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Review Article

The Role of Dystrophin Loss in the *Trypanosoma cruzi* Infection

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Abstract

Dystrophin, an important protein of the dystrophin-glycoprotein complex, has been implicated in the pathogenesis of experimental Chagas disease. It contributes to cell shape, mechanical resistance, contraction and force generation in cardiomyocytes. Dystrophin loss has been associated with end-stage cardiomyopathies and proposed as a common route for myocardial dysfunction and progression to advanced heart failure. One of the most intriguing aspects of chronic Chagasic cardiomyopathy is the long delay after the initial infection to the cardiac manifestations. This has been partially explained by our group in previous studies demonstrating loss/reduction of dystrophin in mice experimentally infected with T. cruzi. The analysis of dystrophin expression showed significant reduction in the acute phase, with the reduction maintained up to the chronic phase. Inflammatory mechanisms could be involved in the dystrophin loss since inflammation has been shown to play a key role in the activation of proteases responsible for dystrophin degradation.

INTRODUCTION

Chagas disease is caused by the protozoan parasite *Trypanosoma cruzi* (*T. cruzi*), which is transmitted when the infected feces of the triatomine vector are inoculated through a bite site or through an intact mucous membrane of the mammalian host [1]. Originally confined to Latin American countries, it had been considered an exotic disease and received less attention from global health policy-makers and the scientific community than it could expect due to its high morbidity and mortality. The migration of infected persons to large urban cities and nonendemic countries changed the epidemiological profile of Chagas disease from a disease of poor rural areas to a globalized problem of large cities in Latin America as well as most of the developed world. By the early 1990s, the World Health Organization considered Chagas disease the most serious parasitic disease in Latin America and as having the greatest economic impact. The number of estimated infected people was approximately 18 million, with a further 100 million under risk. Now, the revised numbers are much reduced, with an estimate of about 10-13 million [2] or, even less, 8-10 million infected people [3].

Although Chagas disease was first described more than a century ago, the course of the disease and its clinical outcomes are still not totally understood. The clinical course of Chagas disease is usually divided into three phases: acute, indeterminate or latent, and chronic. In most cases, the initial infection is asymptomatic. However, a few cases will present acute symptoms and in some instances death may occur in 3%-5% of cases [4,5]. Infected individuals surviving the acute phase enter the indeterminate stage, characterized by a long asymptomatic period before the onset of clinical signs and symptoms. This phase can last 10-30 years or until the end of an individual's life [6]. Approximately 30% of the infected individuals eventually develop late manifestations [5,7,8]. The symptomatic disease affects the heart in 94.5% of patients that are considered to have chronic Chagas cardiomyopathy (CCC), usually between 15 and 50 years of age. Congestive heart failure is the cause of death in 58% of these patients, whereas cardiac arrhythmias and unexpected death affects 36.5%. The remaining manifests as mega-syndromes of hollow viscera, usually megaesophagus and megacolon [9,10]. Different mechanisms have been proposed to explain the pathogenesis of CCC, such as, (1) direct tissue destruction by *T. cruzi* [11-16]; (2) autonomic abnormalities [9,17-22]; (3) microvascular changes [23-29] and (4) autoimmune mechanisms [13,31-38].

One of the most intriguing aspects of chronic Chagasic cardiomyopathy is the long delay after the initial infection to the cardiac manifestations. This has been partially explained by our group in previous studies demonstrating loss/reduction of dystrophin in mice experimentally infected with *T. cruzi*

