-positive. Considering the higher sensitivity of the PCR assay and that ELISA serology can remain positive for a considerable period following effective and specific etiological treatment (Andrade et al., 1991a,b), these results suggest that treatment with DFO could have eliminated the infection in these animals (Miyamoto et al., 2006), however other evaluated have been necessary of this affirmation. The lower frequencies of hemoculture- and PCR-positive results, tests commonly positive in infected mice, taken together with the significantly reduced parasitemia curve and decreased mortality rate recorded in the IT animals, indicates that DFO treatment certainly plays an important protective role during the course of the infection.

3.3. Effect of treatment with DFO on iron levels in the liver

Significantly ($p < 0.05$) lower concentrations of iron were detected on the 14th and 21st d.p.i in the liver of infected mice (IT and INT groups) in comparison with non-infected animals (NIT and NINT groups) (Fig. 3). Pedrosa et al. (1990) reported that treatment of mice with DFO led to a reduction in the iron concentration in the liver of animals infected with *T. cruzi* Y and CL strains, together with a reduction in iron concentration in the spleen of those infected with the Y strain. These authors further established that the levels of iron in the liver and spleen of germfree mice remained unaltered when DFO was administered 15 days post *T. cruzi* infection (Pedrosa et al., 1993). Moreover, in a study of the effect of iron on *T. cruzi* infection in mice, Lalonde and Holbein (1984) demonstrated a reduction in the iron supplies (48% in the liver and 15% in the spleen) of non-infected animals following treatment with DFO or the administration of an iron deficient diet.

According to Dallman (1986), iron deficiency in an organism occurs in three overlapping stages. The first stage is characterised by a reduction in the concentration of serum ferritin, reflecting a loss in the supply of iron in the spleen, liver and bone marrow. The second stage is associated with an increasing capacity of iron linkage and a reduction in the concentration of serum iron, whilst in the final stage the concentrations of hemoglobin and hematocrit decrease and the iron reserves in the spleen and liver became exhausted. In the present study, the reduced concentration of iron found in the liver of infected mice on the 14th and 21st d.p.i. was probably associated with the regulation of iron within the organism as a response to infection by *T. cruzi*.

3.4. Effect of treatment with DFO on iron levels in sera

Significantly ($p < 0.05$) higher concentrations of iron were detected on the 21st d.p.i in serum samples of infected/non-treated mice (INT group; mean value 273.47 μg/dl) in comparison with the NINT (mean value 100.9 μg/dl) and IT (mean value 119.28 μg/dl) groups (Fig. 4). Whilst iron levels in the liver of infected animals (IT and INT groups) had decreased on 14th d.p.i., the serum iron levels remained normal and increased serum concentrations could only be observed on the 21st d.p.i.

Table 1
Results of hemoculture, PCR and ELISA assays conducted during the acute (60th d.p.i.) and chronic (240th d.p.i.) phases of infection in mice that had survived inoculation with *Trypanosoma cruzi* Y strain

Treatment	Animal number	Assays[a]					
		Hemoculture		PCR		ELISA	
		Acute phase	Chronic phase	Acute phase	Chronic phase	Acute phase	Chronic phase
	1	−	−	+	−	+	+
	2	−	−	+	−	+	+
IT[b]	3	−	−	+	+	+	+
	4	−	−	+	−	+	+
	5	−	−	+	+	+	+
INT[c]	6	+	+	+	+	+	+

[a] Assay results shown as positive (+) or negative (−).
[b] IT—animals infected with *T. cruzi* and treated with DFO.
[c] INT—animals infected with *T. cruzi* but not treated with DFO.

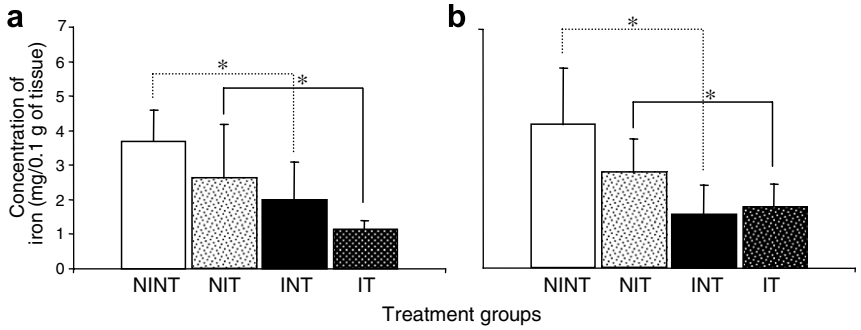

Fig. 3. Levels of iron, measured (a) on 14th d.p.i and (b) on 21 d.p.i, in the liver of Swiss mice that had been inoculated with 500 blood trypomastigotes of *Trypanosoma cruzi* Y strain and had received no treatment (INT) or had been treated with desferrioxamine (IT), together with the corresponding non-infected control groups (NINT and NIT, respectively). Values shown are means ± SD ($n = 20$). Results of two-way ANOVA-infection $^{*}p < 0.05$; treatment: NS; iron *x* treatment: NS, both 14 and 21 days.

