UPREGULATION OF β-CATENIN AND CADHERIN EXPRESSION IN HEARTS OF MICE IN ACUTE AND CHRONIC PHASE OF EXPERIMENTAL *Trypanosoma cruzi* INFECTION

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ABSTRACT

Adherens junctions proteins have also been envolved in pathological mechanism of chagasic myocardiopathy. Aimed to determine the nature of these alterations, ten Swiss Webster male mice, 25 days-old were infected with a Type III strain of *Trypanosoma cruzi* through intraperitoneal (IP) route with 1.0 x 10^4 trypomastigotes/mouse. Five infected mice were killed at 14th day (parasitemia peak) of infection (group A). Another five infected mice (group C), were killed three months after inoculation, representing the chronic phase of the infection. Ten mice were injected through IP route with sterile 0.9% sodium chloride solution and were maintained under the same general conditions of the other groups. Five mice were killed either on day 14 (group CA) and five were killed at the end of the third month (group CC), and served as uninfected controls. Adherens junctions were analyzed by total lysate of the hearts that were immunoblotted for pan-cadherin and β-catenin. Western blot analysis revealed increases of cadherins in both acute (2.1-fold ± 0.89, p= 0.048) and chronic (2.1-fold ± 0.92, p= 0.05) phase versus its respective controls. In relation to β-catenin the results showed the same pattern characterized by increases in its levels also in acute (6.8-fold ± 4.65, p= 0.047) and chronic (3.65-fold ± 1.93, p= 0.033) phase in relation to controls. Thus, these results point to the evolvement of adherens junction proteins with pathological events in hearts of mice infected with *T. cruzi* infection.

KEY WORDS: Adherens junctions, Chagas disease, myocarditis, western blotting.

RESUMO

AUMENTO DA EXPRESSÃO DE β-CATENINA E CADERINA NO CORAÇÃO DE CAMUNDONGOS NA FASE AGUDA E CRÔNICA DA INFECÇÃO EXPERIMENTAL POR *Trypanosoma cruzi*

As proteínas das junções de aderência têm sido associadas ao mecanismo patológico da miocardiopatia chagásica. Objetivando determinar a natureza dessas alterações, infectaram-se dez camundongos machos Swiss Webster com 25 dias de idade por via intraperitoneal com uma cepa tipo III de *Trypanosoma cruzi* na dose de 1,0 x 10^4 tripomastigotas/camundongo. Cinco camundongos infectados foram sacrificados no 14° dia (pico de parasitemia) de infecção (grupo A) e outros cinco camundongos (grupo C) três meses após a inoculação, representando a fase crônica da infecção. Inocularam-se dez animais com solução estéril de cloreto de sódio 0,9%, sendo sacrificados no 14° dia (grupo CA) e cinco ao final do terceiro mês (grupo CC), para servirem como controles não-infectados. As junções de aderência foram analisadas a partir do lisado total dos corações submetido a *western blotting* para pan-caderina e β-catenina. A análise do *western blotting* revelou aumento da expressão de pan-caderina na fase aguda (2,1x ± 0,89, p= 0,048) e crônica (2,1x ± 0,92, p= 0,05) quando comparadas com seus respectivos controles. Em relação à β-catenin, os resultados mostraram o mesmo padrão caracterizado pelo aumento de seus níveis na fase aguda (6,8x ± 4,65, p=

0,047) e crônica (3,65x ± 1,93, p= 0,033) em comparação aos controles. Assim, estes resultados indicam a associação das proteínas de junção de aderência aos eventos patológicos em corações de camundongos infectados por *T. cruzi*.

PALAVRAS-CHAVES: Doença de Chagas, junções de aderência, miocardite, *western blotting*.

