Effects of cyclooxygenase inhibitors on parasite burden, anemia and oxidative stress in murine Trypanosoma cruzi infection

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Keywords
Trypanosoma cruzi; Chagas’ disease; cyclooxygenase; anemia; oxidative stress; nitric oxide.

Abstract
Prostaglandins are known to be produced by macrophages when challenged with Trypanosoma cruzi, the etiological agent of Chagas’ disease. It is not known whether these lipid mediators play a role in oxidative stress in host defenses against this important protozoan parasite. In this study, we demonstrated that inducible cyclooxygenase-mediated prostaglandin production is a key chemical mediator in the control of parasite burden and erythrocyte oxidative stress during T. cruzi infection in C57BL/6 and BALB/c mice, prototype hosts for the study of resistance and susceptibility in murine Chagas’ disease. The results suggested the existence of at least two mechanisms of oxidative stress, dependent or independent with regard to the nitric oxide and cyclooxygenase pathway, where one or the other is more evident depending on the mouse strain.

Introduction
Chagas’ disease is caused by infection with Trypanosoma (Schizotrypanum) cruzi. Murine experimental infection with T. cruzi is associated with severe hematological changes, including thrombocytopenia (Cardoso & Brener, 1980), and neutropenia followed by neutrophilia and eosinophilia (Repká et al., 1985), which may contribute to mortality. Similar hematological alterations have also been described in experimental African trypanosomiasis (Ikede et al., 1977) and are a common characteristic of HIV infection (Claster, 2002) and malaria (Paul & Brey, 2003). Marcondes et al. (2000) reported that experimental acute T. cruzi infection is associated with anemia, thrombocytopenia, leukopenia, and bone marrow hypoplasia and that these alterations can be prevented by nifurtimox (an antitrypanosomal drug) treatment.

The mechanisms responsible for these hematological alterations are not clearly understood. The previous studies revealed that NO does not play a direct role in the development of anemia during T. cruzi infection, but contribute together with TNF-β to oxidative prehemolytic damage of erythrocytes in infected mice (Malvezi et al., 2004). Tribulatti et al. (2005) demonstrated that trans-sialidase (TS) of T. cruzi depletes sialic acid in platelets, increasing its clearance and leading afterwards to thrombocytopenia observed during the acute phase of infection. In addition, it has been shown that IFN-inducible p47GTPase (LRG-47) influences T. cruzi control by simultaneously regulating macrophage microbicidal activity and hemopoietic function (Santiago et al., 2005).

The involvement of cyclooxygenase-mediated prostaglandin production in oxidative stress associated with anemia in early T. cruzi infection has not been examined previously, and the results of studies of its role in the control of parasitism and resistance in T. cruzi infection are discrepant (Celentano et al., 1995; Pinge-Filho et al., 1999; Freire-de-Lima et al., 2000; Michelin et al., 2005).

The aim of this study was to compare the in vivo inhibition of cyclooxygenase-1 (COX-1) and cyclooxygenase-2...
(COX-2) by nonsteroidal antiinflammatory drugs (NSAIDs) with regard to parasitism, resistance, anemia, erythrocyte oxidative damage and **NO production in C57BL/6 and BALB/c mice infected with *T. cruzi* (Y strain). Increased **NO production in C57BL/6 strain, and anemia and increased oxidative stress in erythrocytes in both mouse strains was observed. In C57BL/6 mice, the inhibition of COX reduced survival and oxidative stress in erythrocytes, and increased parasite burden. In BALB/c mice, there was no reduction in oxidative stress with inhibition of COX, but reduced survival and increased parasite burden were observed with COX-2 inhibition.

**Materials and methods**

This study was reviewed and approved by the Internal Scientific Committee and the Ethics in Animal Experimentation Committee of Londrina State University, Londrina, Brazil (Process no 28568/05, CEEA 54/05).

**Mice and reagents**

C57BL/6 and BALB/c male mice were obtained from the mouse breeding facilities of the Department of Immunology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil. Swiss mice were obtained from the breeding colonies of the animal facility of the Center for Biological Sciences at Londrina State University, Londrina, Brazil. Eight- to twelve-week-old male mice was used in all experiments. Mice were maintained under standard conditions in the animal facility in the Department of Pathological Sciences, Center for Biological Sciences, Londrina State University. Commercial rodent diet (Nuvilab-CR1, Nuvital, Campo Mourão, Brazil) and sterilized deionized water were available *ad libitum*. All procedures with the animals were in accordance with the guidelines of the Brazilian Code for the Use of Laboratory Animals.

Indomethacin (1-[p-chlorobenzoyl]-5-methoxy-2-methylindole-3-acetic acid, C19H16CINO4, Sigma, St Louis) was dissolved in absolute ethanol and diluted in RPMI 1640 medium (Roswell Park Memorial Institute, GIBCO BRL) without serum. Separate experiments indicated that 0.005% ethanol did not alter responses in normal mice. Aspirin (2-acetoxybenzoic acid, C9H8O4, Biotec, Brazil) and celecoxib ([4]-fluro-[1H-pyrazol-1-yl]benzenesulfonamide, C17H14F3N3O2S, Pfizer Pharmaceuticals) were dissolved in phosphate-buffered saline (PBS, pH 7.2). All reagents were prepared fresh daily and aliquots were stored at 4 °C until used. Buffers and solutions were prepared in deionized water.

