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Tissue-specific oxidative imbalance and mitochondrial dysfunction during *Trypanosoma cruzi* infection in mice

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Abstract

In this study, we examined the tissue specificity of inflammatory and oxidative responses and mitochondrial dysfunction in mice infected by *Trypanosoma cruzi*. In acute mice, parasite burden and associated inflammatory infiltrate was detected in all tissues (skeletal-muscle>heart>stomach>colon). The extent of oxidative damage and mitochondrial decay was in the order of heart>stomach>skeletal-muscle>colon. In chronic mice, a low level of parasite burden and inflammation continued in all tissues; however, oxidant overload and mitochondrial inefficiency mainly persisted in the heart tissue (also detectable in stomach). Further, we noted an unvaryingly high degree of oxidative stress, compromised antioxidant status, and decreased mitochondrial respiratory complex activities in peripheral-blood of infected mice. A pair-wise log analysis showed a strong positive correlation in the heart-versus-blood (but not other tissues) levels of oxidative stress markers (malonylaldehyde, glutathione-disulfide), antioxidants (superoxide-dismutase, MnSOD, catalase), and mitochondrial inhibition of respiratory complexes (CI/CIII) in infected mice.

Conclusions: *T. cruzi*-induced acute inflammatory and oxidative responses are widespread in different muscle tissues. Antioxidant/oxidant status and mitochondrial function are consistently attenuated in the heart, and reflected in the peripheral-blood of *T. cruzi*-infected mice. Our results provide an impetus to investigate the peripheral-blood oxidative responses in relation to clinical severity of heart disease in chagasic human patients.

Keywords

Chagas disease; mitochondrial dysfunction; oxidant/antioxidant status; tissue-specificity; *Trypanosoma cruzi*

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1. Introduction

Trypanosoma cruzi is the etiologic agent of Chagas disease that is a major human health problem in the southern parts of the American continent. Several decades after the initial infection, >30% of infected individuals develop chronic cardiomyopathy that may lead to congestive heart failure and sudden death [1].

Mitochondria are the prime source of energy, and many of the body functions, including cardiac metabolic and contractile activities, require the mitochondrial generation of ATP. Studies in mice have shown that the altered expression of the transcripts for mitochondrial function-related proteins and increased oxidation of these proteins occur in chagasic hearts [2,3], and are associated with an impaired activity of the respiratory complexes [4,5]. The functional effect of the respiratory complex inhibition was noted to be reduced oxidative phosphorylation and ATP levels in the myocardium of chagasic mice [6].

Besides reduced energy output, mitochondrial defects of the respiratory chain can result in increased electron leakage and production of reactive oxygen species (ROS) that are deleterious to mitochondrial and cellular components [7]. Additionally, chagasic patients are exposed to reactive nitrogen species (RNS) and ROS of inflammatory origin [8]. These reactive oxidants, though important for the control of *T. cruzi*, may also elicit toxicity to host cellular components.

In this study, we investigated whether oxidative cellular damage and mitochondrial dysfunction of the respiratory chain are specific to the heart during *T. cruzi* infection and disease development. For this, we compared the antioxidant/oxidant status and mitochondrial function in tissue from the heart, skeletal-muscle, colon, and stomach of *T. cruzi*-infected mice. We chose to study muscle tissues because *T. cruzi* exhibits myotropic behavior, and muscle cells are the preferred site for infection and parasite replication. Our data show that oxidative damage in acute mice was a bystander effect of parasite-induced inflammatory responses and was widespread in different tissues. The inflammatory processes persisted at a low level in all tissues after the control of acute parasite burden. The finding of persistent oxidative responses in the heart and peripheral-blood of chronically infected mice suggest the pathologic importance of these responses in Chagas disease, and provide an impetus to pursue human studies investigating whether changes in blood correlate with the degree of clinical presentation in the heart of chagasic patients.

2. Materials and Methods

2.1) Mice and parasites

We infected 6-to-8-week-old male C3H/HeN mice with *T. cruzi* trypomastigotes (SylvioX10/4, 10,000/mouse) and sacrificed them during the acute phase (20–35 days post-infection (dpi) corresponding to peak parasitemia and the chronic (150–180 dpi) phase of disease development [9].

2.2) Mitochondria isolation

Freshly harvested tissues (~50-mg) were homogenized, and mitochondria isolated by differential centrifugation [6]. Red blood cells (RBCs) were removed by hypotonic lysis, and the remaining white blood cells (WBCs) were used for mitochondria isolation. Mitochondrial preparations were >96% pure (<3% glucose-6-phosphatase [ER-marker] and acid-phosphatase [peroxisome-marker] activities).

