The benefits of using selenium in the treatment of Chagas disease: prevention of right ventricle chamber dilatation and reversion of *Trypanosoma cruzi*-induced acute and chronic cardiomyopathy in mice

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Cardiac damage is a frequent manifestation of Chagas disease, which is caused by the parasite Trypanosoma cruzi. *Selenium (Se) is an essential micronutrient, the deficiency of which has been implicated in the development of cardiomyopathy. Our group has previously demonstrated that Se supplementation prevents myocardial damage during acute* T. cruzi *infection in mice. In this study, we analyzed the effect of Se treatment in cases of* T. cruzi *infection using prevention and reversion schemes. In the Se prevention scheme, mice were given Se supplements (2 ppm) starting two weeks prior to inoculation with* T. cruzi *(Brazil strain) and continuing until 120 days post-infection (dpi). In the Se reversion scheme, mice were treated with Se (4 ppm) for 100 days, starting at 160 dpi. Dilatation of the right ventricle was observed in the infected control group at both phases of* T. cruzi *infection, but it was not observed in the infected group that received Se treatment. Surviving infected mice that were submitted to the Se reversion scheme presented normal P wave values and reduced inflammation of the pericardium. These data indicate that Se treatment prevents right ventricular chamber increase and thus can be proposed as an adjuvant therapy for cardiac alterations already established by* T. cruzi *infection.*

Key words: selenium - cardiomyopathy - Chagas disease - *Trypanosoma cruzi*

Chagas disease is a neglected parasitic disease that was discovered 100 years ago (Lannes-Vieira et al. 2009). It is caused by the intracellular parasite *Trypanosoma cruzi* and is most frequently associated with cardiac-related manifestations. *T. cruzi* initially invades myocardial cells, resulting in a diffuse and severe neutrophilic and monocytic inflammatory infiltrate in addition to myofibrillar lesions (Bilate & Cunha-Neto 2008). At the acute phase, patients can present with pericardial effusion, dyskinesis or ventricular dilatation detected by echocardiography. Electrocardiographic (ECG) alterations are also observed in patients; these include diffuse ST-T repolarization changes as well as low voltage, conduction problems, left anterior hemiblock and combined blocks (Blum et al. 2008). At the chronic phase, roughly 30% of patients present cardiac dysrhythmia, heart failure and thromboembolism (both systemic and pulmonary) (Rassi et al. 2009). A depressed left-ventricular ejection fraction and increased left-ventricular internal dimensions have been shown to be associated with increased morbidity and mortality (Rassi et al. 2006, Acquatella 2007).

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Evidence has shown that oxidative stress resulting from augmentation of free radical production plays an important role in the pathogenesis of some heart diseases (Gupta et al. 2009). In an experimental model of *T. cruzi* infection, it was reported that increased oxidative stress and antioxidant insufficiency are associated with myocardial oxidative damage and mitochondrial functional decline, findings that might be of pathological significance in Chagas disease (Wen et al. 2004, Gupta et al. 2009).

We have previously investigated the role of selenium (Se), an essential trace element with antioxidant properties, in both human and experimental infection of *T. cruzi*. We demonstrated that a significant percentage of patients with advanced chronic chagasic cardiomyopathy exhibited low Se levels that positively correlated with cardiac insufficiency (Rivera et al. 2002). Moreover, we demonstrated that a Se-deficient diet contributes to an increased susceptibility to this infection in experimental models (de Souza et al. 2002), whereas oral Se supplementation at low doses alleviates heart damage (de Souza et al. 2003). Gomez et al. (2002) have also reported an increased severity of myopathy in Se-depleted chronically infected mice; furthermore, Davis et al. (1998) showed that Se supplementation reduced parasitaemia and mortality in infected mice. Based on these findings and taking into account the association of Se intake with the prevention of cardiac diseases (Korpela et al. 1989, Reeves et al. 1989, Foster & Sumar 1997, Saito et al. 1998), we investigated the effect of Se treatment on the development of chronic cardiopathy in *T. cruzi*-infected mice in both prevention and reversion schemes.