**Trypanosoma cruzi infection**

The Y strain of *T. cruzi* (Silva & Nussenweig, 1953) was maintained by weekly intraperitoneal inoculation of Swiss mice with 2 × 10⁵ blood trypomastigote forms. Infective blood trypomastigotes were obtained from *T. cruzi*-infected mice by drawing blood via cardiac puncture following anesthetization. Motile blood forms were counted and the desired number of parasites (5 × 10⁵) was injected intraperitoneally in C57BL/6 and BALB/c mice. Parasitemia was assessed by counting circulating parasites in 5 µL of blood obtained from the tail vein of infected mice. These data were expressed as the number of parasites mL⁻¹ blood. Parasitemia and survival rates were determined daily, beginning on the 5th day of infection (Brener, 1962).

**Treatment of mice with cyclooxygenase inhibitors (NSAIDs)**

Indomethacin (1.2 mg kg⁻¹), aspirin (50 mg kg⁻¹) and celecoxib (50 mg kg⁻¹) were injected intraperitoneally in mice daily. Groups of six to 10 mice were used. C57BL/6 and BALB/c mice received the first dose 4 h after infection and were treated until they died; parasitemia and mortality were determined in the same period. Untreated *T. cruzi*-infected mice were used as controls. In order to evaluate drug toxicity, normal mice were inoculated only with indomethacin, aspirin or celecoxib for up to 30 days, using the same treatment schedule. The dose of NSAIDs chosen for these experiments was based on studies published previously (Celentano et al., 1995; Pinge-Filho et al., 1999; Freire-de-Lima et al., 2000; Narko et al., 2005).

**Hematological methods**

Erythrocytes and leukocytes from normal and infected mice under ether anesthesia were collected by cardiac puncture with heparinized needles and syringes and counted by standard methods (Dacie & Lewis, 1994). Hemoglobin concentration was determined by the Drabkin method. Hematocrits were obtained by microcentrifugation of capillary tubes filled with heparinized blood (Bain, 1997). For enumerating reticulocytes, 40 µL of heparinized blood were incubated with 20 µL of brilliant cresyl blue for 20 min at 37 °C, and thin blood smears were then prepared on glass slides. After the blood smears were air dried, reticulocytes were counted by light microscopy. All blood analysis and cell counts were performed 12 days postinfection. Platelets were counted in peripheral blood from anesthetized normal and infected mice, which was added to polypropylene tubes containing 3.8% (w/v) sodium citrate (citrate/blood ratio, 1 : 9) (Tribulatti et al., 2005). All the manipulations were carried out at room temperature. The number of platelets and blood cells was determined by manual counting with a Neubauer hemocytometer.

**Bone marrow cell harvest**

Bone marrow cells were harvested by flushing the femoral shafts with ice-cold PBS, as described previously (Tadokoro & Abrahamsohn, 2001). The total number of cells collected...
was determined by manual hemocytometer count. For differential counts, cell suspensions from uninfected and Day 12-infected mice were deposited on a glass slide, stained with May Grünwald–Giemsa, and then counted by light microscopy.

**Histopathological analysis**

On Day 12 of infection, mice treated or not with NSAIDs were sacrificed. The heart was removed, fixed in 10% buffered formalin, and then sectioned. Sections were paraffin embedded, stained with hematoxylin/eosin (H&E), and then examined by light microscopy. The number of parasite nests was counted in 50 microscope fields at ×400 magnification for each tissue section. The mean count was determined for three sections.

**Macrophage cultures and quantification of nitrite (NO₂⁻)**

Peritoneal cells (PC) were prepared from normal and infected (Day 12 postinfection) mice treated or not with NSAIDs. Four days after intraperitoneal injection of 1.0 mL of 5% thioglycollate medium in BALB/c and C57BL/6 mice, cells were harvested from the peritoneal cavity by flushing with cold PBS, centrifuged at 500 × g for 10 min at 4 °C, and resuspended in RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS, Cripton Biotecnologia, Brazil), 50 mg mL⁻¹ gentamicin, 100 μg mL⁻¹ penicillin, 100 μg mL⁻¹ streptomycin and 2 mM glutamine. The PC suspension was dispensed into 48-well tissue culture plates (10⁶ cells mL⁻¹) and the cells were allowed to adhere to the plastic surface for 4 h, followed by washing with warm PBS to remove nonadherent cells. For each experiment, PCs from three mice were pooled.

