

# Characterization of cardiopulmonary function and cardiac muscarinic and adrenergic receptor density adaptation in C57BL/6 mice with chronic *Trypanosoma cruzi* infection

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## SUMMARY

Circulating antibodies in chagasic patients interact with myocardial  $\beta$  adrenergic and muscarinic cholinergic receptors, triggering intracellular signals that alter cardiac function along the course of the disease. However, until now, experimental data in models of chronically infected chagasic mice linking the effects on myocardial  $\beta$  adrenergic and muscarinic receptors to cardiopulmonary dysfunction is lacking. Thus, we studied C57BL/6 mice 8 months after intraperitoneal injection of 100 trypomastigote forms of the Colombian strain of *T. cruzi*. Uninfected mice, matched in age, were used as controls. Histopathological analyses (inflammation and fibrosis) and radio-ligand binding assays for estimation of muscarinic and adrenergic receptor density were performed in myocardium tissue samples. When compared to controls, infected mice had electrical conduction disturbances, diastolic dysfunction, lower O<sub>2</sub> consumption and anaerobic threshold. In addition, hearts of chronic chagasic mice had intense inflammation and fibrosis, and decreased  $\beta$  adrenergic and increased muscarinic receptor densities than normal controls. Our data suggest that chronic *T. cruzi* infection causes alterations in cardiac receptor density and fibrosis deposition which can be associated with cardiac conduction abnormalities, diastolic dysfunction and lower exercise capacity, associating for the first time all these functional and histopathological alterations in chagasic mice.

Key words: Chagas' disease, cardiopulmonary function, cardiac receptors, mouse model.

## INTRODUCTION

Chagas' disease, caused by a haemoflagellate protozoan parasite *Trypanosoma cruzi*, is one of the most common determinants of congestive heart failure and sudden death in Latin America, where it represents a serious health problem, affecting millions of persons (WHO, 1995). Chagas' disease is a complex illness that can evolve in different consecutive phases. The first corresponds to an acute phase occurring after the parasite infection and characterized by intense parasitism and blood parasitaemia. After this, an asymptomatic or indeterminate period, marked by the absence of clinical symptoms is observed. Finally, months or decades after the primary infection, some of the infected individuals enter the chronic phase, which is characterized by chronic myocarditis and the so-called mega syndromes,

affecting the gastro-intestinal tract. Less than 10% of chronic patients present gastrointestinal abnormalities, such as pathological dilations of organs from the digestive tract (Dias, 1989; Dias and Coura, 1997). Approximately 30% of the infected individuals show heart involvement leading to heart failure. Furthermore, the chronic chagasic cardiomyopathy (CChC) is characterized by intense myocarditis and multiple arrhythmias, such as ventricular premature beats, complete right bundle branch block, left anterior fascicular block and atrioventricular block (Koerbele, 1968; Rassi *et al.* 1992).

The mechanism responsible for the development of cardiomyopathy is still controversial and several hypotheses have been proposed. One hypothesis is based on an immune response directed against *T. cruzi* antigens at sites of parasite persistence, leading to a pathogenic inflammatory process (Tarleton, 2001; Higuchi *et al.* 1997). Another hypothesis is that CChC is the result of an auto-immune process triggered in some individuals by *T. cruzi* infection (Cunha-Neto *et al.* 1995; Engman and Leon, 2002). The autoimmunity hypothesis is

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supported by the lack of parasites at lesion sites in the chronic phase of the disease and the presence of autoantibodies in the sera from chagasic patients that recognize a number of cardiac-specific antigens, such as myosin,  $\beta_1$  adrenergic and  $M_2$  muscarinic receptors (Borda *et al.* 1984; McCornick and Rowland, 1989; Sterin-borda *et al.* 1991; Cunha-Neto, 1995; Farias *et al.* 1997; Goin *et al.* 1997). Another mechanism attributes CChC to vascular alterations, which lead to inflammatory and ischaemic changes (Tanowitz *et al.* 1996; Petkova *et al.* 2001). In addition, a neurogenic hypothesis has been proposed, in which autonomic denervation is associated with cardiac disturbances (Davila *et al.* 1989).