SUBJECTS, MATERIALS AND METHODS

Infection and Se supplementation - Male CD1 (WT) (Jackson Laboratories) and Swiss (CECAL-Fiocruz) mice were separated into two cohorts that either received treatment with sodium selenate (Sigma Chemical Co, MO, USA), an inorganic form of Se or no treatment. The first cohort was placed in the Se prevention scheme, aiming to prevent cardiac alteration resulting from *T. cruzi* infection. CD1 mice were treated with sodium selenate added at a concentration of 2 ppm to the drinking water (*ad libitum*) two weeks prior to infection, as previously described (de Souza et al. 2003). Treatment with Se was carried out for 100 days. These groups were named Inf-Se0 (infected untreated, n = 11) and Inf-Se2 (infected and treated with 2 ppm Se, n = 10). Uninfected animals (5 mice/group) supplemented with the same doses (0 and 2 ppm) were used as controls (Cont-Se0 and Cont-Se2). The consequences of infection in terms of parasitaemia and mortality were monitored until 100 days post infection (dpi). The second cohort was placed in the Se reversion scheme to evaluate the effects of Se treatment on cardiac damage already established by the infection. Swiss mice at the chronic phase were treated with sodium selenate for 100 days, starting at 160 dpi. These mice were divided into three groups: infected (Inf), infected and treated with 4 ppm Se (InfSe4) and uninfected (Cont). Two weeks after beginning the Se treatment, the CD1 mice in the Se prevention scheme were infected by intra-peritoneal (ip) injection with 5 x 10^4 bloodstream forms of the Brazil strain of *T. cruzi*, as described by Huang et al. (1999), the same inoculum and *T. cruzi* strain used in the reversion scheme. These mice were fed standard rodent chow. Parasitaemia and mortality were determined and cumulative mortality (CM) and survival rate were calculated.

Magnetic resonance imaging (MRI) - Mice from the Se prevention scheme group was anaesthetized with avertin and a set of Gould ECG leads with custom electrodes were attached to their limbs for cardiac gating. The ECG signal was transmitted to a MRI spectrometer through a cardiac gating box as well as a PC running Ponemah Physiology Suite software. Mice were positioned in a 35 mm home-built MRI coil in a 9.4T GE Omega vertical-bore imaging system. Heart rate and ECG were monitored continuously. Gating trigger delays were controlled by the pulse sequence and were set to acquire images in either systole or diastole. After the imaging experiments, the mice were allowed to recover from the anaesthesia and were returned to their cages in the Animal Institute. All MRI experiments were performed at the Albert Einstein College of Medicine using a GE Omega 9.4T vertical wide-bore nuclear magnetic resonance spectrometer equipped with a microimaging accessory.

Cardiac gated MRI - Scout images of the thorax were acquired. Once the specific region of interest in the heart was identified, images were acquired in diastole. Images were analyzed using MATLAB-based software. Cardiac dimensions were recorded. The left ventricular inner dimension (LVID) corresponds to the average of the septum-lateral and anterior-posterior LVDI. The LV wall is the average of the anterior, posterior, lateral and septum wall dimensions. The right ventricular inner dimension (RVID) is the measurement of the chamber dimension at the widest point of the RV. Cardiac volumes were not calculated from the multi-slice images because there is a slight time shift during each slice acquisition, such that all slices from a single imaging data set are not collected at the same position in the cardiac cycle. Instead, we evaluated all images acquired with different delays, selecting the centre slice of the heart exhibiting the largest LVID to represent the end-diastolic image.