[39,40]. Similar to CCC in humans, cardiac complications due to cardiomyopathy also appear later in life in Duchenne muscular dystrophy and Becker muscular dystrophy, the most common X-linked recessive disorders resulting from mutations in the dystrophin gene that lead to an absence of or defect in the protein dystrophin in striated muscles. In this way, the development of cardiomyopathy in both Chagas disease and congenital dystrophinopathies occurs decades after infection or birth in humans, respectively. Dystrophin and associated glycoproteins form the so-called dystrophin glycoprotein complex (DGC), which contributes to cell shape, mechanical resistance, contraction and force generation in cardiomyocytes [41]. Dystrophin is localized beneath the sarcolemma and links cytoplasmic actin to the extracellular matrix through the membrane spanning glycoproteins (Figure 1) [42]. The reduction of dystrophin has been linked to end-stage cardiomyopathies and proposed as a common route to the induction of cardiomyopathy and heart failure [43,44]. Furthermore, dystrophin loss has been observed in different experimental models of cardiomyopathies, such as post-viral myocarditis caused by Coxsackie virus B [45], myocardial infarction [46], isoproterenol [44,47] and doxorubicin administration [48] and septic cardiomyopathy [49]. Based on these facts, our group was the first to study dystrophin expression in the hearts of mice experimentally infected by *Trypanosoma cruzi* in both acute and chronic stages of the disease.

Dystrophin Expression in Acute and Chronic Stages of Experimentally-Induced *T. Cruzi Infection*

Prado et al. [39], tested the hypothesis that cardiac dystrophin levels were decreased during the earlier phases of the experimental infection by *T. cruzi* in mice and maintained at low levels up to development of cardiomyopathy. Infection with specific strains of *T. cruzi* leads to a cardiomyopathy that evolves from the acute to the chronic phase. To experimentally reproduce this infection, male CD1 mice were infected with the Brazil strain of *T. cruzi*. Control and infected mice were killed 30 days post infection (dpi) (considered acute stage) and 100 dpi (considered chronic stage) and heart disturbances characterized. Densitometric analysis of Western blotting showed a significant reduction of dystrophin expression in the mice hearts at 30 dpi, with the reduction maintained up to 100 dpi. In addition to the quantification by Western blotting, the expression of dystrophin in the cardiac tissue was evaluated by immunofluorescence (IF). The IF revealed that dystrophin labeling occurred in a uniform pattern as a continuous rim at the periphery of most cardiomyocytes from control hearts (Figure 2). However, dystrophin was focally reduced or completely lost in cardiomyocytes of infected mouse hearts. At 30 dpi there were foci of myocytolysis associated with loss of the dystrophin fluorescent signal and infiltration of interstitial cells (Figure 2), asterisk). Additionally, foci of cardiomyocytes showing undamaged actin were observed with markedly reduced/loss of dystrophin fluorescent signal (Figure 2), arrows). The double-stained against dystrophin x actin was to detect cardiomyocyte cytoskeletal actin in order to determine whether dystrophin loss was a consequence of cardiomyocyte death. Spread blocks of cardiomyocytes lacking dystrophin distinctly showed the red fluorescent signal for actin, indicating an absence of correlation between myocyte necrosis and dystrophin loss. At 100 dpi the foci of loss/reduction of dystrophin fluorescent signal were evident (Figure 2), arrows). It has been demonstrated that dystrophin loss destabilizes the DGC present at the sarcolemma, disrupts the physical linkage of the subsarcolemmal cytoskeleton with the sarcolemma causing alterations in calcium homeostasis and increases the membrane permeability, thus impairing contractile