Much of the information about the pathogenesis of Chagas' disease comes from experimental models of chronic chagasic cardiomyopathy, especially using the mouse model. The murine model of CChC reproduces many of the structural, functional and immunological alterations observed in human CChC (Tanowitz *et al.* 1996; Petkova *et al.* 2001; Tarleton, 2001; Engman and Leon, 2002). Until now, however, no comprehensive study has been done analysing the functional, histopathological and receptor density alterations associated with the mouse model of CChC. In this work we investigated the association between the cardiopulmonary function, heart histopathology and muscarinic and  $\beta$  adrenergic receptor density adaptations in chronically infected mice.

## MATERIALS AND METHODS

### *Animals and infection*

Two-month-old C57BL/6 female mice raised and maintained in the animal facilities at the Gonçalo Moniz Research Center – FIOCRUZ, with rodent diet and water *ad libitum* were used in the experiments. They were infected by intraperitoneal injection of 100 trypomastigote forms of the Colombian strain of *T. cruzi* (Frederici *et al.* 1964). Trypomastigotes were obtained by *in vitro* infection of the LCC-MK2 cell line. Parasitaemia was evaluated at different times after infection by counting the number of trypomastigotes in peripheral blood samples (Brener, 1973). All infected animals were studied 8 months after the initial infection with the parasites, at 10 months of age. Uninfected female mice, matched in age, were used as controls. The current investigation conformed to the Guide for Care and Use of Laboratory Animals, published by the US National Institutes of Health.

### *Electrocardiogram*

ECG records were performed using the Bio Amp device PowerLab System (PowerLab 2/20;

ADInstruments, Castle Hill, Australia), recording the bipolar lead I. All animals were anaesthetized by intraperitoneal injection of xilazine at 10 mg/kg body weight and ketamine at 100 mg/kg body weight. Electrocardiographic recordings were obtained after the induction of general anaesthesia. All data were transferred to a computer for further analysis using Chart 5 for Windows software (PowerLab; ADInstruments, Castle Hill, Australia). Wave durations (ms) were calculated automatically by the software after cursor placement. Measurements are average values determined from 14 consecutive ECG records obtained using bandpass filtering (1 to 100 Hz). The sampling rate was 1 kHz. ECG analysis included the following measurements: heart rate; PR interval; P wave duration; QT interval; QTc; atrioventricular block; intraventricular block and other arrhythmias. The heart rate corresponded to P wave frequency calculated from the mean P wave interval duration when AV conduction was not 1:1, or it could be calculated directly by the software using a derivate-based QRS detection algorithm to calculate heart rate by detecting the peak of R waves automatically. As in rodent ECGs T waves are normally not separated from QRS complex, QT interval was measured instead of QRS complex duration (Bestetti and Oliveira, 1990). The QT interval was measured from the beginning of the QRS up to the end of the T wave. Definition of the end of the T wave was the point where the signal returned to the isoelectric line (Wang *et al.* 2000). QTc was calculated dividing the QT interval by the square root of the RR interval. The values of all intervals and waves duration were expressed in milliseconds.

### *Echocardiography*

Echocardiographic analyses were performed using a 2-dimensional echo Doppler cardiogram (Esaote, model CarisPlus, Firenze-Italy). The standard parasternal long and short axis views were obtained with a 10 MHz transducer. All animals were anaesthetized by intraperitoneal injection of xilazine at 10 mg/kg and ketamine at 100 mg/kg body weight. The following parameters were acquired according to the American Society of Echocardiography: diameters of the left ventricle in diastole (LVd) and systole (LVs), septal and posterior wall thickness, shortening fraction (SF), left ventricular ejection fraction and aortic valve area at M-mode and B-mode (AVA), isovolumetric relaxation time (IRT), the early (E) and late (A) peaks of diastolic transmitral flow velocities, E deceleration time (EDT), ejection time (ET), maximal aortic velocity (AoVmax) and flow velocity integral of aortic flow (FVI) using Doppler. The cardiac output (CO) was obtained from the product of FVI by AVA and heart rate.