INTRODUCTION

Cardiomyopathy is an important manifestation of the american trypanosomiasis or Chagas disease, caused by the protozoan *Trypanosoma cruzi*. ANDRADE (1985) classified *T. cruzi* in three types according to its biological behavior and histopathological profile: I, II and III. The type I (Y strain, p.e.) is constituted by strains characterized by slim forms and tropism for macrophage in early stage of infection. Multiplication is fast, showing high parasitemia and mortality (7 to 12 days post infection). Type II (São Felipe strain, p.e.) has large forms predominantly, myocardium tropism, slow multiplication and irregular parasitemia peak (12 to 20 days post infection), when the mortality is higher. Type III (Colombian strain, p.e.) is constituted, predominantly, by large forms with slow multiplication, later parasitemia peak (between 20 and 30 days post infection), low and later mortality (50 days post infection) and evolves mainly skeletal muscle. The type III strains, associated with the silvatic cycle, has also occurred in human patients in north and northeast states of Brazil. In Montalvania (Minas Gerais, Brazil) and neighboring localities of west central Brazil, an overlap between the silvatic and domiciliary transmission cycles determined the concomitance of Types II and III in the same geographical area (LUQUETTI et al., 1986; ANDRADE & MAGALHÃES, 1997).

Cytoskeleton proteins play a key role in many of the events leading to the clinical manifestations of the disease, and changes in the cellular architecture have been directly associated to cardiac dysfunction (MELO et al., 2006). Structural and physiological changes in cardiomyocytes have been demonstrated, including disturbance in the calcium homeostasis (GARZONI et al., 2003), conduction system (CARVALHO et al., 1992), cytoskeletal organization (TANIWAKI et al., 2005) and disruption of actin filaments (TANIWAKI et al., 2005; MELO et al., 2006) which may contribute to the abnormalities in the contraction force evidenced in Chagas disease.

Adherens junctions have also been envolved in pathological mechanism of chagasic myocardiopathy. COLMANETTI et al. (2005) observed dehiscence in intercalated disks, especially in adherens junctions in hamster experimentally infected by *T. cruzi*. SOLER et al (2001) showed a dramatic upregulation of adherens junction protein (neural cell adhesion molecule - NCAM) expression in the intercalated disks of cardiomyocytes in acute and chronic Chagas myocarditis. Besides, the expression and integrity of junctional components are often disturbed in several kinds of heart diseases. TANSEY et al. (2006) demonstrated significant reductions of the intercellular adherens junction proteins after ischemia and reperfusion in heart rabbits. BIANCHI et al. (2001) showed that adherens junctions (VE-cadherin, β-catenin, and γ-catenin) of intercalated disks are partially degraded after 90 minutes of post-cardiopulmonary bypass (CPB) perfusion in pigs and those changes in the steady state of adhrens junction proteins may partially explain myocardium dysfunctions after CPB. Taken together, those findings point to adherens junction as a putative target for studies aimed to clarify biochemical mechanisms involved in heart morbidity, including Chagas disease.

Adherens junctions are essential components of the cardiac cytoarchitecture constituted of a transmembrane (cell-cell contact) proteins known as cadherins, and a set of catenins (α, β and γ), which link the actin cytoskeleton to the membrane (HIRSCHY et al., 2006). Thus, adhrens juntions constitute a system of plaque-bearing adhering proteins that connect the cardiomyocytes with each other at the intercalated disks, allowing transmission of the contractile force across the plasma membrane, enabling the myocardium to function as a syncytium

(FERREIRA-CORNWELL et al., 2002; FRANKE et al., 2006).

The cadherins, whose molecular structure was described firstly by TAKEICHI (1995), are a family of cell surface glycoproteins that mediate calcium-dependent cell-cell adhesion primarily in several tissues of the organism (VLEMINCKX & KEMLER, 1999). Cadherins are single pass transmembrane proteins comprising five extracellular domains (EC1, EC2, EC3, EC4 e EC5), a transmembrane domain and a cytoplasmic domain. The cadherin extracellular domain forms a dimer in parallel arrangement that interacts in amino-terminal end with another dimer arising from the opposite membrane on a neighboring cell creating an adhesion zipper between the cells.

The catenins associated to transmembrane domain mediates the binding between cadherin and actin, yonder cell signalling in response to adhesion. α-catenin is a major anchor of adherens junction to cytoskeleton. In cadherin-catenin complex formation, β-catenin develops a leading role by binding cadherin to α-catenin, that no connect to each other directly. γ-catenin (plakoglobin) binds to α-catenin (KIRKPATRICK & PFEIFFER, 1995)

Adherens junction formation is a multi-step process that starts when cadherin joins to β or γ-catenin after their sinthesis in endoplasmatic reticule. This complex is transported to plasma membrane and α-catenin is added simultaneously to his arrive. In time, the most of cadherin/catenin complexes is recruited to mediate strong cell-cell adhesion (RANSCHT, 1994).