Macrophage monolayers from C57BL/6 and BALB/c mice were incubated in the presence of aspirin (10 mM), indomethacin (10 μM) or celecoxib (10 mM) (Mitchell et al., 1994; Matsuda et al., 2006). Nitrite (NO₂⁻) accumulation in 24-h supernatants of cultured cells was used as an indicator of NO production and was determined by the Griess reaction with sodium nitrite as a standard, as described previously (detection limit: 1.56 μM) (Stuehr & Nathan, 1989). Fifty microliters of supernatant were incubated for 10 min, in the dark and at room temperature, with an equal volume of Griess reagent (1% sulfanilamide, 0.1% N-1-naphthylethenediamine dihydrochloride, 2.5% phosphoric acid). The absorbance was read at 540 nm.

**Evaluation of oxidative stress**

**Determination of oxygen uptake and induction time (Tind) in erythrocytes**

Heparinized blood samples from noninfected and infected mice (Day 12 postinfection) were used for erythrocyte oxidative stress determinations. After removal of the plasma and white cells from whole blood, the remaining erythrocytes were washed twice with a 10 mM sodium PBS, pH 7.4, and then resuspended in the same buffer (1:99, v/v). Both tert-butyl hydroperoxide (2 mM, t-BHT)-induced oxygen uptake and induction time were measured with a Clark-type oxygen electrode at 37 °C (Lissi et al., 1986). The induction time is directly related to the intracellular protective antioxidative capacity, while oxygen uptake is an indirect measure of the susceptibility of erythrocyte membranes to lipid peroxidation elicited by t-BHT (Bainy et al., 1996).

**Tert-butyl hydroperoxide-induced chemiluminescence**

Heparinized mouse blood erythrocytes from different experimental groups were pelleted by centrifugation (800 g, 10 min) at 25 °C and then washed twice with saline. A 1% erythrocyte suspension was prepared with 150 mM NaCl, 10 mM sodium phosphate at the moment of use. The chemiluminescence reaction was initiated by the addition of 20 μL tert-butyl hydroperoxide at a final concentration of 0.6 mM in 1 mL (Gonzales-Flecha et al., 1991; Casado et al., 2007). Chemiluminescence was measured in a Luminometer TD20/20 (Turner Biosystems, Sunnyvale, CA). The results were expressed as Relative Light Unit (RLU mg⁻¹ protein). The entire curve was used as the relative amount of lipid hydroperoxide pre-existing in erythrocyte membranes. Protein concentration was determination by the method of Lowry et al. (1951) modified by Miller (1959).

**Statistical analysis**

Arithmetic means (parasitemia, amastigote nests) and SD of means were calculated. The impact of infection and other treatments were determined by one-way ANOVA. The chemiluminescence curves profile was determined by one-way ANOVA. For profile comparison between chemiluminescence curves was used two-way ANOVA. Survival curves were compared using the Mantel–Haenszel log rank test. Differences were considered statistically significant when \( P < 0.05 \). All statistical analyses were performed using GRAPHPAD PRISM version 4.0 (GRAPHPAD Software, San Diego, CA).

**Results**

**Effects of daily treatment with cyclooxygenase inhibitors on parasitemia, and survival in infected mice**

To address the question of the involvement of prostaglandins in protection during the acute phase of *T. cruzi* Y strain, two murine models of Chagas’ disease were used. They vary
with regard to resistance (C57BL/6) and susceptibility (BALB/c).

Figure 1 shows the parasitemia and the survival rate of C57BL/6 and BALB/c mice infected with T. cruzi, treated or not with different NSAIDs. BALB/c infected with T. cruzi Y strain, were more susceptible than C57BL/6, reaching higher levels of parasitemia and mortality. Parasitic load in the blood peaked 9 days postinfection, which was significantly higher in BALB/c compared with C57BL/6 mice, and declined progressively thereafter. There were statistically significant differences ($P < 0.001$) in survival between the two strains of mice studied (Fig. 1c and d). All of the untreated C57BL/6 mice survived the infection, while all of the untreated BALB/c mice died 26 days postinfection. The treatment of infected C57BL/6 mice with aspirin or celecoxib dramatically increased the blood parasite load starting from the 7th or 9th day of infection, respectively (Fig. 1a, b). BALB/c mice infected with NSAIDs, at 12 days after infection with $T. cruzi$. The treatment of infected BALB/c mice with celecoxib dramatically increased the blood parasite load starting from the 13th day of infection (Fig. 1b, $P < 0.05$). Aspirin and indomethacin treatment showed no significant differences in parasitemia on Days 7, 14 and 21 postinfection. All BALB/c mice infected and treated with celecoxib died on the 18th day (Fig. 1d). BALB/c mice treated with aspirin or indomethacin died from the 22nd day of infection. The mean survival time was a little shorter in the BALB/c mice treated with celecoxib (Fig. 1d, $P < 0.001$).

In addition, we examined the effect of COX inhibition on parasitism in heart tissue taken from C57BL/6 and BALB/c mice treated with NSAIDs, at 12 days after infection with T. cruzi.

On Day 12 postinfection, the heart tissue from C57BL/6 mice treated with celecoxib had threefold more parasite nests than those of untreated mice and those treated with aspirin had twofold more parasites nests than untreated. (Fig. 1e, $P < 0.05$). We observed tendency for an increased number of parasite nests in the heart of infected mice treated with aspirin or celecoxib was half and the same as that in untreated C57BL/6 mice, respectively (Day 30 postinfection), and the mean survival time was a little longer in the C57BL/6 mice treated with celecoxib (Fig. 1c, $P < 0.001$).