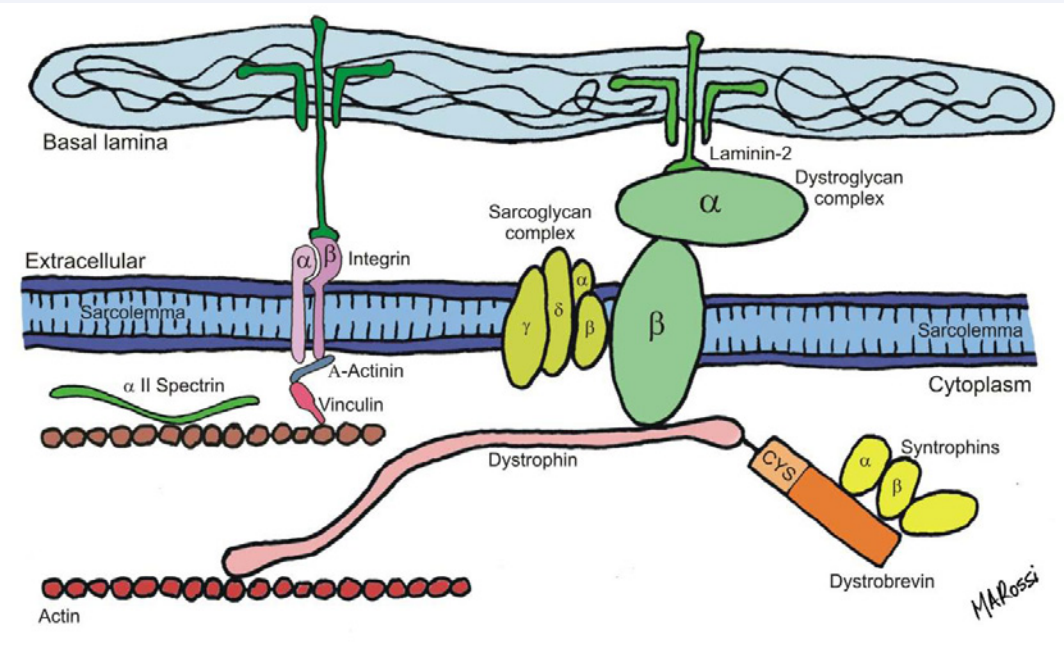

Figure 1 Schematic drawing of dystrophin glycoprotein complex (DGC). The complex forms a transmembrane link between the extracellular matrix (laminin) and the intracellular cytoskeleton (actin filaments), which is thought to mechanically stabilize the plasma membrane of muscle cells.