Since adherens junctions appear to play a pivotal role in cardiomyocyte contraction, the clinical signs of *T. cruzi*, infection could be partially related to biochemical and structural changes in adherens junctions proteins. Therefore, this study aimed to verify the integrity of adherens junction proteins in intercalated disks of mice subjected to experimental infection with a strain of *T. cruzi*, in acute and chronic phase, hoping that the results might contribute to explain the morphological change mechanisms that reflect clinically in chagasic patient and stablish the adherens junctions as biochemistry target to therapeutic intervention, enabling minimize the morbid effects of disease.

MATERIAL AND METHODS

Parasites and mice infection

Trypomastigotes were obtained from blood harvested by heart punction of A/Sn mouse infected with *T. cruzi* isolated from a human patient in chronic phase of Chagas disease examined at the Clinics Hospital of Federal University of Goiás, Brazil, showing heart disease, megaesophagus and megacolon. The strain was considered as type III strain (colombian) according to Andrade's parameter, considering the mainly characteristics (OLIVEIRA et al., 1993).

Twenty male 25 day-old Swiss Webster mice weighing between 150 and 180g were obtained from UFG Animal Facility of the Biological Sciences Institute and maintained on a commercial balanced food (Labina®, Purina do Brasil, Paulínia, São Paulo, Brazil) and water *ad libitum* and housed under controlled conditions. Ten animals were infected through intraperitoneal (IP) route with 1.0×10^4 trypomastigotes/mouse. Infection was confirmed by detection of trypomastigotes in peripheral blood (drawn from tail) 5μL aliquots contained between a glass slide and a coverslip and visualized in 400x magnification microscopic field. Ten animals were injected sterile 0.9% sodium chloride solution through IP route and served as uninfected controls. Thus, three groups of animals were considered for studies: First group (*A from acute*) - constituted by five infected mice, representative of the acute phase of the infection, were slaughtered at 14th day of infection, when parasitemia reached its highest peak (determined previously); Second group (*C from chronic*) – another five infected mice, representative of the chronic phase of the infection, were killed three months after inoculation; Third group – ten uninfected control mice were maintained under the same general conditions of the other groups, and killed either on day 14 (*group CA*, n= 5) or at the end of the third month (*group CC*, n= 5) after the beginning of the experiment.

All animals from all groups were killed after administration of tramadol chloridrate (Sylador®, Sanofi-Aventis, São Paulo, São Paulo, Brazil) followed 15 minutes later by injection of tiletamin-zolazepan association (Zoletil 50®, Virbac, Jurubatuba, São Paulo, Brazil). The heart was removed, sectioned through the interventricular septum into two halves along the great axis and samples were harvested and subjected to histology, immunohistochemistry and western blotting. All animals received care following the Ethical Principles in Animal Experimentation prepared by the Brazilian College of Animal Experimentation (COBEA, 2006).

Protein extraction and immunoblotting assay

Another half of the heart was frozen in n-hexane (Merck, Darmstadt, Germany) pre-cooled in liquid nitrogen (-196°C) and stored in liquid nitrogen until processing. Total homogenate was obtained by cell lysis in sodium acetate buffer (5mM, pH 5.0) associated to protease inhibitors (Protease Inhibitor Mix®, Amersham GE, Piscataway, NJ, USA) for 30 seconds on ice followed by centrifugation at 10,000 g for 3 minutes at 4°C to separate solubilized from unsolubilized protein. The supernatant protein concentration was measured in digital spectrophotometer (model 35D™, Coleman, Santo André, SP, Brazil) at 595 nm wave length using Bradford method (BRADFORD, 1976). Approximately 100 µg of total protein was dissolved in 100µL Laemmli sample buffer (0.25M Tris, 0.192M Glycine, 1% sodium dodecyl sulfate - SDS, pH 8.3) with 5% β-mercaptoethanol and boiled for 5 minutes. Equal amounts of protein for each sample were separated on SDS-10% polyacrylamide gel electrophoresis (SDS - PAGE) in vertical electrophoresis system (Hoefer™ mini-VE complete, San Francisco, California, USA) and transferred onto nitrocellulose membrane (Hybond-ECL®, Amersham GE, Little Chalfon, Buckinghamshire, United Kingdom) using a semi-dry transfer unit (Hoefer™ TE 70, Amersham GE, San Francisco, CA, USA) in 25mM Tris-HCl, 0.19 M glycine, 20% methanol and 0.1% SDS. As protein detection ladder was used broad range Prestained Protein Marker (Cell Signalling Tech, Danvers, MA, USA). Membranes were stained with 0.1% Ponceau S in 5% acetic acid, digitized, and then incubated with 5% non-fat dry milk in TBST buffer (50mmol/L Tris-HCl, pH 8.0, 100mmol/L NaCl and 1% Tween 20) for 1 hour at room temperature to block nonspecific binding. Membranes were probed overnight with rabbit anti-pan-cadherin (anti-E-, P-, N-, R-, VE-cadherin) antibody (Sigma, St. Louis, MO, USA) 1:1000 (v/v) dilution in 2.5% non-fat dry milk in TBST and rabbit anti-β-catenin antibody (BD Transduction Laboratories, Lexington, KY, USA) 1:1,000 (v/v) dilution in 2.5% non-fat dry milk in TBST. After washing in TBST, the membranes were incubated for 30 minutes in 2.5% non-fat dry milk in TBST containing horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (Upstate, Temecula, CA, USA), 1:3,000 dilution (v/v). Peroxidase activity represented by bound of antibodies was visualized using enhanced chemiluminescence by the ECL detection method (THORPE & KRICKS, 1986) and exposed to X-ray films (Kodak, Manaus, AM, Brazil).

