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The cholinergic system in cyclophosphamide induced-Chagas dilated myocardiopathy in *Trypanosoma cruzi* infected rats: an electrocardiographic study.

Marialeycnan Labrador-Hernández[1], Oscar Suárez-Graterol[1], Urimare Romero-Contreras[2], Lila Rumenoff[3], Claudina Rodríguez-Bonfante[4] y Rafael Bonfante-Cabarcas[1].

[1]Unidad de Bioquímica "José Antonio Moreno Yanes", [2]Departamento de Ciencias Morfológicas, [3]Departamento de Patología Médica y [4]Unidad de Parasitología Médica, Decanato de Medicina, Universidad Centroccidental Lisandro Alvarado. Barquisimeto, Venezuela.

Key words: Chagasic cardiomyopathy, *Trypanosoma cruzi,* cyclophosphamide.

Abstract. Chronic Chagasic Cardiomyopathy (CCC) has been related to the cholinergic system by the neurogenic and autoimmune theories. The neurogenic theory explains cardiomyopathy as a result of post-ganglionic parasympathetic denervation. Cyclophosphamide (CP) facilitates the development of autoimmune disease because of a selective depletion of suppressor T cells. In this study we characterized the phenylephrine-induced vasovagal reflex using selective cholinergic drugs, in two rat models: *Trypanosoma cruzi* (TC) infected animals and CCC CP-treated rat model. To achieve this goal, 3 week old-90 Sprague Dawley rats were divided into four groups: Control (C), CP, TC and TCCP; TC and TCCP were inoculated with 1000 trypomastigotes/g; CP and TCCP were treated with CP 20 mg/Kg twice a week for five times. After 6 months, the studied animals underwent electrocardiographic (EKG), radiographic (Rx) and histopathological (HP) assesments. The vagal integrity was evaluated by application of phenylephrine (PE) plus tacrine, while the muscarinic cholinergic function was evaluated using selective M1, M2, M3 and M4 muscarinic antagonists. Our data show show that TCCP rats displayed the highest frequency of EKG, Rx and HP disturbances. TC and TCCP rats exhibited a decreased response to: 1) phenylephrine-induced vagal baroreflex bradycardia; 2) methoctramine-, 4-DAMP- and tropicamide-induced tachycardia; 3) methoctramine-induced QRS shortening, and 4) tropicamide-induced QT prolongation. In conclusion, CP facilitates the development of CCC in

Corresponding author: Rafael Bonfante-Cabarcas. Unidad de Bioquímica "José Antonio Moreno Yanes", Decanato de Medicina, Universidad Centro-Occidental Lisandro Alvarado. Av. Libertador con Av. Andrés Bello, Barquisimeto, Venezuela. Codigo Postal: 3001. Teléfono 58-251-2591854, Fax: 58-251-2591886.
E-mail: rcabarca@ucla.edu.ve

Trypanosoma cruzi infected rats, by promoting parasympathetic disturbances that appear as consequence of alterations on the muscarinic receptor distribution at different neural integration levels.

El sistema colinérgico en ratas infectadas con *Trypanosoma cruzi* con miocardiopatía chagásica inducida por ciclofosfamida: estudio electrocardiográfico.

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Palabras clave: Miocardiopatía chagásica, *Trypanosoma cruzi,* ciclofosfamida.

Resumen. La Miocardiopatía Chagásica Crónica (MCC) se relaciona con el sistema colinérgico por las teorías neurogénica y autoinmune. La teoría neurogénica explica la MCC como resultado de la denervación parasimpática. La ciclofosfamida (CF) facilita el desarrollo de enfermedades autoinmunes por una depleción selectiva de las células T supresoras. En este estudio caracterizamos el reflejo vasovagal inducido por fenilefrina usando drogas colinérgicos, en dos modelos animales: ratas infectadas con *Trypanosoma cruzi* (TC) y ratas con MCC inducida por ciclofosfamida. 90 ratas Sprague Dawley fueron divididas en 4 grupos: Control (C), CF, TC y TCCF; los grupos TC y TCCF fueron inoculadas con 1000 tripomastigotes/g; los grupos CF y TCCF fueron tratados con CF 20 mg/kg dos veces por semana por 5 veces. Después de 6 meses de evolución de la infección, las ratas fueron sometidas a estudios electrocardiográficos (EKG), radiológicos (Rx) e histopatológicos (HP). La integridad vagal fue evaluada mediante fenilefrina y tacrina, la funcionalidad colinérgica mediante antagonistas muscarínicos selectivos. Los resultados mostraron que las ratas del grupo TCCF presentaron mayor frecuencia de trastornos electrocardiográficos, radiológicos e histopatológicos. Las ratas de los grupos TC y TCCF mostraron una respuesta disminuida a: fenilefrina que induce bradicardia refleja; metoctramina, 4-DAMP y tropicamida que inducen taquicardia; metoctramina que induce acortamiento del complejo QRS; y tropicamida que induce un alargamiento del intervalo QT. En conclusión, CF facilita el desarrollo de MCC en ratas infectadas con TC, promoviendo trastornos parasimpáticos que aparecen como consecuencia de alteraciones en la distribución de los receptores muscarínicos a diferentes niveles de integración neural.

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INTRODUCTION

Chagas' disease is a health problem in several Latin American countries, affecting mainly people living in rural areas with poor sanitary conditions (1, 2). The heart is one of the main organs affected during Chagas' disease. The cardiomyopathy, occurring in 25-30% of infected patients, can lead to heart failure and sudden death.

A key Chagas' disease characteristic is an autonomic dysfunction that according to the neurogenic theory, explains the development of the Chagasic cardiomyopathy as

the result of an autonomic imbalance: predominance of the sympathetic system and the loss of parasympathetic input, due to selective and irreversible destruction of postganglionic vagal neurons during the acute phase of the infection (3). One of the methods used to evaluate vagal integrity is through the application of phenylephrine, an α adrenergic agonist, which causes vasoconstriction, increases peripheral vessel resistance and elevates blood pressure. These phenomena activate a vagal reflex that slows down the frequency of myocardial contractions in order to decrease blood pressure. Phenylephrine has been used in humans (4, 5) and animals (6, 7) suffering from Chagas' diseases to demonstrate a disruption of cardiac vagal efferences to the sinus node.

Muscarinic Cholinergic Receptors (MCR) are involved in the vagal baroreflex, both at the integration and at the effectors levels. Integration levels are located in the medulla pons at the vagal nuclei and in the intrinsic cardiac ganglia. Cholinergic receptors are present in three medullary nuclei, the nucleus tractus solitarii (NTS), nucleus ambiguous (AMB) and the dorsal motor nucleus of the vagus nerve (DMV), which have been involved in the baroreceptor reflex activation that induce bradycardia. Administration of carbachol, acetylcholine or pilocarpine into AMB and DMV elicited dose-related decreases in heart rate, similarly eserine injected into these nuclei augmented and prolonged the action of acetylcholine. These effects were completely antagonized by the muscarinic antagonist ethylbenztropine, suggesting involvement of the muscarinic cholinergic receptors in baroreflex-mediated adjustments of the heart rate (8). These findings are supported by data from competitive binding assays using selective muscarinic antagonists on NTS membranes and [^{3}H]-QNB as ligand, which showed a kinetic profile compatible with the presence of an M2 muscarinic receptor (9). However, [^{3}H]-pirenzepine (M1 selective antagonist) binding has also been reported in the subnucleus gelatinosus at presynaptic localization on the vagal afferent terminals, and this binding was reduced by ipsilateral cervical vagotomy (10), while [^{3}H]-PZ binding was not detected at the nucleus tractus solitarius (11).