*Cardio-pulmonary exercise testing analyses*

All animals were weighed before the exam. A motor-driven treadmill chamber (AccuScan Instruments, Model LC2/M, USA) was used to exercise the animals. Room air was pumped into the chamber at a flow rate ( $j$ ) of 680 ml/min. Outflow (same as  $j$ ) was set at 680 ml/min and directed to an oxygen and carbon dioxide analyser (Sable Systems FC-1B O<sub>2</sub> and CA-2A CO<sub>2</sub> Analyzers, USA), to measure the maximal consumption of oxygen (VO<sub>2</sub> max), the production of carbon dioxide (VCO<sub>2</sub>), the respiration exchange ratio (RER) and the anaerobic threshold during exercise. Tests were performed at a 10 degree fixed inclination (ACCUPACER treadmill, ACCUSCAN Instruments, Inc., Columbus, Ohio, USA) and mean room temperature was maintained at 22 °C. Mice were placed in the treadmill chamber for an adaptation period of 5 min before running for 5 min at 4 different velocities (0.1, 0.2, 0.3 and 0.4 m/s) and maintained in the last velocity until exhaustion. Stainless steel grids at the end of the treadmill provided an electrical stimulus to keep the mice running. The criterion for reaching VO<sub>2</sub> max was its levelling off despite increasing running velocity or when animals stayed in the steel grids for more than 10 sec. Another criterion for reaching VO<sub>2</sub> max was a RER above 1.0. VO<sub>2</sub> consumption and CO<sub>2</sub> production were obtained by the following formulae:

$$\text{VO}_2 \text{ max} = (\% \text{O}_2 \text{ max} - \% \text{O}_2 \text{ adaptation}) \times 680 \text{ ml/min/weight (kg)}$$

$$\text{VCO}_2 = (\% \text{CO}_2 \text{ at end} - \% \text{CO}_2 \text{ adaptation}) \times 680 \text{ ml/min/weight (kg)}$$

The anaerobic threshold was determined at the point where the curves for VO<sub>2</sub> levelled off or stabilized and the CO<sub>2</sub> continued to increase. The result was expressed as ml/kg/min of VO<sub>2</sub> consumption.

*Histopathological analysis*

Hearts from chronic chagasic and normal mice were removed and sectioned transversally in 3 segments. One fresh segment was processed for the radio-ligand binding assay. The other segments were fixed in buffered 10% formalin. Sections of paraffin-embedded tissue were stained with standard haematoxylin/eosin and Sirius red staining for evaluation of inflammation and fibrosis, respectively, by optical microscopy. Images were digitalized using a colour digital video camera (CoolSnap, Montreal, Canada) adapted to a BX41 microscope (Olympus, Japan). The images were analysed using the Image Pro Program version 5.0 (Media Cybernetics, San Diego, CA), to integrate the number of inflammatory

cells counted by unit area or the whole fibrotic area. Ten fields were counted per heart from every mouse of each group.

*Preparation of homogenates for the radio-ligand binding experiments*

Heart fragments were minced and suspended in buffer A (25 mmol/l HEPES, 1 mmol/l EDTA, 0.1 mmol/l PMSF, and 5 mmol/l MgCl<sub>2</sub>, pH 7.5) and then homogenized with three 15 sec strokes with a Tissumizer (Tekmar Company, Cincinnati, OH, USA) and 60 sec resting intervals. The suspensions were filtered through cheesecloth and centrifuged at 4 °C for 15 min at 48 000 *g*. The resulting pellets were resuspended in buffer B (similar to buffer A, except for 2 mmol/l MgCl<sub>2</sub>) with the aid of a Teflon-glass homogenizer. Next, the suspensions were centrifuged again for 30 min at 48 000 *g*. Finally, the pellets were resuspended in buffer B and stored at -70 °C as described previously (Giménez *et al.* 2005).

*Radio-ligand binding assay.*  $\beta$ -adrenergic receptor density (B<sub>MAX</sub>) was determined by [<sup>3</sup>H] dihydroalprenolol hydrochloride ([<sup>3</sup>H]-DHA, 120 Ci/mmol, New England Nuclear, Boston, MA, USA) saturation binding assays. The homogenates (150  $\mu$ g of total protein) were incubated in buffer C (25 mmol/l Tris-HCl, 1 mmol/l EDTA and 5 mmol/l MgCl<sub>2</sub>, pH 7.5) with increasing concentrations of [<sup>3</sup>H]-DHA (5–80 nmol/l) at 25 °C for 1 h in a final volume of 400  $\mu$ l. The reaction was stopped by rapid vacuum filtration using a cell harvester (Brandel, Gaithersburg, MD, USA) through GF/B glass fibre filters pre-soaked in 0.3% polyethylenimine followed by 3 fast washes with ice-cold 10 mmol/l phosphate buffer, pH 7.4. Trapped radioactivity was determined by liquid scintillation counting (efficiency of 36%, Packard Instruments CO., Meriden, CT, USA). Non-specific binding was defined as the bound radioactivity in the presence of unlabelled propranolol (10  $\mu$ mmol/l). Specific [<sup>3</sup>H]-DHA binding activity was estimated by subtracting the non-specific binding from the total binding.