Statistical analysis

Immunoblottings were analyzed after digitalization of X-ray films using a flat-bed scanner (Color Page Vivid Pro II™, Genius, Langenfeld, Rheinland, Germany) and Scion Image software (Scion Corporation, Frederick, MD, USA). Ponceau S staining was used to determine proper protein fractionation and equivalent loading. The optical density ratio of the bands to Ponceau S staining was used to correct small uneven loading. Comparison between groups and respective controls were analyzed by one-way ANOVA using Microsoft Excel software (Microsoft Corporation, Seattle, WA, USA). Values are expressed as mean fold change ± standard deviation; a p value $\leq 0,05$ was considered significant.

RESULTS

Western blot analysis revealed increases of cadherin expression in both acute and chronic

phase versus its respective controls as showed in Figure 1. Values of group A (2.1-fold ± 0.89, p= 0.048) and group C increased significantly (2.1-fold ± 0.92, p= 0.05) when compared to its respective controls (Figure 2).In relation to β-catenin, the results showed the same pattern characterized by increases in its levels also in acute and chronic phase in relation to controls (Figure 3). Statistical analysis of densitometry demonstrated significant increases between group A (6.8-fold ± 4.65, p= 0.047) and group CA and group C and group CC (3.65-fold ± 1.93, p= 0.033) (Figure 4).

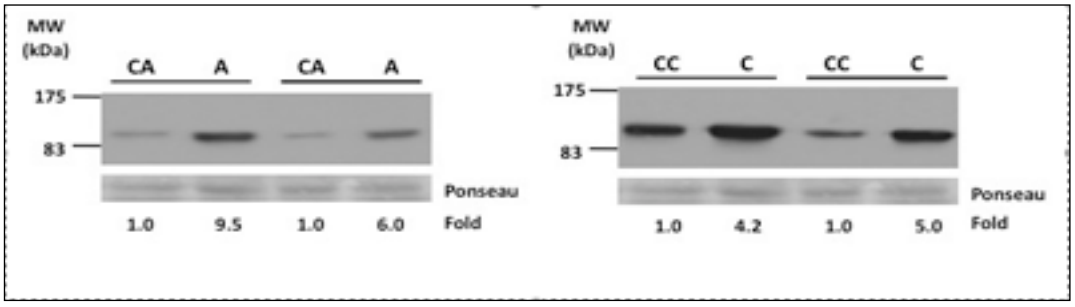

FIGURE 1. Representative immunoblotting of pan-cadherin antibody (130-150 kDa) detection in heart lysates of mice (n=5 per group) infected with *T. cruzi* and killed at 14th day (CA and A - left) and 90th (CC and C - right). Pan-cadherin expression increases in A (2.1-fold ± 0.89, p= 0.048) and C (2.1-fold ± 0.92, p= 0.05) as compared with their respective controls, CA and CC. Ponceau S was used to ascertain proper protein fractionation and transfer.