ECG study - Mice from the Se reversion scheme were tranquilized with diazepam (20 mg/Kg ip) and transducers were carefully placed on the skin in accordance with the chosen preferential derivation (DII). Traces were recorded using a digital system (Power Lab 2/20) connected to a bio-amplifier (Pan Lab Instruments). Traces were analyzed using the Scope software for Windows. We measured the duration of the PR, QRS and QT intervals (QTc) and the P wave in milliseconds (ms) at the end of the experiment (237-259 dpi). The relationship between the QT and RR intervals (RR_0) was individually assessed. To obtain physiologically relevant values for the heart rate-corrected QTc in units of time rather than time to a power not equal to 1, the observed RR_0 was first expressed as a unit-less multiple of 100 ms, giving a normalized RR_0 ($RR_{100} = RR_0/100$ ms). Next, the value of the exponent (y) in the formula $QT_0 = QTc \times RR^y_{100}$ was assessed, where QT_0 is the observed QT and both QT and QTc are expressed in milliseconds. Taking the natural logarithm of each side of the formula $(QT_0) = \ln(QTc) + y\ln(RR_{100})$, the slope of the linear relationship between the log-transformed QT and RR_{100} thus defined the y to which the RR_0 ratio should be raised to correct QT for heart rate (Mitchell et al. 1998).

To avoid measurement bias in MRI and ECG studies, treated and untreated mice received a code number and experts from each area performed measurements and analysis.

Histological examination - Before starting the Se reversion scheme, six infected mice and four uninfected mice were sacrificed at 150 dpi; at the end of the experiment (273 dpi), the surviving mice were sacrificed to collect the heart. The heart tissue was processed in paraffin-embedded sections stained with hematoxilin-eosin. The presence of necrosis was determined when cell debris and a faint coloration were detected in the sections; foci of inflammatory infiltrate (containing at least 10 mononuclear cells) were also investigated.

Statistical analysis - Statistical significance ($p < 0.05$) was evaluated using the Mann Whitney test for the analysis of parasitaemia, cardiac dimension and the log Rank (Gehan-Breslow-Wilcoxon) test for survival of the animals. A *t* test was used for the ECG analysis. The statistical significance of variations in RV dimensions between groups was evaluated using analysis of variance.

These studies were performed in accordance with the guidelines established by the National Institutes of Health

and approved by The Institutional Animal Care and Use Committee of the Albert Einstein College of Medicine and by the Fiocruz Committee of Ethics in Animal Use, license 0099/01 for the research, resolution 6.899/09.

RESULTS

Parasitaemia and mortality - In the Se prevention scheme, all infected mice presented a peak of parasitaemia between 20-24 dpi; this was observed in both supplemented and unsuplemented groups, with no statistically significant differences observed between the two. The median parasitaemia (75th percentile) was similar between Inf-Se0 and Inf-Se2 groups, with values of $11.8 \times 10^5 \pm 4.3$/mL and $7.1 \times 10^5 \pm 3.6$/mL, respectively. Both groups presented a low mortality rate at the acute phase. The CM of the Inf-Se0 group was 18.2% at 40 dpi, whereas all mice supplemented with 2 ppm Se (Inf-Se2) survived beyond that day. At the end of the experiment (120 dpi), the Inf-Se0 and Inf-Se2 groups exhibited a CM of 18.2% and 22%, respectively.

In cohort 2, infected mice presented a peak of parasitaemia at 27 dpi (54.8×10^4 mL) and a CM of 67.9% at 150 dpi. At 160 dpi, infected mice were divided into groups to begin the Se treatment and CM measurements were restarted. Fifty days after Se treatment (240 dpi), the InfSe4 group presented lower CM rates (11%), though this was not significantly different from the Inf group (28.6% CM).

MRI - Cardiac-gated MRI of mice in the Se prevention scheme was performed during the acute and chronic phases (Fig. 1, Table). Compared with Cont-Se0, the RVID of Inf-Se0 mice was 1.3 times higher (3.1 ± 0.2 mm vs. 2.3 ± 0.2 mm; $p = 0.04$) at the acute phase. At the acute phase, the hearts of Inf-Se2 mice were not significantly different from those of Inf-Se0 mice, exhibiting the same infected profile of a significant increase of the RVID compared to Cont-Se2 mice (2.9 ± 0.3 mm vs. 2.0 ± 0.1 mm; $p = 0.01$). During this period of the infection, the variance within groups was 0.1081, 0.0129, 0.3775 and 0.5089 for Cont-SeO, Cont-Se2, Inf-Se0 and Inf-Se2, respectively.