Expression of four different muscarinic receptor transcripts or proteins by the cardiac ganglia, intrinsic neurons and/or by cardiac muscle tissue has been reported (12, 13). Cardiac ganglia contain more than four times more M2 mRNA than what it is found at the presynaptic level in the atria (14). The expression of four different muscarinic receptors by cardiac intrinsic neurons and muscle, provides the molecular basis for the diverse muscarinic actions observed in the heart.

The immunogenic theory, which has been postulated to explain the pathogenic process leading to Chagas' cardiomyopathy, also involves the cholinergic system with reports of the existence of autoimmune antibodies against M2 muscarinic (15) and nicotinic cholinergic receptors (16).

Of interest in the pathogenesis of autoimmune diseases, is the possibility that these pathologies might develop as consequence of regulatory T cells failure to control autoreactive T cell proliferation and B cells antibody production (17, 18). In this line of thought, the development of cardiomyopathy could be due to a non-controlled proliferation of autoreactive lymphocytes, as a consequence of the inhibition of regulatory suppressor T cell proliferation (19). During evolution of Chagas' disease, an immunosuppression phenomenon takes place, which is thought to facilitate the dissemination and establishment of the parasite in the infected host (20, 21). This has been ascribed to many mechanisms, involving regulatory or suppressor T cells

(21-23), γδ cells (24) and adherent cells (20); as well as the presence of parasite suppressive factors such as SAPA, which down-regulates T lymphocyte proliferation as a consequence of T suppressor/cytotoxic cell activation (25).

Cyclophosphamide (CP) has been used in standard protocols for the selective depletion of suppressor T cells in vivo (18), at low doses (20-125 mg/Kg) CP is able to decrease $CD25^{+}CD4^{+}$ regulatory T cells (26, 27) and to induce auto-reactive T lymphocytes (28). CP given at high doses (450 mg/Kg) can produce by itself cardiomyopathy and degenerative vascular changes (29). In relation to Chagas' cardiomyopathy, Andrade et al. (1987) (30) succeeded on enhancing chronic myocarditis, in dogs chronically infected with *Trypanosoma cruzi* (*T. cruzi*) and treated with low doses of CP. This finding suggested that CP interfered with the immunologic suppressor network that is thought to maintain the chronic indeterminate (or latent) phase of *T. cruzi* infection.

Electrocardiography is a useful tool in the study and diagnosis of Chagas' disease, because it is well known that disturbances in the conduction of electrical stimuli are one of the early signs displayed by chagasic patients. Electrocardiographic studies have been performed in chagasic albino rats (31-33) demonstrating that at the acute phase of the disease 48% of the rats develop electrocardiographic disturbances characterized by left axis deviation, intraventricular conduction delay and abnormal Ajmaline's test. In most of these rats, at the indeterminate phase, electrocardiographic disorders ameliorate; however the intraventricular conduction disturbances and alterations on the Ajmaline´s test remained positive in 50% of the animals at the chronic phase of the disease (33).

One of the key issues in defining the pathogenic process leading to Chagas' dilated cardiomyopathy is finding an animal model that resembles human dilated cardiomyopathy. De Souza and col. (34) and Mukjerjee and col. (35) have descrived a heart enlargement on CD1 and C57BL/6 x 129sv T. cruzi Brazil strain- infected mice, respectively, using cardiac magnetic resonance imaging and/or transthoracic echocardiography; however, most of the rat models used to study Chagas' disease, developed only chronic myocarditis without heart enlargement or functional deficits, which is more similar to the indeterminate phase of Chagas' disease than to the chronic dilated chagasic cardiomyopathy.

In this paper, we have characterized electrocardiographicaly the phenylephrine-induced vaso vagal reflex, using highly selective muscarinic antagonists in two rat models: *T. cruzi*-infected rats and *T cruzi*-infected rats treated with low doses of CP (20 mg/Kg five times). This last experimental approach was done based on the autoimmune theory and on the experiments made by others authors on the regulation of the regulatory and autoreactive T cell proliferation (26-28, 36); we hypothesize that CP at low doses favors the autoimmune phenomena, allowing the development of Chagas' cardiomyopathy in *T. cruzi* infected animals.

MATERIAL AND METHODS

Animals

90 three-weeks-old Sprague-Dawley rats were divided in four groups: Control Group (CG, n = 30); Cyclophosphamide treated group (CP, n = 20); *T. cruzi* infected group (TC, n = 20); *T. cruzi* infected-CP treated group (TCCP, n = 20).

Induction of *T. cruzi* infection

At five weeks of age, the animals on the TC and TCCP groups were intradermally inoculated with 1000 para-

sites/g of Y strain (kindly given by Dr. Nestor Añez at Andes University, Venezuela) metacyclic trypomastigotes

Cyclophosphamide treatment

At three weeks of age the rats on CP and TCCP groups received 20 mg/Kg CP intraperitoneally (i.p.), twice a week for a total of 5 times.

Electrocardiographic studies

Electrocardiographic records were done in bipolar configuration, all electrodes were located in the subcutaneous tissue, one above the manubrio-sternal joint, other above xiphoid process and the reference electrode on the abdomen. All electrodes were connected to a BioAmp amplificator (ADInstruments), and analog signals were transformed to digital signals by a PowerLab/8sp interphase (ADInstruments), connected to a personal computer using the Chart v4.2.1 software (ADInstruments). Signal capture frequency was set at 400 events/sec and filtered at 60 Hz.

Pharmacological protocol

Rats were anesthetized with 40 mg/Kg pentobarbital i.p. Baseline electrocardiographic records were taken thirty minutes after anesthesia induction; 1 mg/kg phenylephrine i.p. was given and EKG records taken 10 min after phenylephrine inoculation; subsequently 40 mmol/Kg tacrine i.p. was administered and EKG records performed 10 min after tacrine administration. Low doses of selective muscarinic antagonists were administered i.p. and EKG records taken 10 and 20 min after administration. Finally high doses of antagonists were administered i.p. and EKG records taken 10 and 20 min after high doses antagonists administration. The selective muscarinic cholinergic receptor antagonists used were: pirenzepine (M1 receptor; 10 and 100 nM); methoctramine (M2 receptor; 1 and 10 μM); N-(2-chloroethyl) -4-piperidinyl diphenylacetate N-(2-chloroethyl)-4-piperidinyl diphenylacetate (4-DAMP; M3 receptor; 10 and 100 nM); and tropicamide (M4 receptor, 1 and 10 μM).

Histology

Biopsies of selected heart sections were fixed in PBS-formaline, embedded in paraffin, cut into 4-μm sections, de-waxed, and stained with hematoxylin-eosin.

Radiology

A conventional radiographic unit with a dual-screen, double-emulsion film, mammographic receptor was used. Typical exposure factors were 300 mA, 29 kVp, and 17 ms at a focus-film distance of 76 cm with a 2.11 by 2.41 mm effective focal spot and inherent filtration of 1.2 mm aluminium.