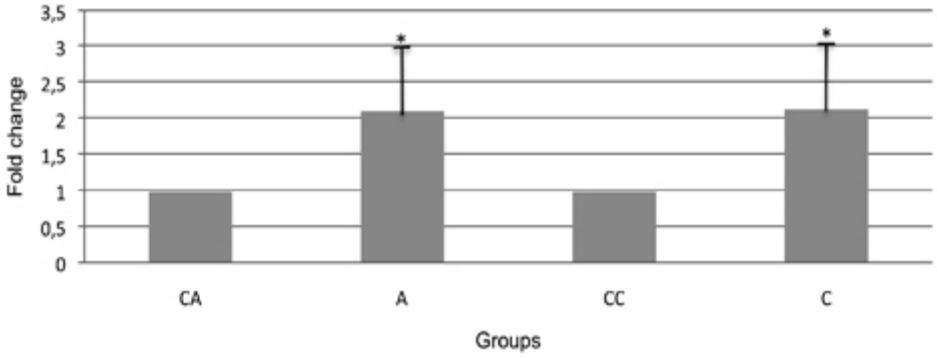

FIGURE 2. Bar graphic illustrating the densitometry analysis of fold means and standart deviations of results obtained in western immunobotting for pan-cadherin detection in heart lysates from mice (n=5 per group) infected with *T. cruzi* in acute (group A and its control CA) and chronic (C and control CC) phase. Bars represent the relative fold change of samples. * p< 0.05 to control group.

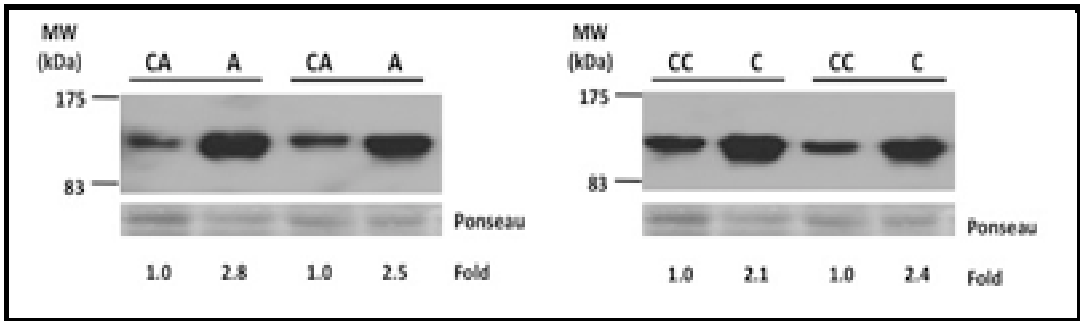

FIGURE 3. Representative immunoblotting of β-catenin (92 kDa) detection in heart lysates of mice (n= 5 per group) infected with *T. cruzi* and killed at 14th day (CA and A - left) and 90th (CC and C - right). The figure shows that β-catenin expression increases in group A (6.8-fold ± 4.65, p= 0.047) and group C (3.65-fold ± 1.93, p= 0.033) as compared with theirs controls, CA and CC. Ponceau S was used to ascertain proper protein fractionation and transfer.

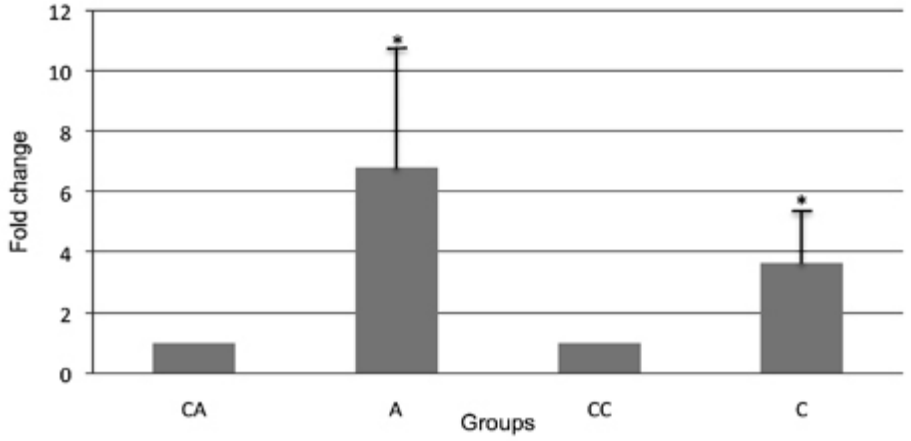

FIGURE 4. Bar graphic illustrating the densitometry analysis of fold means and standart deviations of results obtained in western immunobottings for β-catenin detection in heart lysates of mice (n= 5 per group) infected with *T. cruzi* in acute (group A and its control CA) and chronic (C and control CC) phase. Bars represent the relative fold change of samples. * $p< 0.05$ to control group.