Treatment with Se over a long period of time did not induce any cardiac alterations in Cont-Se2 mice. In relation to the chronic phase (100 dpi), Inf-Se0 mice exhibited a significant enlargement of RVID (Fig. 1B) when compared to Cont-Se0 (Fig. 1A, Table). Critically, the RVID of infected mice treated with Se did not increase any further (Fig. 1D) and remained similar in size to that of the uninfected control mice in the chronic phase (Fig. 1C). The graphical comparison of mean RVID measures (Fig. 1E) clearly shows the effect of Se treatment on the prevention of right ventricular dilatation during experimental Chagas cardiomyopathy. The variance within groups was 0.0034 for Cont-Se0, 0.0757 for Cont-Se2, 0.1933 for Inf-Se0 and 0.0975 for Inf-Se2.

ECG findings - During the period between 237-259 dpi that corresponded to 76-98 days post Se treatment, mice in cohort 2 were subjected to ECG evaluations to study reversion of heart lesions. Se treatment resulted in a significant reversion of the increase of the P wave duration (Fig. 2) in the InfSe4 group (13.8 ± 0.7 ms, n = 7) in comparison to the Inf group (16.0 ± 1.1 ms, n = 8). No difference was observed between the Cont (14.2 ± 1.3 ms, n = 11) and InfSe4 groups.

Histopathological analysis - At 150 dpi, uninfected mice did not present any histological alterations (Fig. 3A), whereas 5-6 Inf mice exhibited pericarditis and 1 exhibited myocarditis in the atrium (Fig. 3B). At the end of the experiment (dpi 273), no important histological findings were made in the hearts of Cont group mice (not shown), whereas two surviving untreated Inf mice exhibited moderate pericarditis (Fig. 3C). In contrast,

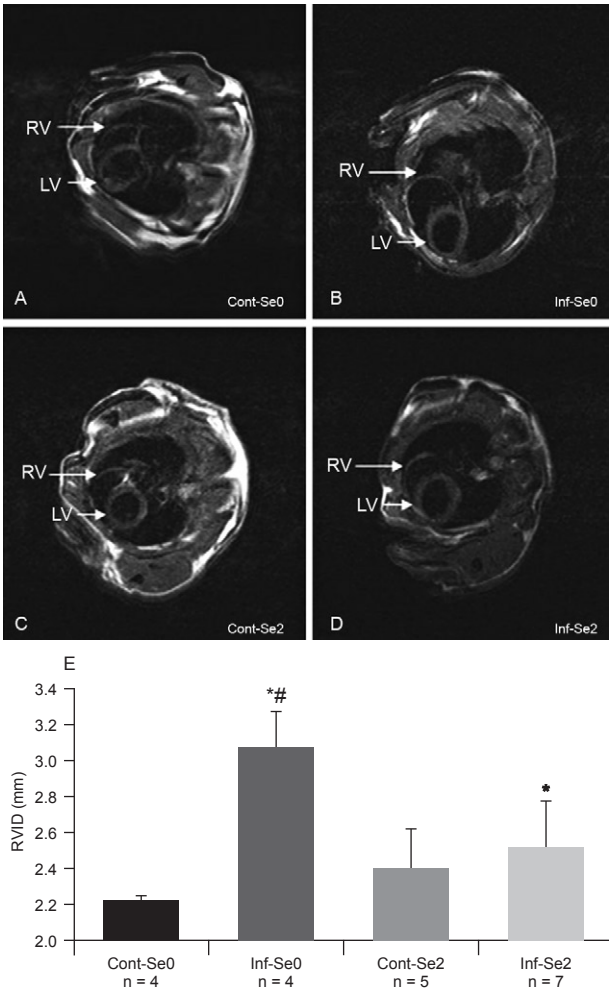

Fig. 1: effect of selenium (Se) supplementation on hearts of mice infected with *Trypanosoma cruzi* at the chronic phase, 100 days after infection. A, C: magnetic resonance imaging (MRI) of non-infected mice supplemented (Cont-Se2) (C) or not (Cont-Se0) (A) with 2 ppm sodium selenate in drinking water; B, D, E: cardiac gated MRI of *T. cruzi* infected mice supplemented (Inf-Se2) (D) or not (Inf-Se0) (B) with the same doses of Se; E: right ventricular inner dimension (RVID) (mean ± standard error of the mean) expressed in millimeters for the different conditions tested; LV: left ventricle; n: number of mice in each experimental group; RVID: right ventricular inner dimension; #: $p < 0.05$ as compared to Inf-Se2. Asterisk means $p < 0.05$ as compared to Cont-Se0.