Muscarinic receptor density was determined by L-[N-methyl-<sup>3</sup>H] scopolamine methyl chloride ([<sup>3</sup>H]-NMS, 84 Ci/mmol, Amersham Biosciences, Piscataway, NJ, USA) saturation binding assays. Accordingly, homogenates (150  $\mu$ g) were incubated in buffer B with increasing concentrations of [<sup>3</sup>H] NMS (10–1200 pmol/l) at 37 °C for 2 h in a final volume of 2 ml. Non-specific binding was defined by unlabelled atropine (2  $\mu$ mol/l) at 37 °C for 2 h. Subsequent procedures were performed as previously described for the  $\beta$ -adrenergic receptor. Estimates of maximal bound (B<sub>max</sub>) and the equilibrium dissociation constant (K<sub>D</sub>) were obtained from least square curve fitting analysis according to

Table 1. Echocardiography analysis in *Trypanosoma cruzi*-infected mice

(LA = left atrium diameter; LVd = left ventricular diameter in diastole; LVs = left ventricular diameter in systole; FS = percentual of fractional shortening; EF = left ventricular ejection fraction at M-mode; EDT = peak early diastolic transmitral flow velocity; E/A = ratio between peak early (E) and late (A) diastolic transmitral flow velocities; IRT = isovolumetric relaxation time; ET = ejection time; AoV max = maximal aortic velocity; CO = cardiac output. Values represent the mean  $\pm$  S.E.M.)

Parameters	Control group <i>n</i> = 5	Infected group <i>n</i> = 6	<i>P</i>
LA (cm)	0.17 $\pm$ 0.02	0.18 $\pm$ 0.03	0.4741
LVd (cm)	0.27 $\pm$ 0.04	0.29 $\pm$ 0.05	0.1866
LVs (cm)	0.11 $\pm$ 0.01	0.15 $\pm$ 0.05	0.0570
Septal wall (cm)	0.07 $\pm$ 0.01	0.08 $\pm$ 0.00	0.0477
Posterior wall (cm)	0.07 $\pm$ 0.01	0.08 $\pm$ 0.01	0.1540
FS (%)	60 $\pm$ 3	53 $\pm$ 13	0.1584
EF (%)	93 $\pm$ 2	86 $\pm$ 12	0.1157
EDT (ms)	29 $\pm$ 10	55 $\pm$ 11	0.0044
E/A	0.97 $\pm$ 0.65	2.18 $\pm$ 0.42	0.0305
IRT (ms)	29 $\pm$ 14	46 $\pm$ 22	0.1143
ET (ms)	75 $\pm$ 11	106 $\pm$ 16	0.0056
AoVmax (m/s)	0.50 $\pm$ 0.13	0.30 $\pm$ 0.17	0.0414
CO (L/min)	0.01 $\pm$ 0.03	0.02 $\pm$ 0.01	0.2340

the rectangular hyperbolic model using the GraphPad Prism 4.03 software (San Diego, CA, USA).

#### Statistical analyses

All continuous variables were presented as mean  $\pm$  standard error, except for receptor density data. Histological data and cardiopulmonary parameters were analysed using Student's *t*-test and Mann Whitney test with Prism Software version 3.0 (GraphPad Software, San Diego, CA, USA). For analysis of receptor density we employed the non-linear regression analysis of radio-ligand saturation experiments. Differences were considered significant if *P* was equal to or less than 0.05.

## RESULTS

#### Altered echocardiographic parameters in chronic chagasic mice

In order to evaluate the echocardiographic disturbances induced by *T. cruzi* infection in the murine model, non-infected and chronic chagasic C57BL/6 mice (8 months after infection) were subjected to echocardiography under anaesthesia. Table 1 summarizes the parameters obtained from both groups. Mice infected with *T. cruzi* had a thicker septal wall, a higher EDT, a higher E/A, a higher ET and a lower AoV max when compared to uninfected controls.