The difference in the parasitemia and survival rates was significant ($P < 0.05$ and $P < 0.001$, respectively). *The difference in the parasitemia and survival rates was significant ($P < 0.05$ and $P < 0.001$, respectively). *t, all animals died.

**Fig. 1.** Effect of cyclooxygenase inhibitors in the evolution of *Trypanosoma cruzi* infection in C57BL/6 and BALB/c mice. Parasitemia (a and b), survival rates (c and d) and number of amastigote nests of cardiac sections (e and f). Groups of ten mice were infected with $5 \times 10^3$ trypomastigote of $T. cruzi$ (Y strain) and treated with indomethacin (1.2 mg kg$^{-1}$), aspirin (50 mg kg$^{-1}$) and celecoxib (50 mg kg$^{-1}$). Mice received the first dose 4 h after infection and were treated until they died. Untreated $T. cruzi$-infected mice were used as controls. *The difference in the parasitemia and survival rates was significant ($P < 0.05$ and $P < 0.001$, respectively). **t, all animals died.
with indomethacin (Fig. 1e, P > 0.05). In the same infection period, BALB/c treated with celecoxib, aspirin and indomethacin had more parasite nests compared with untreated mice (Fig. 1f, P < 0.05).

**Effects of COX-1 and COX-2 inhibition on anemia in T. cruzi-infected mice**

Blood cell counts was conducted and several hematological parameters was measured in uninfected and T. cruzi-infected mice treated or not with NSAIDs, at 12 days postinfection. Based on significant decreases in hemoglobin, hematocrit and erythrocyte count, both mouse strains studied suffered from anemia by the 12th day postinfection (Table 1). The severity of the anemia was greater in C57BL/6 mice. Interestingly, the inhibition of endogenous prostaglandin production did not impact the extent of anemia that developed 12 days postinfection in the two mouse strains (Table 1). *Trypanosoma cruzi* infection in BALB/c and C57BL/6 mice was associated with a significant decrease in total number of bone marrow cells (Table 1). COX-1 and COX-2 inhibition had no effect on bone marrow hypoplasia in either strain of *T. cruzi*-infected mice (Table 1).

**COX-2 inhibition with celecoxib treatment attenuates thrombocytopenia and leukopenia that develop in C57BL/6 infected with T. cruzi**

Because thrombocytopenia and leukopenia are associated with *T. cruzi* infection, the authors asked whether these changes occur in *T. cruzi*-infected mice treated with inhibitors of prostaglandin production. As shown in Fig. 2, platelet and white cell counts indicated thrombocytopenia and leukopenia in both infected mouse strains. Interestingly, only the treatment of infected C57BL/6 mice with celecoxib (selective COX-2 inhibitor) was able to attenuate the thrombocytopenia (Fig. 2a, P < 0.05 when compared with infected group) and leukopenia (Fig. 2b, P < 0.05) observed on the 12th day of infection. None of the treatments with different NSAIDs had an effect on the thrombocytopenia and leukopenia that occurred in BALB/c mice infected with *T. cruzi* (Fig. 2b, P > 0.05 when compared with infected group).

In addition, a significant increase was noted in reticulocytes present in both the C57BL/6 and BALB/c mice (Fig. 2c and f). Only the treatment of C57BL/6 mice with celecoxib increased the percentage of reticulocytes in *T. cruzi*-infected mice (Day 12 postinfection, Fig. 2c, P < 0.05). Celecoxib and indomethacin but not aspirin treatment of BALB/c mice significantly increased the reticulocytosis associated with *T. cruzi*-infection in this susceptible strain of mice (Fig. 2f, P < 0.05).

**Effects of in vivo COX inhibition on differential production of •NO in macrophages from T. cruzi-infected mice**

•NO production was measured to determine whether *in vivo* treatment of infected C57BL/6 and BALB/c mice with NSAIDs could alter the differential production of •NO by C57BL/6 (M-1) and BALB/c (M-2) macrophages during *T. cruzi* infection. C57BL/6 (M-1) macrophages are far more sensitive to the stimulus of IFN-γ plus lipopolysaccharide for the production of NO than are BALB/c (M-2) cells, a feature that might also account for resistance in *T. cruzi* infected mice (Santos et al., 2006). It was observed that macrophages from BALB/c responded poorly to infection with *T. cruzi* with regard to spontaneous production of •NO compared with