TABLE

Cardiac dimensions in mice in the acute and chronic phases of *Trypanosoma cruzi* infection with or without selenium (Se) supplementation

	Cont-Se0	Cont-Se2	Inf-Se0	Inf-Se2
Acute phase				
RVID (mm)	2.3 ± 0.2[a]	2.0 ± 0.1	3.1 ± 0.2[c]	2.9 ± 0.3[b]
IV	1.00	0.86	1.34	1.26
LVID (mm)	4.3 ± 0.3	4.0 ± 0.1	3.9 ± 0.2	3.9 ± 0.2
IV[a]	1.00	0.94	0.91	0.91
n	4	5	7	6
Chronic phase				
RVID (mm)	2.2 ± 0.0[a]	2.4 ± 0.1	3.2 ± 0.2[c,d]	2.5 ± 0.1
IV	1.00	1.09	1.45	1.13
LVID (mm)	4.0 ± 0.2	4.2 ± 0.2	4.3 ± 0.1	4.2 ± 0.20
IV	1.00	1.05	1.07	1.05
n	4	4	7	5

a: results in average ± standard error of the mean; *b*: $p < 0.05$ as compared to uninfected animals supplemented with 2 ppm Se (Cont-Se2); *c*: $p < 0.05$ as compared to uninfected animals (Cont-Se0); *d*: $p < 0.05$ as compared to infected animals treated with 2 ppm Se (Inf-Se2); Inf-Se0: infected untreated animals; IV: index of variation; LVID: left ventricular inner dimension; n: number of survivors mice in each group during the acute and chronic phases; RVID: right ventricular inner dimension.

two out of three InfSe4 mice did not present any histological alterations in the heart (Fig. 3D) and only one presented minimal pericarditis.

DISCUSSION

In the present study, we observed the course of infection of two outbred mice lineages (CD1 and Swiss) by the Brazil strain of *T. cruzi* to study the potential beneficial effect of Se treatment in experimental Chagas disease; the experiments described revealed a striking effect of Se treatment on both the prevention and reversion of heart functional and histopathological damage. Based on our previous report (de Souza et al. 2003), we conducted the treatment study using the inorganic form of Se, sodium selenate. To investigate the preventative effects of Se, we administered a dose of 2 ppm (the lowest concentration assayed in preliminary experiments, data not shown) to CD1 mice infected with Brazil strain *T. cruzi* for long-term follow-up studies. For the Se reversion scheme, we used 4 ppm Se, a dose that was sufficient to prevent cardiac necrotic lesions in acute Swiss mice (de Souza et al. 2003). The course of the Brazil strain infection was similar in both cohorts, which presented low mortality rates and peaks of parasitaemia from 20-27 dpi, allowing monitoring of the mice until the late stage of the chronic phase. Se did not influence the survival of infected mice, as we reported before (de Souza et al. 2003); however, it exhibited positive effects on heart function.