*T. cruzi* infection induces lower resistance to exercise in mice. To investigate whether *T. cruzi* infection compromises the exercising capacity of infected mice, treadmill test and respiratory gas analyses were

performed in age-matched infected and non-infected mice. Chronic chagasic mice endured less exercise duration and had lower VO<sub>2</sub> max, higher RER, and lower anaerobic threshold when compared to controls (Table 2).

#### Chronic chagasic mice develop functional and structural abnormalities

To demonstrate the involvement of *T. cruzi* infection in the development of cardiac electric abnormalities we recorded the electric extracellular activity from chronic chagasic and normal mice by electrocardiogram. All infected mice had arrhythmias such as intraventricular conduction disturbances, atrio-ventricular block and atrioventricular dissociation (Fig. 1A, Table 3). Electrocardiographic analyses in control C57BL/6 mice had no abnormalities (Fig. 1B, Table 3).

Histopathological analysis of heart sections demonstrated a 2-fold increase in the number of inflammatory cells in infected mice (Fig. 2A) in relation to normal mice. With regard to heart fibrosis, infected mice had significantly more fibrotic areas when compared to control mice (Fig. 2B; \* *P* < 0.05, Student's *t*-test and Mann-Whitney test).

#### Muscarinic and $\beta$ receptor densities are altered in *T. cruzi*-infected mice

To determine the density of the muscarinic and  $\beta$  adrenergic cardiac receptors, we performed radio-ligand saturation experiments. As shown in Table 4, maximal [<sup>3</sup>H] NMS binding to cardiac mAChR is

Table 2. Results of cardiopulmonary exercise testing

(VO<sub>2</sub> max = maximum consumption of oxygen; VCO<sub>2</sub> = carbon dioxide production; RER = respiratory equilibration rate (=VO<sub>2</sub> /VCO<sub>2</sub>). Values represent the mean ± S.E.M.)

Parameters	Control group n = 5	Infected group n = 6	P
Exercise duration (min)	13 ± 2	5 ± 2	0.0001
VO <sub>2</sub> max (ml/kg/min)	56 ± 18	27 ± 10	0.0041
VCO <sub>2</sub> (ml/kg/min)	68 ± 14	53 ± 15	0.0600
RER	1.28 ± 0.30	2.06 ± 0.46	0.0088
VO <sub>2</sub> at anaerobic threshold (ml/kg/min)	42 ± 12	20 ± 6	0.0068