Data analysis

Quantitative data are presented as the mean ± SEM. Statistical significance of the differences observed between groups were determined using ANOVA test followed by Dunet or Bonferroni post-tests, accepting as significant a $p < 0.05$

RESULTS

Cardiomyopathy evidences

Rats belonging to the TCCP group exhibited many electrocardiographic disturbances (Fig. 1) that were not observed in the other groups. For instance, atrial flutter (panel A), atrial fibrillation (panel B), bundle branch block (panel C), ventricular extrasystoles (panel D), and low voltage QRS complex (compare QRS amplitudes on panels A, B, C, D to the QRS amplitude on panel F, which is from a healthy rat). In order to quantify these qualitative findings, we calculated the average number of electrocardiographic disturbances for each

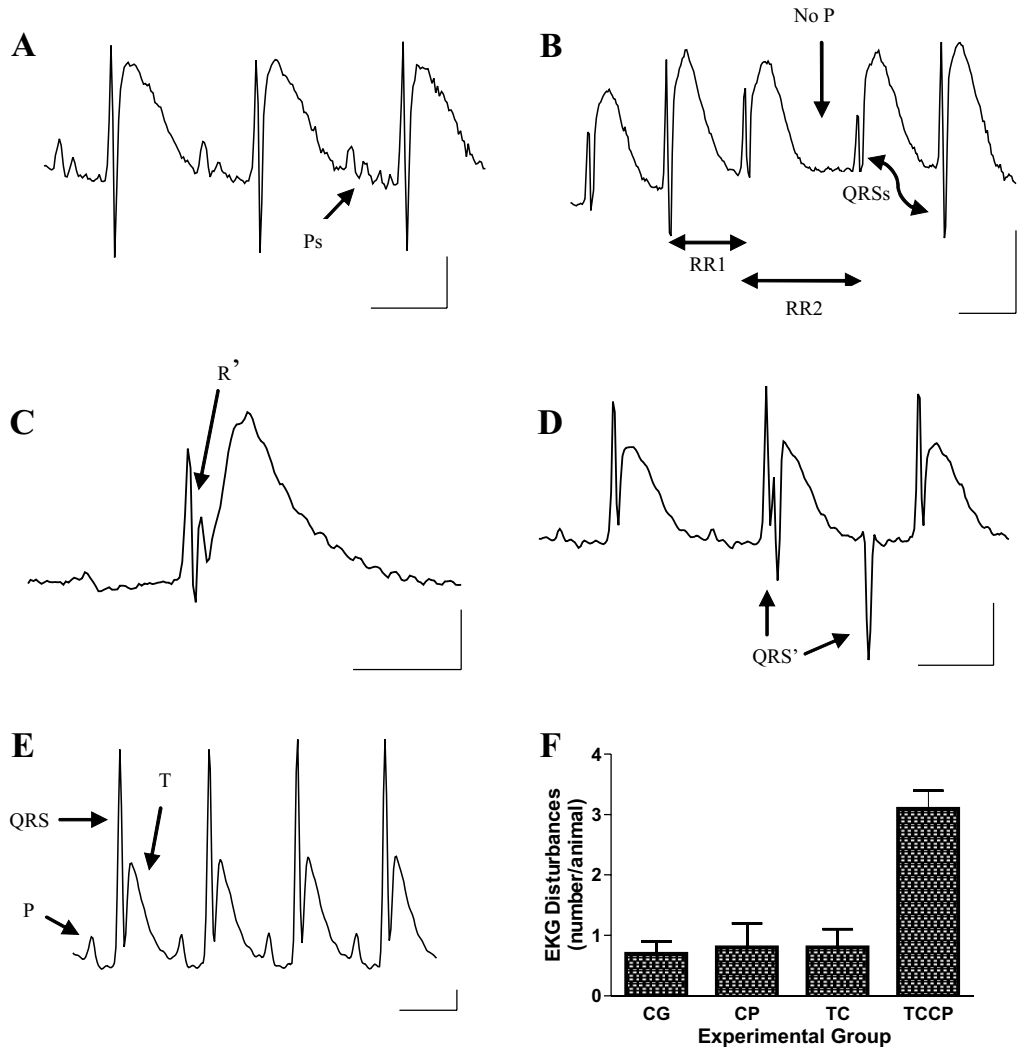

Fig.1. Electrocardiographic disturbances. The horizontal bar calibration indicates 100 msec and the vertical ones indicates 100 μV to all EKG records. Electrocardiographic records were done in bipolar configuration under penthobarbital anesthesia. Signal capture frequency was set at 400 events/sec and filtered at 60 Hz. Panel E show a p wave, QRS complex and T wave on a normal record taking from a healthy rat, illustrating standard morphologies and amplitudes (pointed out by p, QRS and T arrows), as well as PR, QRS and QT segment normal lengths. An electrocardiographic trace compatible with atrial flutter is displayed at panel A, this abnormality is characterized by the presence of train of p waves (pointed out by Ps arrow) preceding the QRS complex. Atrial fibrillation characterized by a variable RR segment (pointed out by RR1 and RR2 double arrows), absence of p wave (pointed out by No P arrow) and QRS complexes with different morphologies (pointed out by QRSs arrows) is displayed at panel B. Bundle branch block characterized by QRS complex displaying double R (pointed out by R' arrow) is showed at panel C; this trace also displays a prolonged PR segment. Two ventricular extrasystoles (pointed out by QRS' arrows), one at the end of the second QRS complex and the other located between the second and the third cardiac cycle is illustrated at panel D. Transformed qualitative data into semi quantitative data is presented at panel F (to each rat observed electrocardiographic disturbance was given 1 point; data represents the average number of disturbances for each group).

animal first, and then average them for each group, the results are shown in the Fig. 1 (bar right bottom figure), where it is observed that rats on the TCCP group had significantly ($p < 0.05$) more EKG disturbances (3.1 0.3 events/animal) than those rats on the CG (0.7 0.2 events/animal), CP (0.8 0.4 events/animal) or TC (0.8 0.3 events/animal) groups.

Rats infected with *T. cruzi* and treated with CP (TCCP group) had significantly higher PR segment prolongation (63.30 1.37 msec), than that obtained for control rats (56.96 0.62 msec), CP treated rats (56.94 0.58 msec) or *T. cruzi* infected rats (56.21 0.76 msec) (Fig. 2). Similar results were obtained for the QT segment, where the TCCP group showed a significantly greater prolongation (90.42 2.63 msec) than that observed on CG (75.68 1.05 msec), CP (73.61 1.57 msec) or TC (77.86 1.26 msec) groups. Rats belonging