### Table 1. Hematological values and bone marrow cells counts¹ in Trypanosoma cruzi-infected mice**

<table>
<thead>
<tr>
<th>Group</th>
<th>Hemoglobin (g.dL⁻¹)</th>
<th>Hematocrit (%)</th>
<th>Erythrocytes (x 10⁹mL⁻¹)</th>
<th>Bone marrow cells (x 10⁵mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>14.4 ± 0.4</td>
<td>45.0 ± 0.6</td>
<td>7.1 ± 0.3</td>
<td>5200 ± 1110</td>
</tr>
<tr>
<td>I</td>
<td>12.0 ± 1.0*</td>
<td>29.8 ± 2.2*</td>
<td>5.6 ± 0.7*</td>
<td>3560 ± 1370*</td>
</tr>
<tr>
<td>I+Celecoxib</td>
<td>10.5 ± 2.0*</td>
<td>34.0 ± 5.0*</td>
<td>6.1 ± 0.3*</td>
<td>3240 ± 1170*</td>
</tr>
<tr>
<td>I+Aspirin</td>
<td>10.3 ± 1.0*</td>
<td>32.8 ± 1.6*</td>
<td>5.9 ± 0.2*</td>
<td>2790 ± 790*</td>
</tr>
<tr>
<td>I+Indomethacin</td>
<td>10.3 ± 1.0*</td>
<td>32.7 ± 3.2*</td>
<td>5.8 ± 0.4*</td>
<td>2630 ± 730*</td>
</tr>
<tr>
<td>BALB/c</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>14.5 ± 1.4</td>
<td>44.6 ± 2.2</td>
<td>8.0 ± 0.1</td>
<td>4720 ± 830</td>
</tr>
<tr>
<td>I</td>
<td>12.0 ± 1.5*</td>
<td>35.6 ± 2.0*</td>
<td>5.5 ± 0.2*</td>
<td>2660 ± 1370*</td>
</tr>
<tr>
<td>I+Celecoxib</td>
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<td>34.7 ± 1.5*</td>
<td>6.6 ± 0.1*</td>
<td>2150 ± 660*</td>
</tr>
<tr>
<td>I+Aspirin</td>
<td>10.0 ± 1.1*</td>
<td>31.8 ± 2.9*</td>
<td>5.3 ± 0.3*</td>
<td>2740 ± 890*</td>
</tr>
<tr>
<td>I+Indomethacin</td>
<td>10.2 ± 1.6*</td>
<td>33.4 ± 4.0*</td>
<td>6.1 ± 0.4*</td>
<td>2060 ± 210*</td>
</tr>
</tbody>
</table>

*Significantly different (P < 0.05) when compared with noninfected group. The treatment with NSAIDs did not effect on anemia or on bone marrow hypoplasia in either strain of *T. cruzi*-infected mice (P > 0.05).
**Groups of mice were infected (I) with 5 × 10⁹ *T. cruzi* or infected and treated with celecoxib, aspirin or indomethacin. Normal mice (N). Values represent the mean ± SD and are representative of two independent experiments, using five to 12 mice per group.
¹Day 12 after *T. cruzi* infection.
macrophages from C57BL/6 (Fig. 3). In C57BL/6 mice on the 12th day of infection, aspirin treatment caused a decrease in NO production by M-1 macrophages, while celecoxib treatment increased NO production in these cells (Fig. 3a, P < 0.05). Indomethacin treatment had no effect on NO production in M-1 (Fig. 3a, P > 0.05), when compared with the production of NO in M-1 from infected and untreated mice. In parallel, we found that all NSAID treatments studied increased NO production in M-2 macrophages when compared with untreated infected mice (Fig. 3b, P < 0.05).

**Erythrocyte oxidative stress**

Oxygen uptake by erythrocytes is directly associated with the susceptibility of the erythrocyte membrane to undergo lipid peroxidation elicited by t-BHP and is proportional to previous oxidative stress experienced by the erythrocytes in vivo. Oxygen uptake by erythrocytes from C57BL/6 and BALB/c mice was significantly increased 12 days following infection (Fig. 4, P < 0.05). The treatment with the NSAIDs used (celecoxib and aspirin) significantly diminished erythrocyte oxygen uptake in both strains of infected mice (Fig. 4a and c, P < 0.05).

Induction time (T\text{ind}) is directly related to the intracellular protective antioxidant capacity of erythrocytes. *Trypanosoma cruzi* infection in C57BL/6 and BALB/c mice resulted in a significant reduction in T\text{ind} (Fig. 4b and d, P < 0.05). Treatment of C57BL/6 mice with NSAIDs completely abrogated the infection-associated decrease in T\text{ind} (Fig. 4b, P < 0.05). Prior to infection, T\text{ind} was significantly longer in erythrocytes isolated from BALB/c mice compared with C57BL/6 mice. Only the treatment with celecoxib protected against the reduction of antioxidant capacity of BALB/c erythrocytes on Day 12 postinfection (Fig. 4d, P < 0.05).