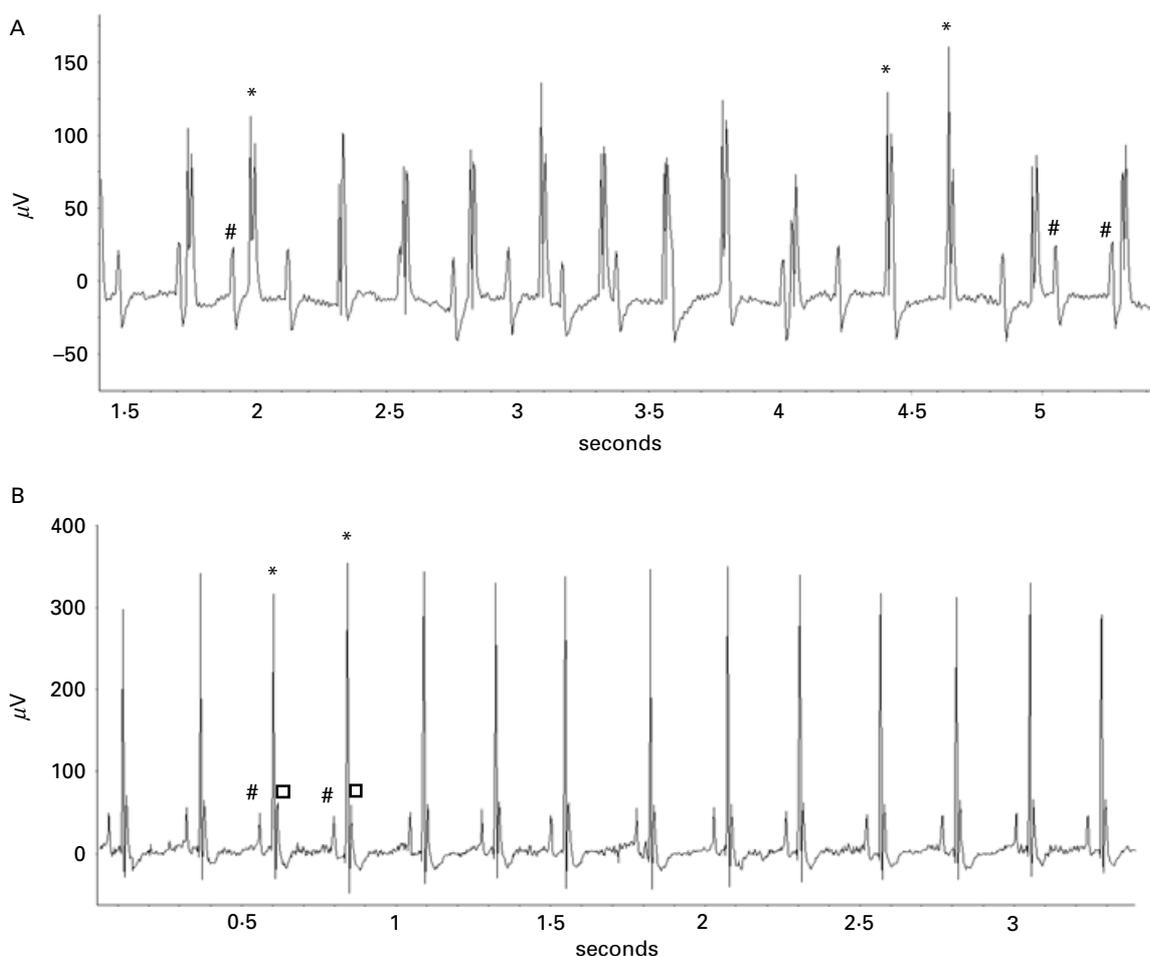


Fig. 1. ECG recordings from C57Bl/6 mice. (A) ECG of a *Trypanosoma cruzi*-infected mouse recorded 8 months after infection showing total AV conduction block and intraventricular conduction disturbances. P wave frequency was 350 bpm. (B) ECG recorded in a control C57Bl/6 mouse with regular sinus rhythm and beat rate of 240 bpm. #, P wave; \*, QRS complex; □, T wave.

increased in the *T. cruzi*-infected group as compared to controls (226.3 and 123.4 fmol/mg of protein, respectively). The affinity of [<sup>3</sup>H] NMS to the receptor in infected mice, although showing a tendency, was not significantly different from the control group (197.0 and 91.7 pmol/l, respectively). In contrast to what was observed with the muscarinic receptor density, maximal [<sup>3</sup>H]-DHA binding for the *T. cruzi*-infected group was decreased in comparison

to the control group (22.7 fmol/l and 31.5 fmol/mg of protein, respectively). As with the cardiac mAChRs, no differences were observed for the equilibrium dissociation constant (K<sub>D</sub>) (Table 4).

DISCUSSION

Chronic chagasic cardiomyopathy in mice is a well-studied model to investigate the physiopathology of

Table 3. Eletrocardiographic parameters measured in the experimental groups

(Values represent the mean  $\pm$  S.E.M. of the studied mice. In the infected group the heart rate was calculated by P wave interval when AV conduction was not 1 : 1. and PR intervals were only measured in animals were AV conduction was 1 : 1 ( $n=4$  for the infected group). AVD, atrioventricular dissociation; IVCD, intraventricular conduction disturbance; AVB, atrium-ventricular block; ASI, acute subendocardial ischemia; SB, Sinusal blockage. \*  $P<0.05$  compared to *Trypanosoma cruzi*-infected mice, Student's *t*-test. Some animals developed more than one type of cardiac conduction disturbances.)