2.3) Biochemical activities

Superoxide dismutase (SOD) activity_{420nm} in tissue and blood homogenates and MnSOD activity in isolated mitochondria was measured by a decrease in O₂^{•-}-dependent pyrogallol oxidation. Catalase (CAT) activity_{240nm} was measured by H₂O₂ reduction. Glutathione-peroxidase (GPx) activity_{340nm} was measured, using tert-butyl-hydroperoxide substrate, by GSH oxidation coupled to NADPH utilization by glutathione reductase [10]. CI-complex activity_{340nm} in isolated mitochondria was monitored by NADH (200-μM) oxidation with 80-μM 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone (DB) electron acceptor (±6.35-μM rotenone). Complex-CIII activity_{550nm} was quantitated by 60-μM DBH₂ (60-μM) oxidation with 50 μM cytochrome-c electron donor (±3.75-μM antimycin) [5].

2.4) Oxidants/antioxidants

We measured malonylaldehyde (MDA) levels by a TBARS assay [11,12]. The 2,4-dinitrophenylhydrazine (DNP)-derivatized protein carbonyls and 3-nitrotyrosine (3NT) contents were detected by Western blotting (WB) using an anti-DNP and anti-3NT antibody, respectively. Glutathione (GSH, GSSG) content was determined by a DTNB-GSSG reductase recycling assay [10].

2.5) Parasite detection

Tissues (50-mg) were subjected to Proteinase K lysis, and total DNA was isolated by phenol:chloroform extraction and ethanol precipitation method. Total DNA (50-ng) was used as a template in a PCR reaction with *T. cruzi* 18S rRNA-specific oligonucleotides. Amplicons were resolved/visualized by agarose gel electrophoresis. Murine *GAPDH* was amplified as a control [9].

2.6) Histology

Tissues were fixed in 10% formalin, embedded in paraffin, and hematoxylin/eosin-stained sections (5-μm) visualized by light microscopy [9]. Tissues were graded for type/distribution of inflammatory infiltrate, amount of inflammation (rare-mild-moderate-severe), parasite-foci (absent-rare-scattered-abundant), and other pathologic changes (e.g., calcification, necrosis, fibrosis, vascular-thickening).

2.7) Data analysis

Data are expressed as mean ± SD ($n \geq 9$ animals/group). ANOVA and Student's *t*-tests were employed to determine the significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, infected-versus-normal).

3. Results

3.1) Oxidant/antioxidant status

We performed WB with anti-3NT antibody to evaluate the extent of protein-nitration in chagasic tissues. Protein-3NT formation was maximal in plasma and heart tissue and moderate in skeletal muscle and stomach tissue of infected mice (Fig-1). No statistically significant difference in protein-3NT levels was observed in the colon tissue of infected-versus-normal mice. Likewise, WB with anti-DNP antibody showed a substantial increase in DNP-derivatized carbonyl-proteins in the heart, plasma, and stomach tissue of acutely- and chronically-infected mice. In other tissues, a marginal rise in carbonylation of 45-kDa (skeletal muscle) and 70-kDa (colon) proteins was observed in acute mice, but this increase was abolished during chronic phase (data not shown).

Lipid peroxidation, measured by examination of the MDA contents, was increased by 2–3-fold in the heart, plasma, and stomach of infected mice (Fig-2A), the maximal increase was observed in the plasma (211%) and stomach (217%) of chronic mice. The MDA level was not changed in the colon and skeletal muscle of infected mice.

The GSSG/GSH index is a good indicator of oxidant/antioxidant imbalance (Fig-2B). We noted a GSSG/GSH ratio (x100) of 7.7, 16, and 20.5 in the heart and 8.0, 4.6, and 34.9 in plasma of normal, acutely-infected, and chronically-infected mice, respectively. This increase in GSSG/GSH status was a consequence of a 42–112% increase in GSSG and a 28–76% decrease in GSH contents in the heart and plasma of infected mice (Table-1S, supplement data). No statistically significant change in GSSG/GSH ratio was noted in infected skeletal and colon tissues. In acute stomachs, GSSG/GSH ratio was increased as a consequence of ~40% increase in GSSG level (Fig-2B, Table-1S).