Tert-butyl hydroperoxide-induced chemiluminescence was used to determine the integrity of nonenzymatic antioxidant defenses and the levels of lipoperoxides in erythrocytes of mice subjected to *T. cruzi* infection. Figure 5a shows
Prostaglandin is a key mediator in T. cruzi infection

![Fig. 3. NO₂ accumulation in cultures of macrophages from Trypanosoma cruzi infected-mice (Day 12 after infection). Macrophage monolayers (2 x 10⁵ cells 200 μL⁻¹) from C57BL/6 (M-1) and BALB/c (M-2), mice were incubated at 37 °C in 5% CO₂ in the presence or absence of indomethacin (1.2 mg kg⁻¹), aspirin (50 mg kg⁻¹) or celecoxib (C) (50 mg kg⁻¹) during 12 days. After 24 h the spontaneous accumulated NO₂ was determined in the supernatant by the Griess reaction (see ‘Material and methods’). Results are expressed as the mean ± SD from 10 animals per group, and are representative of three independent experiments.

*Significantly different (P < 0.05) when compared with noninfected group. **Significantly different (P < 0.05) when compared with untreated infected group.

In addition, the results of studies that utilized inhibitors of cyclooxygenase to determine the role of prostaglandins in the control of parasitism and resistance to T. cruzi infection are discrepant (Celentano et al., 1995; Pinge-Filho et al., 1999; Freire-de-Lima et al., 2000; Michelin et al., 2005).

Three NSAIDs was tested: aspirin, that inhibits COX-1 more than COX-2 and the inhibition is irreversible (Frolich, 1997); indomethacin a dual COX-1 and COX-2 inhibitor (Süleyman et al., 2007; and celecoxib, a COX-2 selective inhibitor (Mitchell et al., 1994) that inhibit COX-2 375 times more strongly than COX-1 (Süleyman et al., 2007). It was demonstrated that in early infection treatment with aspirin, indomethacin or celecoxib increased dramatically parasitemia and reduced survival rate of T. cruzi-infected, resistant C57BL/6 mice. The inhibition of COX-2 with celecoxib produced the same result in parasite-infected susceptible BALB/c mice. Although the blockade of COX-1 with aspirin or with indomethacin tended to decrease the number of parasites in the blood of BALB/c, it was not capable of altering the survival rate of these mice.

In agreement with the authors’ results, Celentano et al. (1995) observed that treatment with cyclooxygenase inhibitors (aspirin or indomethacin) increased mortality rates of C3H/HeN mice infected with T. cruzi (K98 strain). However, in contrast to the authors’ findings and those of Celentano and colleagues, others have found that the inhibition of the prostaglandin synthesis abolishes parasitemia and delays the mortality of susceptible mice infected with T. cruzi (Freire-de-Lima et al., 2000). Besides, it was reported that high levels of PGE₂ are produced by macrophages (Monamaris et al., 1998), spleen cells from T. cruzi-infected mice and the inhibition of cyclooxygenase by indomethacin resulted in marked reduction of PGE₂ (Pinge-Filho et al., 1999). Anyway, studies are necessary to investigate new anti-inflammatory agents that do not impair the control of parasite burden while retaining the properties to relieve symptoms, which would have a long-term benefit for chagasic patients.

These differences are not easy to explain, and the effects of NSAIDs on T. cruzi infection have been baffling since the original paper of Celentano et al. (1995).

In T. cruzi infection, BALB/c mice are susceptible and show higher IL-4 production and parasitemia than in resistant C57BL/6 mice (Hoff et al., 1993). It is possible that the inhibition of prostaglandin production observed in our studies did not induce alterations in the production of IL-4 in BALB/c infected with T. cruzi, but might have inhibited the development of protective Th1 response in C57BL/6 mice. In fact, recent studies showed that NSAIDs selectively inhibit IFN-γ and TNF-α production in spleen cells Michelin et al. (2005) and natural killer (NK) and γδT cells (Inaoka et al., 2006). However, the high lethality after NSAID treatment could be related to a marked systemic increase in TNF-α production in a situation analogous to that in the nonhemolysed fraction of erythrocytes from C57BL/6 infected mice there is a time-dependent progressive increase in total chemiluminescence with a shift to the left of the maximal emission that reached a level double that of control. Treatment with COX inhibitors reduced emission significantly by c. 50%. The emission levels for infected BALB/c mice increased about 100%, with the curve also showing a shift to the left. Treatment with the COX inhibitors did not reverse these changes (Fig. 5b).

Discussion

Although it has been shown that prostaglandins, together with NO and TNF-α, participate in a complex circuit that controls lymphoproliferative and cytokine responses in T. cruzi infection (Pinge-Filho et al., 1999) the possible involvement of cyclooxygenase-mediated prostaglandin production in oxidative stress associated with anemia in early T. cruzi infection has not been previously investigated.
that observed in *T. cruzi*-infected C57BL/6 (Pinge-Filho et al., 1999) and IL-10−/− mice (Hunter et al., 1997). In addition, there is a strong difference between BALB/c and C57BL/6 mice in their ability to produce PGE2 (Kuroda & Yamashita, 2003). All these findings together may explain the discrepancy between our results and those of other authors.

The experiments revealed that on Day 12 of infection, treatment with celecoxib and aspirin but not indomethacin increased the number of parasite nests compared with untreated infected C57BL/6 mice. Interestingly, the heart tissue of infected BALB/c (Day 12 postinfection) mice treated with the COX-1 antagonist (indomethacin), had eightfold more amastigote nests than did untreated infected mice. Celecoxib or aspirin increased heart parasitism but to a lesser extent.