Cardiac-gated MRI readily detects alterations in both RV and LV and its non-invasive nature makes it a suitable technique for serial in vivo study of the structure and function of the hearts of infected mice (Hoit 2001, Jelicks et al. 2002). We recently proposed that enlargement of the RV may represent a marker for chagasic cardiomyopathy in mice (Huang et al. 1999, de Souza et al. 2005) and some studies have addressed the possible reduction of the severity of this cardiomyopathy using various strategies (Jelicks et al. 2002, Goldenberg et al. 2008). In the present study, the Se prevention scheme was able to significantly reduce the infection-induced increase of the RVID at the acute and chronic phases of *T. cruzi* infection. Although the analysis of variance within the Cont-Se0 group was 0.0034 at the chronic phase, this group presented an average RVID of 2,226 mm with a standard deviation of 0.050 mm. The mechanisms underlying the effect of Se on the prevention of heart enlargement are currently under investigation. To our knowledge, this is the first MRI-based description of the preventive effects of Se treatment on ventricular dilatation. The disease progression observed in the experimental model used in this study may be analogous to the slow changes that occur during the early chronic phase of infection in humans; therefore, Se treatment could be used to halt disease progression in patients at this stage of infection.

Our findings using the Se prevention scheme encouraged us to study the effectiveness of Se treatment in reducing cardiac alterations already established in the chronic phase of infection as a model for chronic human Chagas heart disease. In these experiments, we initiated Se treatment starting at 160 dpi, 10 days after heart alterations can be observed histologically

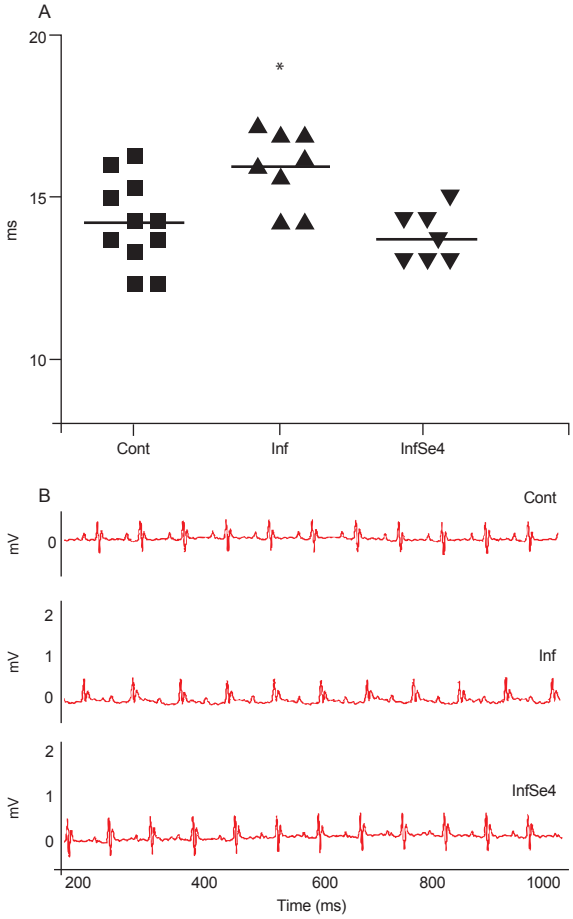

Fig. 2: P wave of mice submitted to selenium (Se) reversion scheme. A: P wave duration expressed in individual values with means; B: electrocardiographic recordings of three representative mice; Cont: uninfected; Inf: infected; InfSe4: infected and treated with 4 ppm Se. Asterisk means $p < 0.05$ as compared to Cont and InfSe4.