The activities of antioxidant enzymes, i.e., SOD, MnSOD, and CAT, were decreased by 31–54%, 16–57%, and 35%, respectively, in heart, and by 17%, 14–30%, and 49%, respectively, in blood of infected mice (Fig-3A–C). A maximal decline was observed during the chronic stage. GPx activity was not changed in the heart tissue and increased by ~3-fold in peripheral-blood of mice during the acute stage (Fig-3D). Other tissues of infected mice exhibited an elevated or unchanged antioxidant status (Fig-3). For example, in skeletal-muscle of infected mice, CAT and GPx activities were increased by 72% and 113%, respectively, in acute stage and normalized during chronic stage. We observed no statistically significant change in CAT and GPx activities in the infected colons, and in GPx activity in infected stomachs. CAT activity was decreased by ~40% in infected stomach during the acute stage, but normalized at chronic stage. The SOD/MnSOD activities were not statistically different in the skeletal muscle, colon, and stomach of infected mice. Overall, the data presented in Fig 1–Fig 3 show that 3NT, carbonyls, MDA, GSSG/GSH (oxidative/nitrosative stress markers) levels were significantly increased in the heart and peripheral-blood, moderately altered in the stomach, and unchanged in the colon and skeletal muscle of mice during the acute and chronic phase of infection and disease development. The increase in oxidative/nitrosative stress in the heart and peripheral-blood was associated with a SOD/MnSOD insufficiency and glutathione antioxidant imbalance.

3.2) Respiratory complexes

CI and CIII complex activities were, respectively, decreased by 35–49% and 52–58% in heart and by 33–73% and 80% in blood of infected mice (Fig-4) [5]. CI activity was decreased by 30%, 32%, and 44% in the skeletal, colon, and stomach tissue, respectively, of acutely-infected mice and normalized during chronic phase (Fig-4A). CIII activity was not statistically changed in infected skeletal, colon, and stomach tissues (Fig-4B). The pattern of tissue-specific change in mitochondrial complex activities remained constant, whether we expressed the specific activity with-respect-to mitochondrial protein or citrate synthase activity. These data suggest that mitochondrial function was transiently perturbed in several tissues of mice at the acute stage and remained compromised in the heart and peripheral-blood during the chronic disease stage.

3.3) Parasite burden and inflammatory responses

We monitored parasite burden by a semi-quantitative PCR using *Tc18S*rRNA-specific primers. In acute mice, a strong *Tc18S*-specific signal was amplified in all tissues: skeletal muscle (28-cycles), heart (30-cycles), and blood, colon and stomach (35 cycles) (Fig-5A). In chronic stage, a similar level of *Tc18S*-specific signal was detectable in all tissues (35 cycles). Similar levels of murine *GAPDH* amplification verified the PCR amplification efficiency was consistent and equal amount of DNA was used in all reactions. These data suggest that parasite burden in

acute tissues was in order of skeletal muscle>heart>colon = stomach, while at chronic stage, a low but detectable level of parasite burden persisted in all tissues.

Histological studies showed acute inflammatory infiltrate in all tissues by 10 dpi that peaked during 30–35 dpi. Acute inflammation (focal and diffused) was more intense in heart and skeletal muscle (Fig-6B/E) than that observed in other tissues (Fig-6H/K). Parasite nests were found most in the heart and skeletal muscle (Fig-6B/E) than other tissues (Fig-6H/K) of acutely-infected mice. During chronic stage, parasite nests were rarely detected in any of the tissues. The pathologic sequelae in the chronic heart included moderate, diffused inflammation (predominantly polymorphonuclear neutrophils) and fibrosis in chronically infected mice (Fig-6C). Mononuclear cell infiltration (macrophages and a substantial number of T cells) was noted in all tissues. The mononuclear cells were rather abundant, considering the parasite burden was controlled. In skeletal muscle, inflammatory foci were more focal than diffused (Fig-6F). In colon of chronic mice, inflammatory cells were detected in mucosa, epithelial cells, and smooth muscle (Fig-6I). In chronic stomach, inflammatory infiltrate was present in muscle coat and submucosa (Fig-6L). Overall, chronic inflammatory infiltrate was lower than that noted in acute stage, but persisted in all tissues (Fig-6).