Therefore, this is the first indication that cyclooxygenase-mediated prostaglandin production plays a critical role in heart-protective immunity against *T. cruzi* in an experimental Chagas’ disease model. Notably, NSAIDs are used clinically for fever control during *T. cruzi* infection. If the results are extrapolated to a clinical setting, treatment with NSAIDs may produce more severe disease, and the authors’ findings argue against the use of NSAIDs as potential drugs for therapy in the acute phase of Chagas’s disease (Freire-de-Lima et al., 2000).
by macrophages from the peritoneal cavity of T. cruzi-infected mice.

Confirming previous reports (Santos et al., 2006), it was observed that macrophages from BALB/c (M1) T. cruzi infected-mice respond poorly with regard to •NO production compared with macrophages from C57BL/6 (M2) infected-mice. The inhibition of •NO production by aspirin in macrophages from C57BL/6 on Day 12 postinfection, could explain the increased parasitemia and death of these animals in early infection with T. cruzi. Interestingly, the same treatment provoked an increase in •NO production in infected BALB/c mice, and this could explain the control of parasitemia in early infection in aspirin-treated, infected BALB/c -mice.

Treatment with indomethacin had no effect and celecoxib increased •NO production in macrophages from infected C57BL/6 mice. The longer survival of infected C57BL/6 mice treated with celecoxib compared with indomethacin can be explained by the difference in •NO production. The increase in •NO production by macrophages from BALB/c infected mice treated with celecoxib or indomethacin was not enough to reduce parasitemia or increase survival rate whose baseline levels were lower than in C57BL/6 mice.

Oxidative stress studies in Chagas’ disease have recently been conducted (Wen et al., 2004, 2006; Zacks et al., 2005) but possible pathogenetic mechanisms, particularly involving •NO, were not addressed. Erythrocyte GSH concentration and GPx were reduced with disease progression, suggesting the involvement of systemic oxidative stress in patients with Chagas cardiomyopathy (Pérez-Fuentes et al., 2003; de Oliveira et al., 2007). Antioxidants such vitamin E and C administered to these patients reversed the oxidative status (Zacks et al., 2005; Macao et al., 2007). Oxidative stress in heart but not in skeletal muscle of mice infected with T. cruzi evaluated by lipid peroxide and carbonylated protein production both in acute and chronic phases has been reported (Wen et al., 2004). In this latter study, the authors proposed mitochondrial dysfunction as a cause of reactive oxygen species generation leading to progression of chagasic cardiomyopathy (Wen et al., 2006). Extensive DNA damage in cardiac myocytes and in spleen cells has also been found in T cruzi infection (Ribeiro et al., 2007).

In the present study, we extended the above investigation by examining the oxidative stress in erythrocytes of mice sensitive (BALB/c) and resistant (C57BL/6) to T. cruzi infection and the effect of cyclooxygenase inhibitors on that oxidative damage. For this purpose, tert-butyl hydroperoxide-initiated chemiluminescence, a very sensitive method, was employed to evaluate the integrity of nonenzymatic antioxidant defenses and to quantify erythrocyte membrane peroxidation. The basis for this assay is the assumption that previous attack of erythrocytes by reactive oxygen and nitrogen species (i.e. ONOO− and •OH) generates membrane lipid peroxides and depletes low-molecular weight antioxidants in cells resulting in a higher chemiluminescence emission (Casado et al., 2007). Additionally, in our experimental model, besides the suggested contribution of oxidative stress that takes place in important organs such as heart, liver, spleen (Cardoni et al., 1990; Ribeiro et al., 2007), other sources of free radicals such as circulating neutrophils (Docampo et al., 1983), macrophages (Cardoni et al., 1997; Pinto et al., 2002), arachidonic acid cascade (Cardoni & Antúnez, 2004) and endothelial cells (Cuzzocrea et al., 2006) can play a more significant role in systemic and erythrocyte prehemolytic lesion. Our results reveal that infection with T. cruzi, was associated with significantly increased chemiluminescence levels in erythrocytes of both C57BL/6 and BALB/c mice, but C57BL/6 mice had higher erythrocyte chemiluminescence levels than did BALB/c mice. This peroxidative injury was confirmed by the increased oxygen uptake and decreased induction time in both strain mice. The findings described here and our previous results