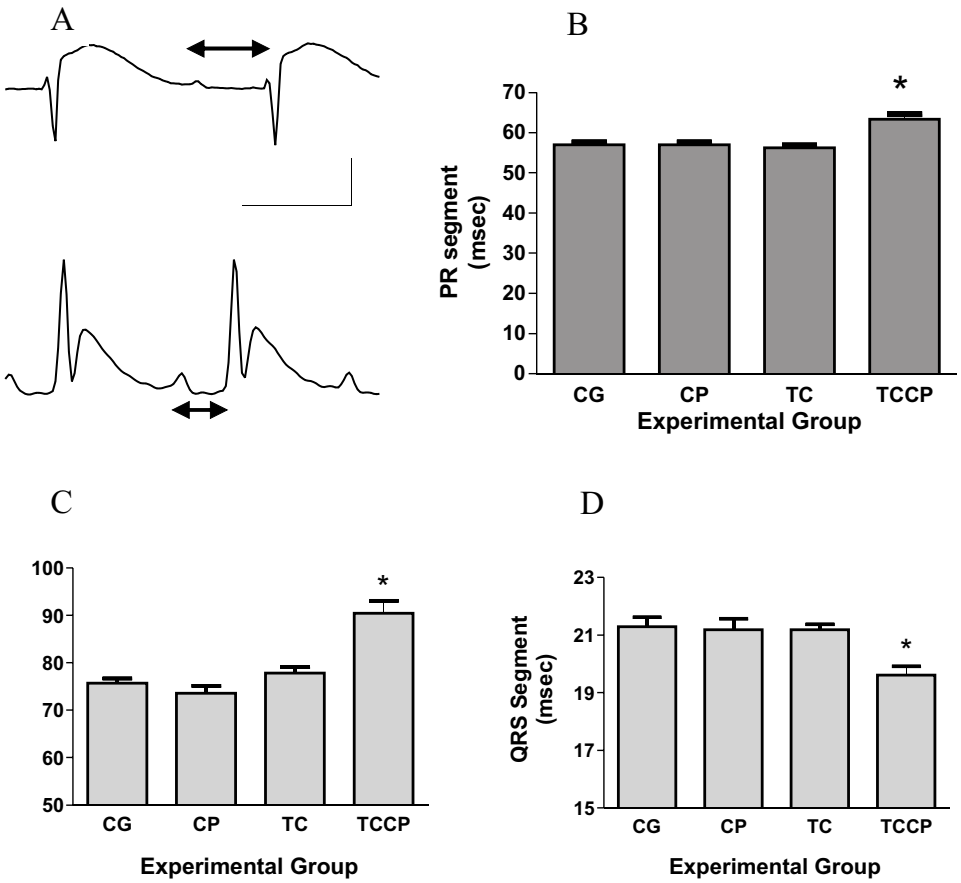

Fig. 2. PR, QRS and QT segment length. At the bottom of panel A is shown an electrocardiographic trace obtained from a healthy normal rat, while on the top is a trace obtained from a *T. cruzi*-infected CP-treated rat where a prolonged PR segment can be observed (compare the two double arrows). Panel B shows the average values for each experimental group: the TCCP group displayed a significantly higher PR segment length compared with the other groups. Panel C put in view the QRS segment length average values for each experimental group: the TCCP group displayed a significantly lower QRS segment length. Panel D presents the QT segment length average values for each experimental group: the TCCP group displayed a significantly higher QT segment length. The horizontal bar calibration indicates 100 msec and the vertical one indicates 100 μV. *means $p < 0.05$.

to the TCCP group also displayed a significantly shorter QRS complex (19.61 0.31 msec) as compared with the CG (21.28 0.33 msec), CP (21.18 0.39 msc) or TC (21.18 0.19 msec) groups (see Fig. 2).

T. cruzi-infected CP-treated rats (TCCP) displayed a more diffuse and larger mononuclear infiltrate and fiber muscle degeneration than *T. cruzi* infected rats, which displayed only a slight or moderate mononuclear infiltrate (Fig. 3). Radiographic studies revealed that *T. cruzi*-infected CP-treated rats also displayed heart enlargement (cardiomegaly), which was not observed in the other groups (Fig. 3).

Electrocardiographic characterization of the cholinergic system

Heart rate. Animals' basal heart rates under pentobarbital anesthesia were similar amongst all groups, oscillating between 338 to 351 bpm. Phenylephrine was able to induce bradycardia in all groups, but this was significantly less pronounced on *T. cruzi* infected rats. Tacrine slightly potentiated the phenylephrine-induced bradycardia in the control and CP-treated rats. In contrast, tacrine induced slight tachycardia in all *T. cruzi*-infected rats; which was significantly higher on the TCCP group (Table I).

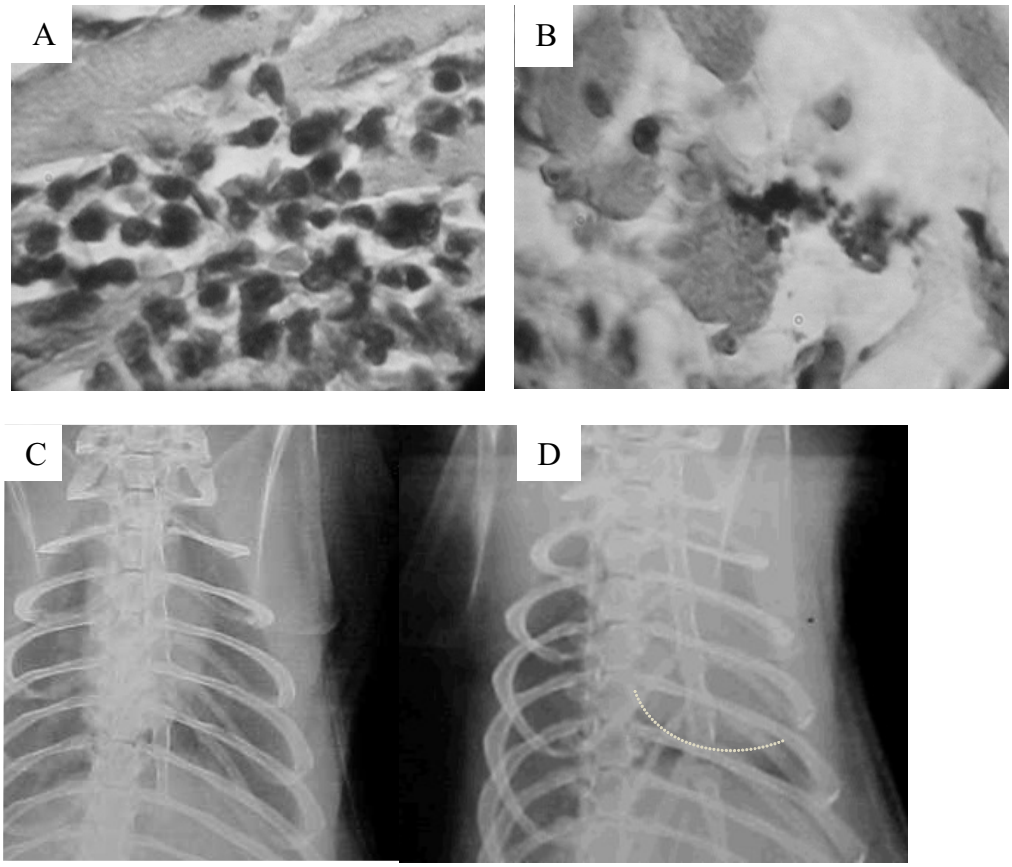

Fig. 3. Histopathological and radiographic evidences of Chagasic cardiomyopathy. Fragments of heart were processed by conventional paraffin embedding and hematoxylin-eosin staining. Histological sections 400X magnification are shown at A and B panels, they display a mononuclear infiltrate (A) and hyaline muscle fiber degeneration (B), respectively. At B and C panels are shown a postero-anterior chest radiographs from healthy rat (C) and from a *T. cruzi*-infected CP-treated rat displaying cardiomegaly (D). The dotted line at D underlines the position of heart base and apex.