3.4) Correlation analysis

A pair-wise log analysis identified a strong positive correlation for the disease state-specific changes in the heart-versus-plasma levels of oxidative stress markers (MDA, GSSG), and heart-versus-blood level of antioxidants (CAT, SOD, MnSOD) and respiratory complex (CI, CIII) activities (Fig-1S, Table-2S, supplement data). The changes in oxidant/antioxidant status and mitochondrial function in the heart were not strongly correlated with those observed in the skeletal muscle, colon, and stomach tissues of infected mice.

4. Discussion

In this study, our objective was to determine the tissue-specificity of oxidative responses during *T. cruzi* infection. We have found that in acute stage, oxidative injurious processes are widespread in all tissues and paralleled with the detection of parasites and infiltration of inflammatory infiltrate. After control of the acute parasite burden, a mild-to-moderate level of diffused inflammatory responses remained; however, the mitochondrial function and oxidant/antioxidant status were normalized in skeletal muscle, colon, and stomach tissues of infected mice. In contrast, the heart, that is also the site of clinical chagasic disease, continued to exhibit extensive cellular oxidative decay and mitochondrial loss of function. A distinctive pattern of antioxidant/oxidant imbalance and mitochondrial decay was presented in the peripheral-blood. These data show the pathological importance of oxidative overload and mitochondrial dysfunction in Chagas disease development, and provide an impetus to utilize peripheral-blood for understanding the oxidative pathologic mechanisms in human Chagas disease.

We have used the SylvioX10/4 strain of *T. cruzi* that exhibited myotropic behavior. Our data show *T. cruzi* infected various tissues of C3H/HeN mice, as evidenced by the detection of *Tc18SrRNA* signal, and parasite foci and extensive inflammatory infiltrate in the heart, skeletal muscle, colon, and stomach sections (Fig-5/ Fig 6). The inflammatory infiltrate in the acute stage mainly consisted of phagocytic cells (e.g. macrophages) and neutrophils. The oxidative burst (ROS release) of macrophages, iNOS-dependent NO release, and myeloperoxidase (MPO) activity of neutrophils produce cytotoxic oxidants. For example, $O_2^{\cdot-}$ reacts with NO, forming toxic peroxynitrite ($ONOO^-$). H_2O_2 , through the Haber-Weiss and Fenton reactions, and $O_2^{\cdot-}$, through reaction with HOCl, generate a hydroxyl ($\cdot OH$) radical that is considered the most potent oxidant in biological systems [13]. Besides their role in killing parasites, these oxidants may also affect the host cellular components. Accordingly, we observed an increase in oxidative stress biomarkers, i.e., protein carbonyls, protein-nitrotyrosine, and MDA adducts,

in different tissues of acutely-infected mice. We inferred from these data that acute oxidative damage is mainly a bystander effect of inflammatory responses elicited by the parasite.

It is noteworthy that despite both exposure to parasites and associated inflammation, the acute tissue exhibited a variable extent of oxidative damage that did not directly correlate with the number of parasites and amounts of inflammatory infiltrate. For example, tissue parasitism and associated inflammation were heaviest in skeletal muscle and heart tissue compared to that found in stomach and colon tissues (Fig-5/ Fig 6). However, the heart and stomach sustained the maximum oxidative damage in mice at the acute infection stage (Fig-1/ Fig-2). Given that the acute clinical disease mainly manifests as myocarditis [14] and megaesophagus [15], our data lead to the implication that oxidative damage of the heart and stomach might be of pathological significance in acute Chagas disease.

During the chronic stage, parasitic foci were rarely detectable by histological studies in any of the examined tissue of infected mice. Sterile immunity was not generated, as we were able to amplify *Tc18SrRNA* sequence from various tissues (Fig-5), and parasite-specific antibodies persisted throughout the course of infection and disease development (data not shown). Similarly, inflammatory infiltrate persisted in different tissues, though focal inflammatory foci were mainly detected in the skeletal muscle (Fig-6). Considering that inflammatory responses and parasite burden during the progressive chronic phase were detected in all examined tissues, it would be an oversimplification to suggest that cardiac pathology is merely an outcome of infection and inflammation, or parasite persistence that is sufficient to drive an ongoing host immune response targeted against *T. cruzi*. An unvarying high degree of oxidative damage mainly persisted in the myocardium of infected mice during the chronic phase, as evidenced by high levels of MDA, protein carbonyl, and GSSG contents in the heart compared to findings in the skeletal muscle and colon tissue (Fig1–Fig3). The deleterious effects of 4-HNE-induced oxidative modifications of lipids and proteins have been linked to the etiology and/or progression of several human diseases, including hypertrophic cardiomyopathy [16] and ischemia-perfusion-related cardiac injuries [17]. We propose the persistent activation of oxidative injurious processes plays an important role in heart-specific tissue damage in Chagas disease.