![Fig. 5. Time course curve of tert-butyl hydroperoxide-initiated chemiluminescence in erythrocytes from C57BL/6 (a) and BALB/c (b) mice. Animals were infected with 5 x 10⁴ trypomastigotes of Trypanosoma cruzi (Y strain). Four hours after the infection, mice received aspirin (50 mg kg⁻¹), indomethacin (1.2 mg kg⁻¹) or celecoxib (50 mg kg⁻¹) by the intraperitoneal route. Normal mice and untreated T. cruzi-infected mice were used as controls. *Significantly different (P < 0.05) when compared with noninfected group. **Significantly different (P < 0.05) when compared with untreated infected group.](image-url)
(Malvezi et al., 2004) together indicate that acute infection of mice with T. cruzi leads to oxidative damage to erythrocyte membranes by both NO-dependent and NO-independent pathways. Accordingly, in C57BL/6 mice, a good responder to iNOS induction, the nitrite levels in peritoneal macrophages increased about five times after 12 days of infection. Such increase was not seen in BALB/c mice, a known poor responder to iNOS induction. Increased NO production has been observed in j77.4 murine macrophages (Bergeron & Olivier, 2006) and in spleen cells after T. cruzi infection (Docampo et al., 1983). In susceptible BALB/c mice, however, no increase in NO was observed after infection with T. cruzi. On the contrary, ROS secreted by peritoneal macrophages during the chagasic infection of susceptible mice was higher than that observed in the resistant mice (Russo et al., 1989). These data indicate that C57BL/6 mice have higher NO levels while BALB/c mice show higher O$_2^-$ levels. As in the interpretation given to the comparison between C57BL/6 and Swiss mice in the previous study (Malvezi et al., 2004), erythrocyte oxidative injury found in the present study could be the result of NO reacting with O$_2^-$ yielding ONOO$^-$ or of the Fenton reaction producing OH near the outer leaflet of erythrocyte membrane, both of which lead to oxidative injury. At high NO levels, the reaction between NO and O$_2^-$ is favored because the rate constant of this reaction is higher than that between O$_2^-$ and H$_2$O$_2$ (Halliwell & Gutteridge, 1999). Moreover, because COX inhibitors efficiently inhibited the formation of membrane lipid peroxides, the cyclooxygenase pathway might have been involved in this process either increasing NO generation or directly producing additional reactive oxygen species (Halliwell & Gutteridge, 1999). These events could be occurring in erythrocytes of C57BL/6. At low NO, O$_2^-$ should be reacting with H$_2$O$_2$ to give OH. This could be the case in BALB/c as seen in C57BL/6 NOS$^{-/-}$ (Malvezi et al., 2004).

The involvement of cyclooxygenase-mediated prostaglandin production in oxidative stress induced by T. cruzi infection has not been previously investigated. All three cyclooxygenase inhibitors employed, reduced significantly the levels of chemiluminescence and oxygen uptake and increased the induction time in C57BL/6 mice infected with T. cruzi. Despite the variable levels of oxygen uptake, increased lipid peroxides and decreased induction time in erythrocytes from infected BALB/c mice were consistently unaffected by cyclooxygenase treatment. These results indicate that arachidonic acid metabolism via cyclooxygenase plays a significant role, at least in part, in oxidative damage in erythrocytes of C57BL/6 mice infected with T. cruzi. The oxidative stress seen in the BALB/c strain, however, does not depend on cyclooxygenase pathways. This profile was not demonstrated in macrophages producing NO. Except for aspirin, the other more selective drugs did not inhibit the NO increase in peritoneal macrophages of infected C57BL/6. The NO levels in BALB/c on the contrary were significantly increased. This is in accordance with the findings showing that in vivo inhibition of prostaglandin release by selective inhibition of COX-2 has only minimal effects on NO production (Salvemini, 1997; Hamilton & Warner, 1998). It is possible that there are at least two mechanisms of oxidative stress in T. cruzi infection whose predominance depends on the strain. In C57BL/6 mice with high NO generation, both NO and ROS appear to be involved possibly through ONOO$^-$ production. This notion is supported by our results showing an increased level of chemiluminescence and NO and by the fact that aminoguanidine, a selective inhibitor of iNOS, reverses erythrocyte lipid peroxidation in this strain (Malvezi et al., 2004). Additionally, the reduction in erythrocyte lipid peroxides but not NO production by cyclooxygenase inhibitors in infected C57BL/6 mice, means that arachidonic acid metabolism via the cyclooxygenase pathways could be involved in ROS generation. In BALB/c with low NO levels production, the oxidative injury of erythrocytes depends almost exclusively on reactive oxygen species such OH and singlet oxygen. These findings are supported by the evidence that C57BL/6 iNOS$^{-/-}$ infected with T. cruzi, shows high oxidative stress levels (Malvezi et al., 2004). Moreover, because our results showed that cyclooxygenases inhibitor were not able to reduce oxidative stress, it is suggested that in this strain oxidative damage in erythrocytes does not depend on either NO or cyclooxygenase-mediated prostaglandin formation.

In conclusion, the results indicate that T. cruzi infection induces prehemolytic lesions in the erythrocyte membrane through oxidative stress. This injury can account, at least in part, for the anemia observed in this disease. The mechanism by which the oxidative stress occurs depends on the strain. In susceptible mice with low NO generation, oxidative injury results almost exclusively due to ROS production and does not depend on cyclooxygenase pathway activation. In resistant mice with high NO production, oxidative injury depends on the NO reaction with O$_2^-$ giving ONOO$^-$. The observed decrease in oxidative stress by inhibitors of cyclooxygenase suggests that O$_2^-$ may be generated in part by this pathway.

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Disclosures

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