DISCUSSION

In myocardium, cadherin/catenin complex is primarily localized to adherens junctions in intercalated disks where it serves as an attachment site for myofibrils, in addition to its structural role in maintaining myocyte adhesion. The disruption and expression of many of junctional components are often perturbed in cardiovascular disease as confirmed by FUJIO et al. (1995) and DUPONT et al. (2001). In view of these reports, in the present study, we turned our attention to the intercalated disks of the myocardium, which play an important role in cardiac contractile function.

In relation to adherens junctions disruption, our immunoblotting results did not show smaller-molecular-weight fragments of cadherins and β-catenin in acute and chronic phase in total heart lysates. BIANCHI et al. (2001), carrying out bichemical and confocal microscopy analysis of adherens junctions in hearts after cardiopulmonary bypass (CPB), revealed partial degradation of these proteins, pointing to a molecular mechanism leading to cardiomyocyte dysfunction often observed in patients subjected to CPB. However, our results suggest that disruption of adhrens junctions proteins does not seem to be the leading cause of heart malfunction due to *Trypanosoma cruzi*, although this parasite is associated to miofibrillar proteins rupture in *T. cruzi* experimental infection (TANIWAKI et al., 2005).

In this study, western blotting analysis of heart lysates of mice infected with *T. cruzi* clearly showed upregulation of cadherins and β-catenin expression in acute and chronic phase. However, the fold change mean was similar for pan-cadherin in both phases and β-catenin overexpression was higher in acute than chronic stage when compared to respective controls. Our observations add to the myriad of reports stating that heart damage is frequently associated to deletion, overexpression, mutation or exchange of genes encoding adherens junctions components (BIERKAMP et al., 1996; RUIZ et al., 1996; SOLER et al., 2001; FERREIRA-CORNWELL et al., 2002; PERRIARD et al., 2003; GROSSMANN et al., 2004).

Upregulation of cell-cell adhesion molecules expression by host cells have been shown to act as receptors for the binding and invasion of infectious agents to selective tissues, for example, E-cadherin is the receptor for *Listeria monocytogenes* in epithelial cells (MENGAUD et al., 1996) and NCAM is the receptor for the targeting of cells by the rabies virus (THOULOUZE

et al., 1998) and *T. cruzi* (SOLER et al., 2001). Thus, the increases of cadherins and β-catenin expression in Chagas myocarditis reported here suggest that cardiomyocytes adherens junctions may act as a receptor for tissue targeting and cellular invasion by *T. cruzi* in this experimental infection. The higher fold change in β-catenin expression compared to control in acute phase in relation to chronic phase may indicate its higher involvement as ligant and invasion receptor in this stage when the myocarditis showed more intense heart lesions (not shown).

Increased adherens junctions proteins expression, as demonstrated by this study, was also reported by FERREIRA-CORNWELL et al. (2002). Their investigation showed that increased cadherin expression in hearts was accompanied by increased levels of β-catenin in transgenic mice, implying that excess cadherin/catenin complexes may alter the contractile dynamics by changing the stoichiometry of the cadherin/myofibril connection, leading to alteration of the myofibril connection and less efficient force transduction across the plasma membrane. The dissipation of contractile force could lead to a compensatory response (i.e. hypertrophy), and consequently cardiomiopathy. Besides, according to LI et al. (2006), the junctional complexes must be properly organized within the intercalated disc to mediate normal mechanical and electrical coupling between the cardiomyocytes to preserve normal cardiac function. Thus, when this organization is perturbed leads to cardiac arrhythmia. Both cardiomiopathy and arrhytmia are usual findings observed in patients with Chagas disease.

The results of this investigation point to the role of adherens junction proteins as potential targets or triggers to unleash the pathological events and its unrollings in hearts associated to *T. cruzi* infection.

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