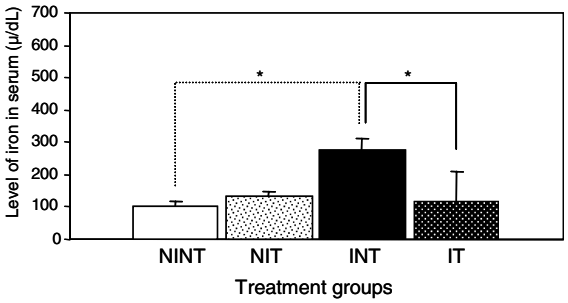

Fig. 4. Levels of iron, measured on 21 d.p.i, in serum of Swiss mice that had been inoculated with 500 blood trypomastigotes of *Trypanosoma cruzi* Y strain and had received no treatment (INT) or had been treated with desferrioxamine (IT), together with the corresponding non-infected control groups (NINT and NIT, respectively). Values shown are means ± SD ($n = 12$). Results of two-way ANOVA-infection $^{*}p < 0.05$; treatment: NS; iron *x* treatment: NS, both 14 and 21 days.

It appears that some parasite forms provoke a large release of iron from the liver, leading subsequently to an increase in the level of the heavy metal in the serum of infected animals (IT and INT). The lower serum iron levels in the IT group compared with the INT group would then be correlated with the chelation of plasma iron by DFO.

Letendre (1985) observed that during infection there was a decrease in the levels of circulating iron, occasioned by its diminished release from the mononuclear phagocyte system, which restricted the amount of iron available to extracellular parasites. However, the beneficial aspects of this process are questionable in the case of intracellular parasites such as *T. cruzi* that lodge in the mononuclear phagocyte cells of the endothelial reticulum system. In the study conducted by Pedrosa et al. (1990), a reduction of serum iron in mice treated with DFO and infected with *T. cruzi* strains Y, CL and YuYu could not be confirmed. Furthermore, Lalonde and Holbein (1984) demonstrated that no significant changes occurred in the serum iron levels of non-infected mice that had been treated with DFO, while treated/*T. cruzi* infected animals presented iron supplies that were sufficient to maintain a normal immune response. It is important to note, however, that the protocols regarding inoculum and DFO administration used by these authors were different from those employed in the present work.

A reduction in serum iron concentration was not observed in the present study presumably because amastigote forms of the intracellular parasite, established in the cells of the mononuclear phagocyte system but unable to obtain the necessary iron for survival, stimulated a release of heavy metal into the serum. Iron present in the serum would then bind to transferrin and become available to the intracellular parasite by receptor-mediated endocytosis.

3.5. Effect of treatment with DFO on hemoglobin levels

Significant ($p < 0.05$) differences were observed on the 21st d.p.i. in the hemoglobin levels between the IT (7.14 µg/dl) and NIT (10.9 µg/dl) groups, but no significant differences were observed between the other groups (Fig. 5).

According to Bothwell et al. (1979), the primary iron pool in the blood of a vertebrate host is associated with hemoglobin, and hemoglobin levels are commonly employed in order to evaluate iron deficiency in hosts that have been submitted to iron deficient diets, or to infection,

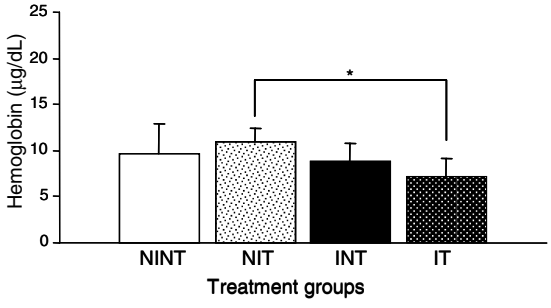

Fig. 5. Hemoglobinemia, determined on 21 d.p.i, of Swiss mice that had been inoculated with 500 blood trypomastigotes of *Trypanosoma cruzi* Y strain and had received no treatment (INT) or had been treated with desferrioxamine (IT), together with the corresponding non-infected control groups (NINT and NIT, respectively). Values shown are means ± SD ($n = 24$). Results of two-way ANOVA-infection $^{*}p < 0.05$; treatment: NS; iron *x* treatment: NS, both 14 and 21 days.

or to treatments that trigger alterations in iron levels. Changes in hemoglobin levels have been reported in various trypanosome infections (Esievo et al., 1982; Igbokwe and Anosa, 1989), including infection by *T. cruzi,* and are associated with a reduction in the number of platelets (Cardoso and Brener, 1980; Ruiz et al., 1989), erythrocytes (Cardoso and Brener, 1980) and hematocrit values (Lalonde and Holbein, 1984). Moreover, Marcondes et al. (2000) observed that the acute phase of experimental infection by *T. cruzi* is characterised by anaemia, thrombocytopenia, leucopoenia and bone marrow hypoplasia. However, the mechanisms responsible for these hematological alterations are not fully understood.

Pedrosa et al. (1990) evaluated iron deficiency in mice and correlated the effect of this deficiency with the evolution of Chagas' disease. The authors showed that the hypohemoglobinemia presented by the host was permanent in animals fed on an iron-free diet, probably because the low supplies of the heavy metal were not enough to compensate for the erythropoiesis that follows anaemia. In animals infected with CL and Y strain and treated with DFO, the haemoglobin levels recovered suggesting that this treatment does not interfere with the supplies required for erythropoiesis. Hence, the reduction in hemoglobin levels observed in the IT group compared with the NIT group may be explained by the presence of infection by *T. cruzi* in association with the action of DFO in the dose employed in the present work.

The data reported in this study suggest that the prolonged treatment with DFO of *T. cruzi* infected mice reduces the availability of iron to the infecting parasites leading to a reduction in parasitemia and mortality. Further study of this system could be of significant value in understanding the effect of iron reduction during *T. cruzi* infection.

Acknowledgments

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