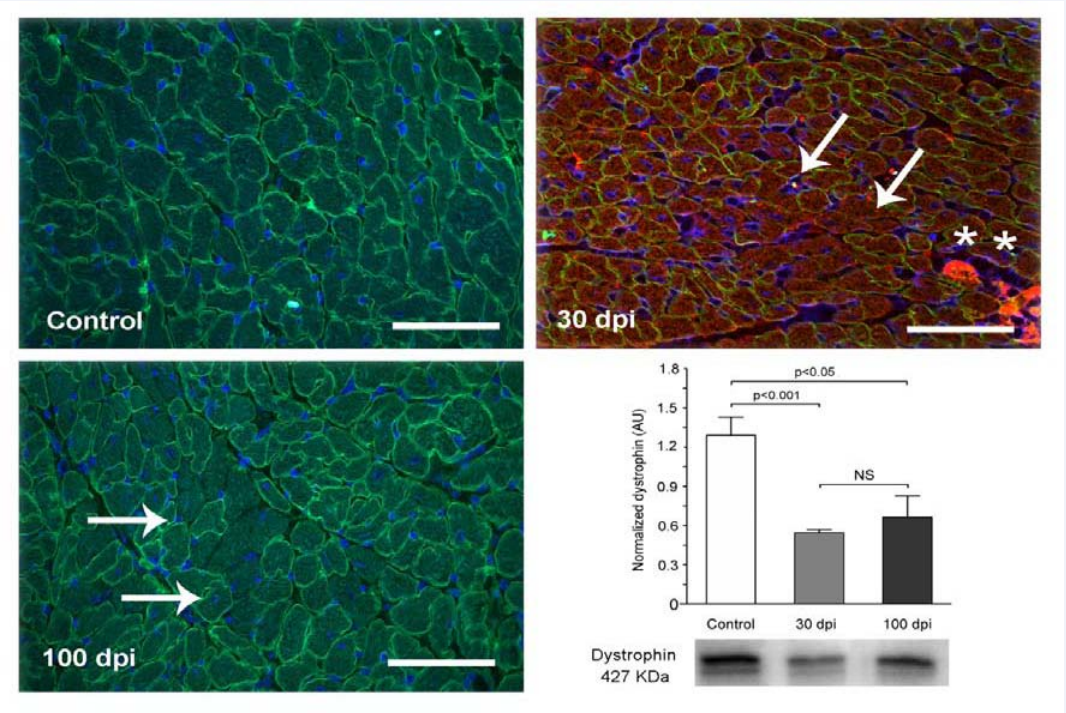

Figure 2 Representative images of IF for dystrophin (green fluorescence) and actin (red fluorescence) in control and T. cruzi-infected mice and immunoblotting quantification. The IF study in control showed that dystrophin labeling occurred in a uniform pattern as a continuous rim at the periphery of most cardiomyocytes. Dystrophin was focally lost in cardiomyocytes of infected mouse hearts. At 30 dpi there were foci of myocytolysis associated with loss of the dystrophin fluorescent signal and infiltration of interstitial cells (asterisk). Additionally, foci of cardiomyocytes showing undamaged actin were observed with markedly loss of dystrophin fluorescent signal (arrows). The double-stained against dystrophin x actin was to detect cardiomyocyte cytoskeletal actin in order to determine whether dystrophin loss was a consequence of cardiomyocyte death. Spread blocks of cardiomyocytes lacking dystrophin distinctly showed the red fluorescent signal for actin, indicating an absence of correlation between myocyte necrosis and dystrophin loss. At 100 dpi, foci of dystrophin loss were evident (arrows). Bars=60 microns.

transmission that results in contractile dysfunction [50].

Morphological and functional cardiac alteration: Since dystrophin loss has been linked to alterations of contractile force transmission, the next step was to characterize the morphological and functional alterations in these hearts.

At 30 dpi there was an intense and diffuse myocarditis characterized by lymphomononuclear interstitial infiltrate, disruption of myofibers, multiple pseudocysts of amastigotes, enlargement of the interstitial space and perivascular inflammatory infiltrate, mainly in the right ventricle (Figure 3), H&E). By 100 dpi, the number of lymphomononuclear inflammatory cells was reduced and no parasites were detected (Figure 3), H&E). The number of interstitial mononuclear cells was more pronounced at 30 dpi when compared to 100 dpi, especially in the right ventricle (Figure 3), graph). The analysis of picrosirius red-stained sections revealed mild myocardial fibrosis manifested by an increased amount of pericellular collagen (endomysial matrix) and mild perivascular fibrosis. These findings were more evident in the right ventricle. The increase in the volume fraction of fibrosis was significant only at 100 dpi in both ventricles (Figure 3), graph).

Magnetic resonance imaging (MRI) and echocardiography have been used with increasing frequency in experimental Chagas disease. There are many advantages related to the application of these imaging methodologies [51-55]. They are noninvasive and do not require exposure to radioactivity or contrast agents. They also permit the serial monitoring of individual mice, without the necessity of sacrifice. This reduces the number of mice required for the validation of results since each mouse can be imaged several times through the course of disease progression and heart alterations can be evaluated at multiple time points. The MRI study showed no significant difference in the left ventricular internal diameter in infected mice compared with uninfected controls. However, the inner dimension of the right ventricle was significantly dilated from 30 to 100 dpi (Figure 4), arrow). In the evaluation of systolic function by echocardiography, the left ventricle ejection fraction, evaluated by B-mode, did not show any change at 15 dpi in comparison with controls. However, there was a 23% reduction in the left ventricle ejection fraction at 30 dpi, 20% at 60 dpi and 20% at 100 dpi in comparison with respective controls (Figure 4), graph).