Experimental group ( <i>n</i> )	Heart rate (bpm)	PR interval (ms)	RR interval (ms)	QT interval (ms)	QT <sub>c</sub>	Cardiac conduction disturbances
Infected ( $n=6$ )	315 $\pm$ 69.2*	81 $\pm$ 8.0*	213 $\pm$ 68.6*	41 $\pm$ 5.8*	2.9 $\pm$ 0.73*	AVD ( $n=2$ ) IVCD ( $n=4$ ) AVB ( $n=4$ ) ASI ( $n=4$ ) SB ( $n=1$ )
Control ( $n=5$ )	223 $\pm$ 40.2	49 $\pm$ 4.4	277 $\pm$ 55.0	31 $\pm$ 2.3	1.8 $\pm$ 0.22	—

Table 4. [<sup>3</sup>H]-NMS and [<sup>3</sup>H]-DHA saturation binding parameters from control and *Trypanosoma cruzi*-infected mice heart homogenates

Ligand/Binding Parameter	Muscarinic receptor antagonist ([ <sup>3</sup> H]-NMS)			$\beta$ -Adrenergic receptor antagonist ([ <sup>3</sup> H]-DHA)		
	Control group	<i>T. cruzi</i> -infected group	<i>P</i> *	Control group	<i>T. cruzi</i> -infected group	<i>P</i> *
B <sub>MAX</sub> (fmol/mg of total protein)	123.4 $\pm$ 8.8	226.3 $\pm$ 15.4	0.004	585.9 $\pm$ 43.5	372.5 $\pm$ 33.4	0.018
K <sub>D</sub> §	91.7 $\pm$ 24.7	197.0 $\pm$ 40.6	0.091	22.7 $\pm$ 8.5	31.5 $\pm$ 10.6	0.811

\* Statistical difference between the means was determined by the unpaired Student's *t*-test (two tails).  $P<0.05$  was considered significant.

§ K<sub>D</sub> values for [<sup>3</sup>H]-NMS are given in pmol/l and for [<sup>3</sup>H]-DHA in nmol/l.

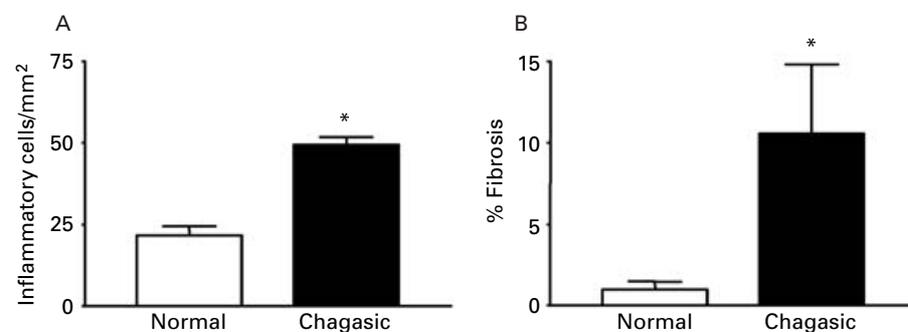


Fig. 2. Morphometrical analyses of heart sections from normal and infected mice. Quantification of inflammatory cells (A) and of fibrosis (B) in heart sections of C57Bl/6 control mice and *Trypanosoma cruzi*-infected C57Bl/6 mice. Values represent the mean  $\pm$  S.D. of 6 mice/group (A) and 8 mice/group (B). \*  $P<0.05$ , compared to normal group, Student's *t*-test and Mann-Whitney test.

the disease because it resembles the human illness characterized by inflammatory mononuclear cell infiltration, electro- and echocardiographic abnormalities and reactive cardiac antibodies (Gorelik *et al.* 1992; Mirkin *et al.* 1994; Goin *et al.* 1999; Garcia *et al.* 2005). In our study we simultaneously evaluated, for the first time, mechanical, electrical, biochemical (increase in M<sub>2</sub> cardiac receptor density) and structural cardiac abnormalities, and myocarditis in *T. cruzi*-infected mice.

Doppler echocardiography is an important non-invasive tool for the assessment of diastolic function. Doppler inflow velocity-derived variables remain the cornerstone of diastolic function evaluation. The 4 useful variables obtained from mitral flow interrogation are peak early diastolic transmitral flow velocity (E), peak late diastolic transmitral flow velocity (A), early filling deceleration time (DT) and A-wave duration. We used the analysis of the first 3 variables. In our study, mice infected with *T. cruzi* had a ratio

E/A >1, a prolonged EDT and normal FS and left ventricular EF. These findings are compatible with severe diastolic dysfunction or restrictive pattern, in which a severe decrease in compliance leads to further elevation of left atrial pressure and results in very high E wave, low A wave and progressive shortening of EDT. In chagasic patients, diastolic heart failure is associated with a marked increase in morbidity and all-cause mortality independent of the presence of systolic failure.