Two systems, one that scavenges free radicals and another that produces free radicals, coexist in tissues. Oxidative damage is a consequence of the extent of oxidative stress and the antioxidant capacity. It is documented that different tissues are provided with antioxidant defense systems having differing degrees of effectiveness, and the overall antioxidant capacity of the tissues correlates with the level of the components of the major antioxidant system [18]. The findings of the present study show that the glutathione antioxidant capacity (GSH and GPx) of the normal murine heart and stomach was higher than that of skeletal muscle and colon tissues. Others have shown that in the rat, the antioxidant contents are in the order of liver>heart>muscle [19]. These observations support the idea that heart and stomach are, indeed, equipped with an antioxidant defense system similar to or better than that of other tissues. Despite this, acute and chronic hearts (and acute stomachs) exhibited an increased GSSG/GSH ratio (Fig-2B). These data imply that persistence of oxidative stress in chagasic hearts is an outcome of antioxidant depletion and oxidant/antioxidant imbalance. Under such conditions, an antioxidant system is not able to provide protection from the ROS-induced oxidative modification of lipids and proteins. A passive antioxidant response to increased oxidative stress in experimental models of ischemia/reperfusion [20] supports our hypothesis.

Considering that all tissues were exposed to parasite-induced inflammatory oxidative stress in infected mice, other factors must contribute to increased ROS production and/or increased susceptibility to oxidative stress-induced damage in heart tissue. It is well known that ROS are generated at several subcellular sites [21] and particularly in mitochondria [22]. In effect, ~2%

of the O_2 consumed by mitochondria is converted to $O_2^{\cdot-}$ due to spontaneous electron leaks from the respiratory chain. Activated skeletal and intestinal muscles intermittently require mitochondria as an energy source, while cardiomyocytes are constantly dependent upon mitochondrial functions for their energy requirement for maintaining the contractile and other metabolic activities. According to energy demand, a ~30% cell volume of cardiomyocytes is provided by mitochondria, while in other tissues mitochondria constitute only 3–6% of cell volume [23]. Thus, maximal O_2 consumption, as would be expected based upon the number of mitochondria in the heart, would produce substantial $O_2^{\cdot-}$ in the heart through electron leakage from the respiratory chain. Thus, it can be inferred that even in normal conditions, heart tissue is maximally exposed to ROS of mitochondrial origin. Besides this, inefficient functioning of the respiratory complexes, as documented in chagasic hearts in this and previous studies [5], would result in an inadequate coupling of the respiratory chain with oxidative phosphorylation and an excessive release of electrons to molecular oxygen, leading to an increased mitochondrial ROS production. We have recently found the rate of mitochondrial $O_2^{\cdot-}$ generation was substantially increased in cardiac tissue of infected mice [6], and associated with oxidation of several subunits of the respiratory complexes [24]. The active-site thiol and heme proteins within respiratory complexes are particularly vulnerable to ROS [25]. The oxidative modification/degradation of heme proteins of the complexes release iron, the catalyst of the Fenton reaction, resulting in the formation/release of $\cdot OH$ radicals [26–28]. Together, these observations suggest that, under disease conditions, mitochondria are vulnerable to oxidative stress, as well as to becoming the site of an increasing order of ROS production. We surmise that the acute inflammatory oxidative stress-induced mitochondrial injuries initiate a feedback cycle of ROS production and oxidative overload that causes sustained oxidative damage in the myocardium. A compromise in mitochondrial antioxidant enzyme activity (MnSOD) in chagasic myocardium would further exacerbate the mitochondrial ROS toxicity.

Our data show that blood served as a useful tissue capable of detecting and responding to the changes induced in the body during the course of *T. cruzi* infection and disease development. White blood cells are the main constituents of the immune system, and both red and white blood cells are dynamic components of the circulatory system capable of interacting with all of the cells, tissues, and organs in a body. The changes in oxidative stress, antioxidant imbalance, and mitochondrial dysfunction were detectable in the blood of infected mice. Notably, a strong positive correlation was detected for the disease state-specific changes in the heart-versus-blood level of oxidative stress markers (MDA, GSSG), antioxidants (CAT, SOD, MnSOD), and respiratory complex (CI, CIII) activities. We also noted that the depletion of antioxidants was strongly correlated with a loss in respiratory complex activities in the heart and blood (individually or in combination) of infected mice. This is an important observation, as the blood provides valuable information regarding the status of the heart disease. Cardiac biopsies to obtain research samples is an invasive procedure that is restricted by the ethical concerns of harming the patient, and the size of the biopsies may not be sufficient to obtain meaningful molecular and biochemical results. In contrast, blood is easily accessible from human patients, and collection of blood samples requires relatively non-invasive techniques, thereby alleviating discomfort to the patient.