TABLA I
EFECTS OF PENTHOBARBITAL (PB), PHENYLEPHRINE (PE) AND TACRINE (TA) ON HEART RATE

	PB		PE				TA					
	BPM		BPM		% PB		BPM		% PB		% PE	
CG	351	5.17	273	4.63	77.9	1.12	260	4.98	74.2	1.45	95.6	1.75
CP	348	6.78	276	6.33	79.4	1.65	265	8.18	77.3	2.66	97.6	2.20
TC	348	6.24	294	4.08*	85.0	1.56*	300	6.93*	86.6	2.46*	101.9	2.04
TCCP	338	7.70	280	10.73	81.9	2.30	282	12.10	84.3	2.42*	102.9	2.58*

Data are presented as heart rate absolute numbers (BPM) or as percentage respect to PB (% PB) or to PE (% PE). indicates $p < 0.05$ when PB BPM values is compared with PE and TA BPM values; * indicates $p < 0.05$ when the values obtained from one group are compared with those of the other groups. BPM means beats per minute, PB means penthobarbital, PE means phenylephrine, TA means tacrine, CG means control group (healthy rats), CP means cyclophosphamide-treated rats, TC means *T. cruzi*-infected rats and TCCP means *T. cruzi*-infected cyclophosphamide-treated rats.

Muscarinic cholinergic antagonists counteracted the bradycardia induced by phenylephrine in all groups, but this effect was less pronounced on TC and TCCP groups when using methoctramine, 4-DAMP and tropicamide (Fig. 4); this difference was statistically significant for tropicamide on both groups and for 4-DAMP on the TC group. Low doses of tropicamide had only a minor effect on the CP group (Fig. 4).

PR interval. As stated above, TCCP rats had a prolonged PR interval under pentobarbital anesthesia basal conditions (see Fig. 2). Phenylephrine induced a prolongation of the PR interval in all groups, which was slightly potentiated by tacrine (Table II). Muscarinic cholinergic antagonists counteracted this effect induced by phenylephrine, shortening the PR interval length as follow: pirenzepine on the TC and TCCP groups, methoctramine on the CG and CP groups, and tropicamide on the CG and TC groups. An exception was observed for pirenzepine in the CP group, where high doses induced a significantly longer PR interval (Fig. 5). These effects were dose-related and statistically significant.

QRS segment. EKG records showed that TCCP rats had a QRS complex significantly shorter under pentobarbital anesthesia basal conditions (Fig. 2). Phenylephrine increased the complex length in all groups, however, this effect was significantly less pronounced on *T. cruzi*-infected CP-treated rats (Fig. 6). Tacrine potentiated the effect of phenylephrine but there were no significant differences amongst experimental groups (Fig. 6). Muscarinic cholinergic antagonists did not have significant effects on the QRS complex length, with the exception of methoctramine, which diminished the duration of the QRS complex on the CG and CP groups, but not on TC and TCCP groups (Anova test, $p < 0.05$, with the Bonferroni post-test correction, $p > 0.05$); (Table III).

QT interval. *T. cruzi* infected-CP treated rats under pentobarbital anesthesia displayed the longest QT segment (Fig. 2). Phenylephrine increased the QT segment length on all groups with no significant differences observed amongst groups. Tacrine potentiated the effect of phenylephrine, with a significantly greater effect on the TCCP group. Pirenzepine, methoctramine and tropicamide decreased QT length in all groups in a dose related manner; tropicamide displayed the highest potency on the CG group, approaching an effect of 25% and 27% for the 1 and 10 μM doses, re-

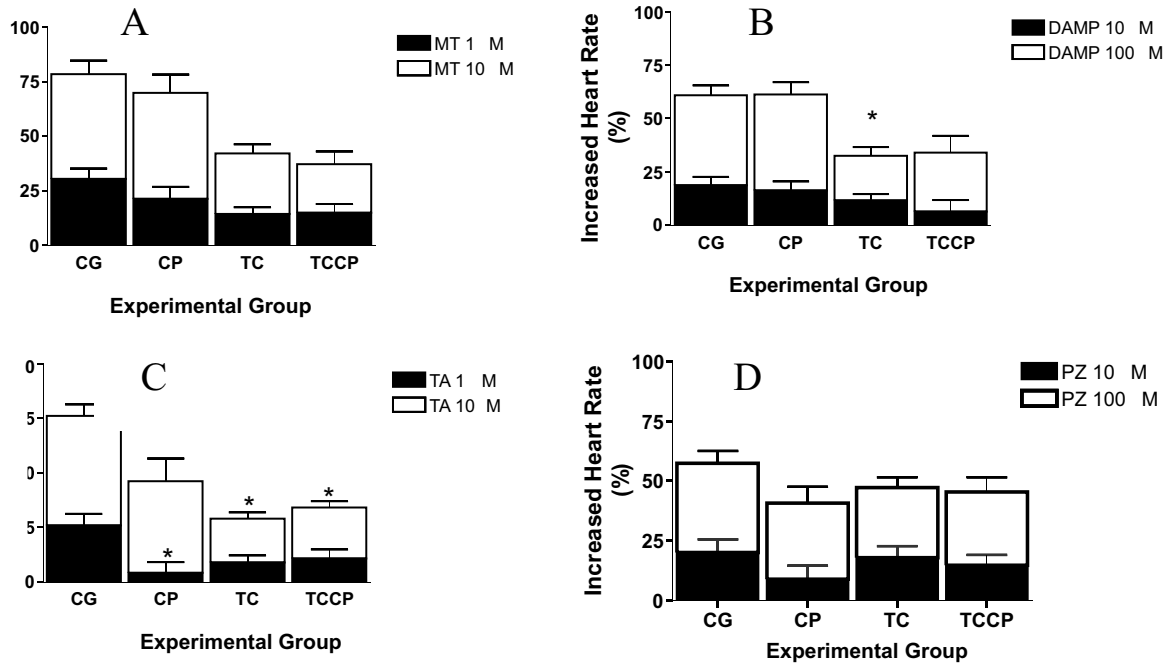

Fig.4. Effects of muscarinic selective antagonists that counteracts phenylephrine plus tacrine-induced bradycardia. Rats were anesthetized with penthobarbital 40 mg/Kg i.p., followed 30 minutes later by i.p. application of phenylephrine and tacrine 40 mmol/Kg on 10 min interval schedule. After these, low and a high dose of antagonists were successively administered at 20 min intervals. Results represent the normalized data, expressed as percentage of heart rate, taking as a 100% the data obtained after administration of tacrine. Each panel correspond to data obtained for methoctramine (MT), 4-DAMP (DAMP), tropicamide (TA) and pirenzepine (PZ). Empty and fill bars represent data obtained at high and at low doses of the antagonists, respectively. Drug concentrations are indicated in each panel. MT (panel A), 4-DAMP (panel B) and TA (panel C) counteracted phenylephrine plus tacrine induced-bradycardia, however, this effect is less pronounced on *T. cruzi*-infected rats (TC) and on *T. cruzi*-infected CP-treated rats (TCCP), as compared with healthy rats (CG) and CP-treated rats (CP), significant differences were observed for 4-DAMP and TA. PZ (panel D) counteracted the phenylephrine plus tacrine-induced bradycardia, displaying similar effects for all the groups assayed. * indicates $p < 0.05$.