Impairment in diastolic filling may be related to both abnormalities of active relaxation of the myocardium and passive elastic properties as a result of hypertrophy, myocardial ischaemia, decreased adrenergic tone, or increased myocardial connective tissue. Accordingly, the *T. cruzi*-infected group had decreased adrenergic receptor density and an increase in inflammation and fibrosis, factors that may explain the diastolic dysfunction observed in the present study. We did not find differences in isovolumic relaxation time (IVRT) between the groups. It is possible that the inability of the left ventricle to fill in early diastole and a progressive elevation of left atrial pressure results in an early opening of the mitral valve and return of the IVRT to values observed under normal conditions.

During cardiopulmonary exercise testing our mice showed reduced exercise duration, lower  $\text{VO}_{2\text{max}}$  and anaerobic threshold. Depletion of  $\beta$  receptors could be one of the explanations for the low functional capacity during cardiopulmonary exercise testing in our infected mice. Other possibilities are: impairment in relaxation increases myocardial wall tension in diastole, and loss of cardiac elastic properties causes a reduction in compliance, both of which may lead to an increase in pulmonary venous pressure. Patrianakos *et al.* (2004) showed that patients with restrictive pattern had not only lower cardiopulmonary exercise test but also higher N-terminal pro-atrial (ANP) and N-terminal pro-brain natriuretic peptide (BNP) levels [27]. They also found a significant correlation between BNP levels and maximal oxygen consumption. A reasonable explanation is that patients with elevated left ventricular diastolic pressures at rest are prone to marked elevation of capillary wedge pressure during exercise and this may be the reason for the observed correlation between natriuretic peptides and exercise capacity.

ECG data demonstrate an increased P wave frequency associated with augmented PR and QT intervals in infected mice. This suggests that the increased heart rate is not the result of higher adrenergic activity, as indicated by the lower beta-adrenergic receptor density detected in our experiments. If this was the case one should expect increased P wave rate associated with decreased PR and QT intervals. Therefore we speculate that the increase in heart rate can result from local

mechanisms related to a higher degree of inflammation observed in hearts from infected mice. In this regard the mouse model seems to differ from the human case, where bradycardia is the most common finding.

Sera from chagasic patients contain antibodies that can react '*in vitro*' with plasma membrane of living heart cells inducing functional changes and modifying their  $\beta$  adrenergic and cholinergic receptor activities (Masuda *et al.* 1998; Hernández *et al.* 2003). In the present study radio-ligand binding assays showed an increased density of muscarinic receptors only in the infected group. Many reports demonstrated a relevant role for  $M_2$  receptors in the development of cardiac conduction abnormalities (Masuda *et al.* 1998; Hernández *et al.* 2003), thus suggesting that the increased expression of  $M_2$  receptor may explain the various arrhythmias detected in the infected group.

More recently, Giménez *et al.* (2005) created murine models of autoimmune cardiomyopathy by gene gun DNA dermal bombardment using plasmids encoding the entire sequence of the  $M_2$  muscarinic acetylcholine and the  $\beta_1$  adrenergic receptors. In this study, cardiac contractile impairment was most noticeable in the  $\beta_1$  adrenergic receptor-immunized animals. Interestingly, muscarinic receptor density in hearts of both  $M_2$  AChR and  $\beta_1$  adrenergic receptor-immunized mice was significantly up-regulated. On the other hand,  $\beta_1$  adrenergic receptor density was similarly decreased in both experimental groups by a 2-fold factor. In line with these previous studies, we observed an increase in muscarinic receptor density and a decrease in  $\beta_1$  adrenergic receptor density in the murine model of Chagas' disease. Alternatively, the up- and down-regulation of muscarinic and adrenergic receptors, respectively, may be due to cardiac autonomic dysfunctions known to occur in chagasic cardiomyopathy.

In conclusion, the present study analyses a series of functional, biochemical and histopathological parameters in mice with chronic chagasic cardiomyopathy showing the typical inflammation and fibrosis, but also an up-regulation of muscarinic  $M_2$  receptors and a down-regulation in  $\beta_1$  adrenergic receptors, all of which are compatible with the conduction disturbances, diastolic dysfunction and lower exercise capacity observed in these animals.

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