In summary, we demonstrate that acute oxidative overload in *T. cruzi*-infected mice is a bystander effect of inflammatory responses elicited by the parasite. As the acute parasite burden is controlled, these inflammatory and oxidative injurious processes subside in skeletal muscle, colon, and stomach tissues. However, mitochondrial functional decay, resulting in increased ROS production, persists in the heart and contributes to a sustained oxidative pathology of chagasic disease. Further, we have observed that the alterations of parameters, e.g., MDA content, 3-NT level, protein carbonyls, GSH/GSSG ratio, SOD/MnSOD activity, and respiratory complex activities, in the heart and blood of infected mice have the same pathologic

tendencies. This is an important finding, as peripheral-blood is the easiest available tissue for human studies, and will be useful for understanding the role of mitochondrial defects and oxidative overload in the initiation and/or progression of cardiac pathology in human chagasic patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

CAT, catalase
 CI, NADH ubiquinone oxidoreductase
 CIII, ubiquinol cytochrome c oxidoreductase
 GPx, glutathione peroxidase
 GSH, glutathione
 GSSG, glutathione disulfide
 HNE, 4-hydroxy-2-nonenal
 MDA, malonyldialdehyde
 3NT, 3-nitrotyrosine
 RNS, reactive nitrogen species
 ROS, reactive oxygen species
 SOD, superoxide dismutase
 TBARS, thiobarbituric acid reactive substances
T. cruzi, *Trypanosoma cruzi*

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Figure 1. Protein tyrosine nitration in different tissues of *T. cruzi*-infected mice

T. cruzi-infected C3H/HeN mice were sacrificed during the acute (20–35 dpi) and chronic (150–180 dpi) stages, and various tissues were harvested. Tissue homogenates and plasma were resolved by SDS-PAGE, transferred to membrane, and Western blot analysis performed using mouse antibody to 3-nitrotyrosine (panels A–D). Membranes were stained with Coomassie blue to confirm equal loading of protein samples (panels E–H).

Figure 2. Oxidant status in various tissues of *T. cruzi*-infected mice

Shown are (A) the MDA content measured by TBARS assay, and (B) the GSSG/GSH ratios derived from the measurement of GSSG and GSH contents by a modified DTNB-GSSG reductase recycling assay. Abbreviations: He-Heart, Pl-Blood, Sk-Skeletal, Co-colon, St-Stomach.

Figure 3. Antioxidant enzyme activities in various tissues of *T. cruzi*-infected mice
Spectrophotometric assays were employed to measure the specific activity of the antioxidant enzymes. (A) Superoxide dismutase (SOD), (B) MnSOD, (C) Catalase (CAT), and (D) Glutathione peroxidase.

Figure 4. Respiratory complex activities in different tissues of *T. cruzi*-infected mice

C3H/HeN mice were infected with *T. cruzi* and sacrificed during the acute (grey bars) and chronic (black bars) stages. Mitochondria were isolated from different tissues and blood cells by differential centrifugation, and the activity of the CI (A) and CIII (B) respiratory complexes was measured by spectrophotometry.

Figure 5. Tissue parasite burden in infected mice

(A) Total DNA was used as a template in a PCR amplification of *T. cruzi* 18S rRNA-encoding sequence. Specificity of PCR for *Tc18S* was confirmed by the detection of no signal when total DNA from normal mice was used as a template. Densitometric analysis of *Tc18S* signal, normalized with murine *GAPDH*, is shown in **panel B**.

Figure 6. Histological analysis

Tissue sections from normal and *T. cruzi*-infected mice were subjected to hematoxylin/eosin staining, and visualized by light microscopy (original magnification: 20X). Shown are the representative micrographs from the heart (A–C), skeletal muscle (D–F), colon (G–I), and stomach (J–L) tissues of normal (A/D/G/J), acutely-infected (B/E/H/K), and chronically-infected (C/F/I/L) mice. Parasitic nests are marked with arrows (B/E).