TABLA II
EFECTS OF PENTHOBARBITAL (PB), PHENYLEPHRINE (PE) AND TACRINE (TA) ON PR SEGMENT LENGTH

	PB		PE			TA					
	msec		msec		% PB	msec		% PB		% PE	
CG	56.8	0.7	61.5	0.7	108.4	0.9	63.1	0.8	111.3	1.4	102.7	1.0
CP	56.9	0.6	60.7	0.8	106.7	1.4	63.1	0.8	111.2	1.7	104.1	1.6
TC	56.2	0.8	61.8	0.8	110.0	0.76	62.9	0.9	112.0	1.1	101.8	1.0
TCCP	63.3	1.4 *	68.3	1.7*	108.1	1.82	73.1	2.32*	116.0	2.5	106.4	2.4

Data are presented as absolute numbers (msec) or as percentage respect to PB (% PB) or to PE (% PE). Indicates $p < 0.05$ when PB BPM values is compared with PE and TA BPM values; * indicates $p < 0.05$ when the values obtained from one group are compared with those of the other groups. PB means penthobarbital, PE means phenylephrine, TA means tacrine, CG means control group (healthy rats), CP means cyclophosphamide-treated rats, TC means *T. cruzi*-infected rats and TCCP means *T. cruzi*-infected cyclophosphamide-treated rats.

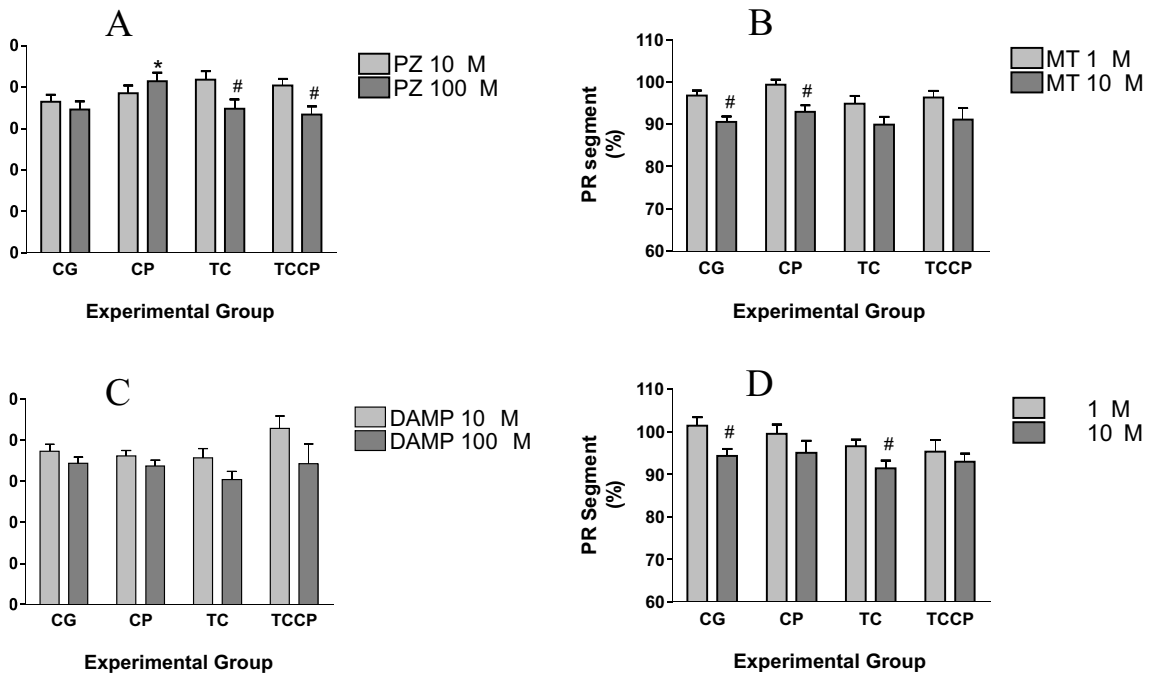

Fig. 5. Effects of selective muscarinic antagonists, on counteracting phenylephrine plus tacrine-induced a prolongation of PR interval. Penthobarbitol 40 mg/Kg i.p. were given to the rats, followed 30 minutes later by i.p. application of phenylephrine 1 mg/kg and tacrine 40 mmol/Kg successively, at 10 min interval; low and a high doses of antagonists were then administered successively at 20 min intervals. Results represent the normalized data expressed in percentage of PR length, assuming as a 100% the data obtained after tacrine treatment. Each panel represents data obtained for pirenzepine (PZ, panel A), methoctramine (MT, panel B), 4-DAMP (DAMP, panel C) and tropicamide (TA, panel D). Empty and fill bars represent data obtained at high and at low doses of the antagonists, respectively. Drug concentrations are indicated in each panel. PZ, MT, 4-DAMP and TA shortened PR segment, an effect related to the doses applied, however, high dose of PZ increased the PR segment on CP-treated rats. * indicates $p < 0.05$ when groups are compared; indicates $p < 0.05$ when antagonist doses are compared in each group.

spectively. No response to 1 μM tropicamide was observed on TCCP group. 4-DAMP displayed the lowest potency and the effect was not related to the doses assayed (Figs. 6 and 7).

DISCUSSION

In this paper we presented electrocardiographic, radiographic and histopathological evidences that CP is able to induce a chronic chagasic dilated cardiomyopathy in rats infected with *T. cruzi*. To our knowledge, this is the first report showing dilated cardiomyopathy induced by CP in *T. cruzi* infected rats. Using a pharmacological approach with phenylephrine and tacrine, we found that rats chronically infected with *T. cruzi*, with or without CP treatment, showed disturbances in the cholinergic system.

CP is an alkylating agent used in cancer therapy for its antiproliferative properties. High CP doses in rats produced histologic and biochemical changes compatible with those of cardiomyopathy (29). At low doses, CP shows no cardiotoxic effects, but is able to induce immune