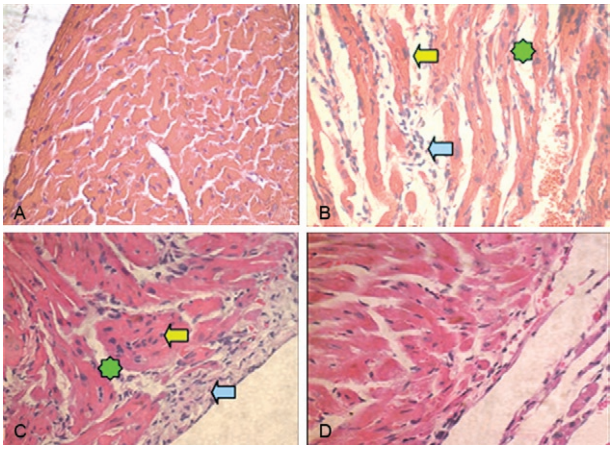

Fig. 3: histopathological analysis of mice at the late stage of the chronic phase of *Trypanosoma cruzi* infection. Paraffin-embedded Swiss mice hearts were sectioned and subjected to hematoxylin and eosin staining. A-D: heart, pericardium and myocardium. Uninfected mice show normal pericardium and myocardium structures (A, magnified 40X) in contrast to infected mice at 150 days post infection (dpi), that show mild to moderate mononuclear inflammatory infiltrate (blue arrow), edema (green asterisk) and reactive changes in muscle cells (yellow arrow) (B, magnified 40X). At 273 dpi (magnified 40X), non-treated infected mice present mild to moderate mononuclear inflammatory infiltrate, edema and reactive changes in muscle cells, while histology of infected mice treated with 4 ppm selenium (D, magnified 40X) shows minimal pericarditis.

(Chandra et al. 2002, Huang et al. 2003). Treatment with Se for 76-98 days reversed atrial alterations, observed in all infected non-treated mice; this effect was manifested as a normalization of the P wave duration that is typically prolonged in infected-untreated mice. As we were particularly interested in measuring the RVID using MRI, we focused on acquiring images at end diastole that showed the fullest expansion of the ventricular chambers. We did not acquire images at end systole and therefore could not accurately calculate the ejection fraction from the MRI data.

It has been reported that mice infected with the Brazil strain of *T. cruzi* presented inflammation and fibrosis of the myocardium at 150 dpi (Chandra et al. 2002, Huang et al. 2003). In the present work, infected mice at the late stage of the chronic phase presented pericarditis with a discrete infiltrated focus around the coronary (more precisely, close to the sinoatrial node). The pericarditis could affect the normal electric stimulation. We suggest that, even if discrete, the inflammation could impair the initiation of electric stimulation of the atrium and the beginning of the stimulation in ventricles, subsequently affecting the contraction of ventricles. Unfortunately, the histology could only be evaluated in the small number of surviving infected mice; however, it was possible to observe that Se treatment reversed the pericarditis in two out of three infected mice, whereas all of the non-treated infected mice exhibited this alteration.

The beneficial effects of treatment with the inorganic form of Se were demonstrated in experimental models with several cardiac injuries. Dietary supplementation of 100 µg Se (sodium selenite) in patients submitted to total parenteral nutrition was shown to revert arrhythmias and cardiomegalies and lead to an increase in left ventricle ejection fraction (Saito et al. 1998). In addition, the incidence of Keshan disease, an endemic dilated congestive myocardiopathy in areas of Se deficiency in China and Russia, has been shown to be prevented by oral Se supplementation at a dosage of 150-300 µg/week (Reeves et al. 1989). It should be noted that Se supplementation has also been suggested as a strategy for prevention of myocardial diseases in other studies of human cardiopathy (Korpela et al. 1989). This strategy may be classified as either supplementation or treatment depending on the concentration of Se used and on the country-specific legal regulations on drugs that are different, for example, between the USA (Food and Drug Administration) and Brazil (National Health Surveillance Agency). Thus, we opted to describe the presented effects as resulting from Se treatment.

We conclude that Se treatment prevents right ventricular enlargement in experimental Chagas disease models and even after the establishment of cardiac damage, Se treatment reverses the inflammation in the pericardium close to the atrioventricular node. These data indicate that Se can be considered at least as an adjuvant for the treatment of cardiac alterations caused by *T. cruzi* infection. A clinical trial investigating the effectiveness of Se supplementation in preventing heart dilation at adequate doses should be encouraged; particularly in endemic areas with high risks of *T. cruzi* infection and Chagas disease progression in chronic cases. We have recently shown that either an inorganic (sodium selenate) or an organic form (Se-methylselenocysteine) of Se were able to reduce intestine lumen diameter and increase intestinal motility at the chronic phase of infection (de Souza et al. 2010). Based in our experimental findings reported both here and in previous works, a clinical trial is in preparation to evaluate if Se treatment is capable of (i) impairing the progression of ventricular dysfunction in patients with mild heart dysfunction and (ii) improving cardiac function in patients with moderate heart dysfunction.

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