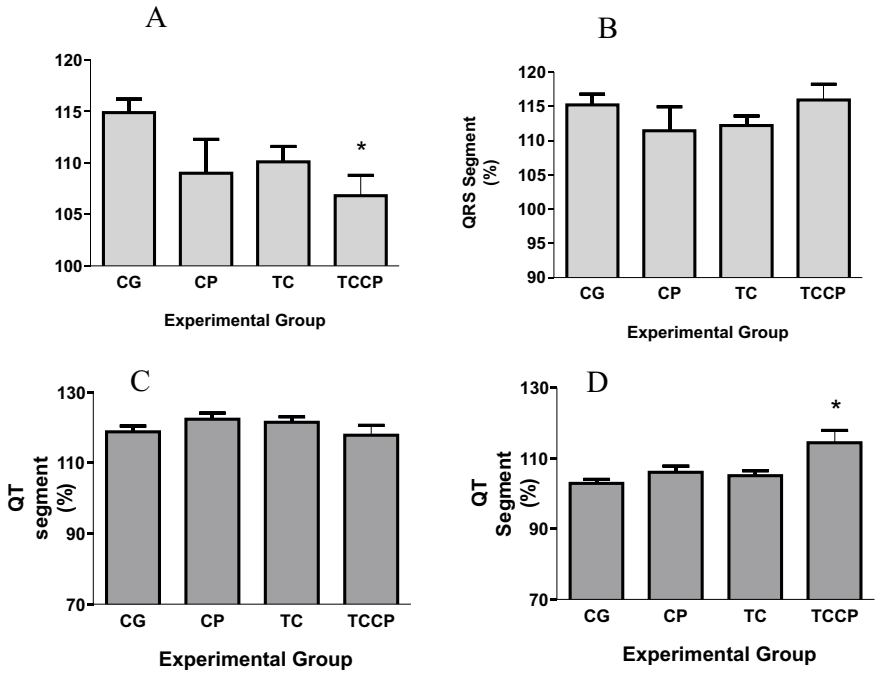

Fig. 6. Effects of phenylephrine and tacrine on QRS and QT segments length. At panels A and C the effect of phenylephrine on QRS and QT segments are shown, respectively, as a percentage respect to the values obtained after penthobarbital application. The phenylephrine-induced prolongation of QRS complex length, which it was greatest on healthy rats (CG group) and lowest in *T. cruzi*-infected CP-treated rats (TCCP group), also phenylephrine-induced prolongation of QT segment length, however, the differences were not significant between experimental groups. At panels B and D the effects of tacrine on QRS and QT segment length are shown, respectively, as the percentage respect to the values obtained after phenylephrine application. Tacrine also induced prolongation of the QRS complex length but differences observed between groups were not significant. On healthy rats (CG), CP-treated rats (CP) and in *T. cruzi*-infected rats (TC) tacrine induced shortening of QT segment length, however, on *T. cruzi*-infected CP-treated rats a prolongation of QT segment was observed, a difference was significantly ($p < 0.05$) when compared against the other groups. * indicates $p < 0.05$ compared to the control group.

TABLA III
EFECTS OF PIRENZEPINE, METHOCTRAMINE, 4-DAMP AND TROPICAMIDE ON QRS COMPLEX LENGTH

	Pirenzepine				Methoctramine				4-DAMP				Tropicamide			
	10 nM		100 nM		1 μM*		10 μM*		10 nM		100nM		1 μM		10 μM	
CG	97.6	2.5	93.6	2.3	96	2.4	87.8	2.5	99.1	2	94.8	1	94.3	1.9	99.5	2.4
CP	100.5	2.3	98.8	2.4	95	1.9	91	3.1	98.9	3.1	92.7	2.3	96.2	2.9	99.2	3.6
TC	101.1	2.0	97.1	1.5	100	3	97.7	2.7	92.1	1.7	96.5	2.2	92.4	1.6	102	3.2
TCCP	96.67	2.5	95.6	3.4	114	6.3	98.1	3	97.3	1.7	94.8	2.4	99.5	3.6	94.6	2.9

Values presented are the percentual values respect to the values obtained after tacrine treatment. * indicates $p < 0.05$ obtained by ANOVA test when the results obtained from one drug are compared between all groups together, however using Bonferroni post-test corrections there are no significant differences between all possible pair of groups. CG means control group (healthy rats), CP means cyclophosphamide-treated rats, TC means *T. cruzi*-infected rats and TCCP means *T. cruzi*-infected cyclophosphamide-treated rats.

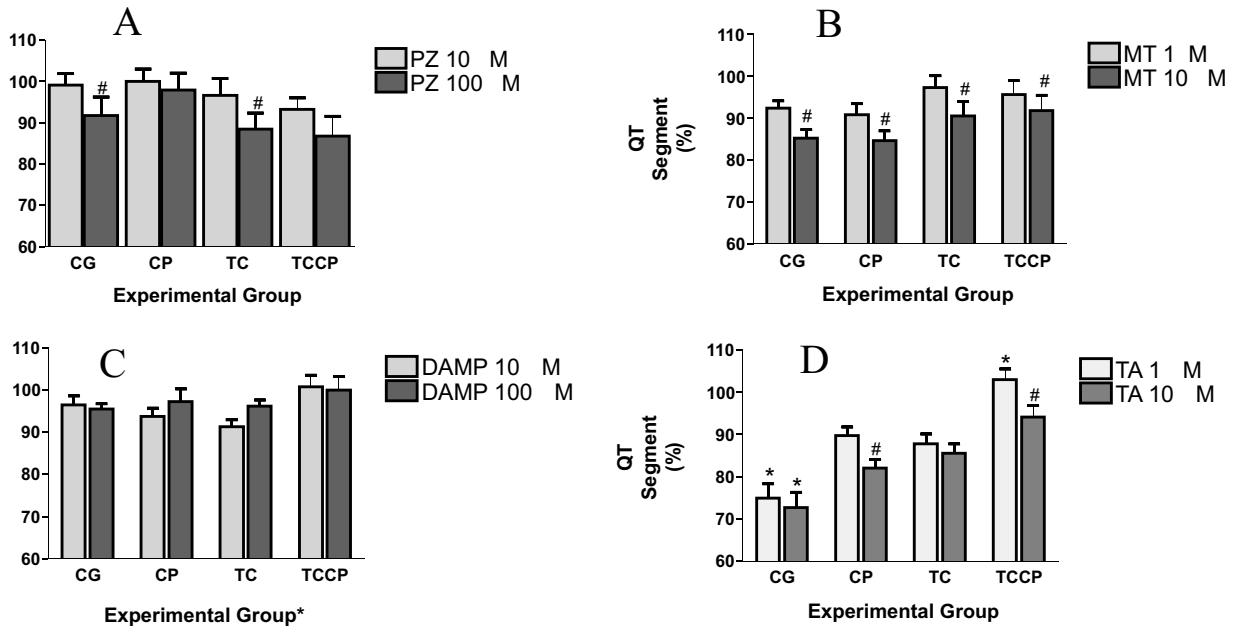

Fig. 7. Effects of muscarinic selective antagonists in counteracting the phenylephrine plus tacrine-induced prolongation of QT length. Rats were anesthetized with penthobarbitol 40 mg/Kg i.p. followed 30 minutes later by successively i.p. application at 10 min intervals of phenylephrine 1 mg/kg and tacrine 40 mmol/Kg; subsequently low and a high doses of antagonists were administered successively at 20 min intervals. Results represent the normalized data expressed in percentage of QT length, assuming as 100% the values obtained after tacrine treatment. Each panel represents data obtained for pirenzepine (PZ, panel A), methoctramine (MT, panel B), 4-DAMP (DAMP, panel C) and tropicamide (TA, panel D). Empty and fill bars represent data obtained at high and at low doses of the antagonists respectively. Drug concentrations are indicated in each panel. PZ, MT and TA shortened the PR interval, an effect related to the applied doses; an exception was observed for DAMP which effects in all groups were not dose related. The effect of TA for both doses was higher in the healthy rats and shorter in *T. cruzi*-infected CP-treated rats. * indicates $p < 0.05$ when groups are compared, indicates $p < 0.05$ when antagonist doses are compared in each group.

disregulation consisting of the selective depletion of suppressor T cell function in vivo (18) and the induction of autoreactive T lymphocytes (28).

Low doses of CP had been used previously on *T. cruzi*-infected animals. In dogs chronically infected with *T. cruzi*, CP treatment induces a chronic diffuse myocarditis, characterized by focal fibrinoid, coagulative and lytic necrosis with fiber disintegration associated with a mononuclear infiltrate; without CP treatment, *T. cruzi* infected dogs develop only a mild focal myocarditis represented by the accumulation of lymphocytes in the interstitial connective tissue (30).

In this paper we demonstrate that *T. cruzi*-infected CP-treated rats develop chronic chagasic cardiomyopathy, characterized by electrocardiographic abnormalities such as atrial flutter or fibrillation, ventricular extrasystoles, prolonged PR, a bundle branch block and low voltage QRS; radiographic evidence of cardiomegaly; and histologic evidence of a diffuse mononuclear infiltrate and hyaline fiber degeneration. *T. cruzi*-infected rats not treated with CP displayed only a focal mononuclear infil-

trate, but neither electrocardiographic abnormalities nor cardiomegaly, indicating that these animals develop an indeterminate form of Chagas' disease. Uninfected rats treated with CP had no cardiac changes, indicating that CP alone, at low doses, is unable to cause cardiomyopathy, and merely enhances the pathogenic process induced by *T. cruzi* antigens, facilitating the evolution of a dilated cardiomyopathy.

The CP-enhanced *T. cruzi* pathogenic effects could be analyzed in relation to the capacity of CP to selectively depress T regulatory cells, thus allowing proliferation of autoreactive T cells or, alternatively, to cause immunosuppression, facilitating the proliferation and dissemination of the parasite. CP has been widely used for the selective depletion of suppressor or regulatory T cells *in vivo* (18). It decreases the number, percentage and the function of $CD25^+$ $CD4^+$ regulatory T cells (26, 27) and induces autoreactive T lymphocytes (28). Depression of regulatory T cells is associated with the induction of autoimmune diseases in both animals and human (18, 36).

This line of thought promotes the theory that autoimmunity causes chagasic cardiomyopathy, through either the loss of tolerance to auto antigens induced by *T. cruzi* or to the existence of cross reactivity between cellular proteins and parasite antigens (molecular mimesis). It has been proposed that *T. cruzi* and heart antigens are similar, thus chagasic cardiomyopathy reflects an autoimmune process (37).

Chagas' disease, by itself, is able to induce immunosuppression, which is thought to facilitate the dissemination and establishment of the parasite in the infected host (20, 21). This has been ascribed to many mechanisms, including the involvement of regulatory or suppressor T cells (21, 22) as well as the presence of parasite's suppressive factors such as SAPA, which is able to down-regulate T lymphocyte proliferation as a consequence of T suppressor/cytotoxic cell activation (25). CP could directly potentiate the immunosuppressive phenomena induced by *T. cruzi,* by suppressing regulatory T cells or indirectly by allowing a higher parasites' proliferation rate within the host.

Parasite persistence must be a necessary phenomenon that allows setting up pathogenic mechanisms. Recently, it has been demonstrated an absolute correlation between parasite persistence in tissue measured by in situ polymerase chain reaction analysis and the presence of disease in the cardiac muscle (38). Persistence of the parasite is a consequence of the host failure to clear the infection, resulting in infection-induced immunity plus autoimmune tissue damage (39), that could explain development of those pathological myocardial changes observed in Chagas' disease.

In this paper, we have evaluated the autonomic nervous system using the phenylephrine-dependent activation approach; moreover, we potentiated the phenylephrine effect by inhibiting the acetyl cholinesterase enzyme with tacrine. This approach allowed us to test the functional reserves of acetylcholine. Our results clearly showed that *T. cruzi*-infected and *T. cruzi*-infected CP-treated rats had an impairment of the vagal baroreflex, expressed as a reduced bradycardia response, as compared with uninfected healthy and CP-treated rats, indicating that CP by itself did not induce autonomic impairment. The fact that tacrine induced tachycardia instead of bradycardia on *T. cruzi*-infected and *T. cruzi*-infected CP-treated rats, suggested that these animals could have limited reserves of acetylcholine, maybe due to a diminished number of nerve terminals, disturbances in storage or release, or alterations in the function of acetylcholinesterase.

Histological studies have revealed that chagasic hearts with evidence of severe

chronic myocarditis, have severe parasympathetic denervation (40), and this parasympathetic dysautonomia is an independent and early phenomenon in Chagas' disease that may precede the left ventricular systolic dysfunction (41). Integrity of the vagus nerve fibers in rats with acute chagasic myocarditis has also been examined by direct stimulation; it was demonstrated that, at low frequency stimulation, chagasic animals had a lower negative chronotropic response as compared with control animals, while at high frequency stimulation, the negative chronotropic response was similar. The authors suggested that these results may represent a decreased excitability and higher stimulation threshold, probably secondary to the acute inflammatory process and background sympathetic tone (42). Morphometric studies have revealed myelin damage and axonal swelling of the vagus nerve myelinated fibers in chagasic rats (43).

Muscarinic cholinergic receptors are present in the three medullary nuclei involved in the baroreceptor reflex activation-induced bradycardia. Administration of carbachol, acetylcholine, pilocarpine and eserine into these nuclei, elicited dose-related decreases of heart rate and potentiate the action of acetylcholine. These effects were completely antagonized by the muscarinic antagonist ethylbenztropine, suggesting an involvement of muscarinic cholinoreceptors in baroreflex-mediated adjustments of the heart rate (8). It has been suggested that M2 muscarinic receptor subtype is the predominant cholinergic receptor on NTS membranes (9). M1 muscarinic receptors predominate at vagal afferent terminals presynaptic localization in the subnucleus gelatinosus (10), while the nucleus tractus solitarius do not have M1 receptor (11). The expression of four different muscarinic receptor transcripts or proteins by the intrinsic cardiac neurons and cardiac muscle has been reported as well as (12, 13). Ganglia contain more M2 mRNA than what is found in the atria and they are located mainly at the presynaptic level (14).

In this paper we found that the muscarinic selective receptor antagonists were able to antagonize phenylephrine-tacrine activate-baroreflex mediated bradycardia. Rats infected with *T. cruzi,* with or without CP treatment, showed a decreased response to methoctramine, 4-DAMP and tropicamide, but not to pirenzepine. This effect was demonstrated for methoctramine in both groups by lengthening of the QRS complex, and for tropicamide in the *T. cruzi*-infected CP-treated rats by the QT interval. These results suggest that M2, M3 and M4 muscarinic receptor subtypes in the central nuclei, in the cardiac ganglia or in the heart muscle have different distribution. M1 subtype density appeared to be very low, as pirenzepine exhibited overall the lowest response. Differential expression of the four muscarinic receptors in the cardiac intrinsic neurons and in the cardiac muscle could provide a molecular basis for the altered muscarinic actions observed in the chagasic hearts.

In conclusion, CP is able to facilitate the development of cardiomyopathy in *T. cruzi*-infected rats. This effect could be associated with the capacity of CP to deplete regulatory T cells or that of facilitating the proliferation of *T. cruzi*, which also induces an immunosuppressing effect that potentiates the CP-induced depletion of regulatory T cells. The phenylephrine-tacrine pharmacological approach allowed us to demonstrate that *T. cruzi*-infected and *T. cruzi*-infected CP-treated rats have autonomic parasympathetic disturbances; however, the magnitudes of these disturbances were similar in both groups, which represent different progression phases of the disease, indicating that the autonomic disturbance is an

early phenomenon that appears before the development of Chagas' cardiomyopathy. The effects observed for the selective antagonists suggested that chagasic rats have alterations on the muscarinic receptors distributions in the central vagal nuclei, cardiac intrinsic ganglion or cardiac muscle cells.

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