Dystrophin loss, inflammation and mortality rate: Although dystrophin gene mutations represent the primary cause in Duchenne and Becker muscular dystrophies, it has been demonstrated that the secondary processes, involving persistent inflammation with high levels of proinflammatory cytokines, likely sustain and exacerbate the progression of these diseases [56]. A previous study demonstrated that Duchenne patients with evidence of active myocarditis associated with myocyte damage

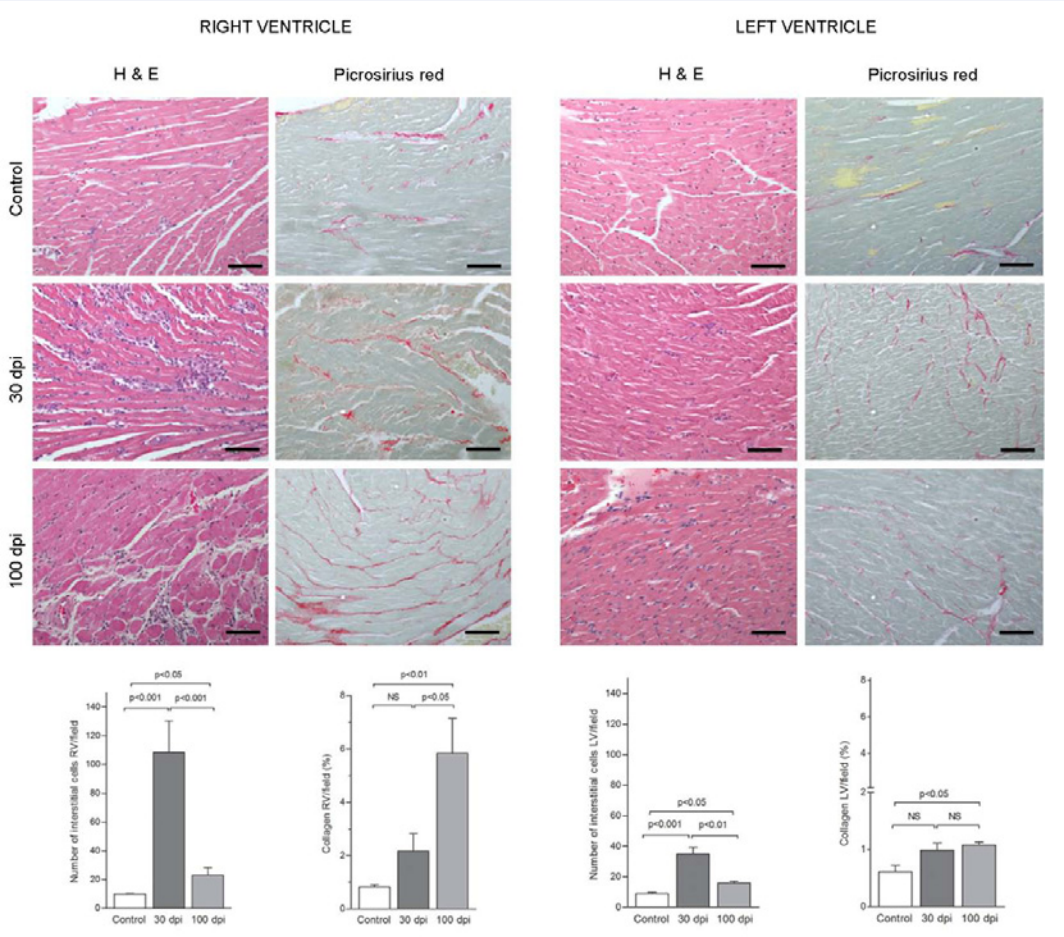

Figure 3 Hematoxylin and eosin (H&E) and picrosirius red staining and picrosirius red-polarized light of right and left ventricles from control mice and infected at 30 and 100 dpi. At 30 dpi there was an intense and diffuse myocarditis mainly in the right ventricle. By 100 dpi, the number of inflammatory cells was reduced and no parasites were detected. The analysis of picrosirius red-stained sections revealed mild myocardial fibrosis and perivascular fibrosis, mainly in the right ventricle. Graphs show the number of interstitial cells and collagen quantification in both ventricles. Bars=100 μm.

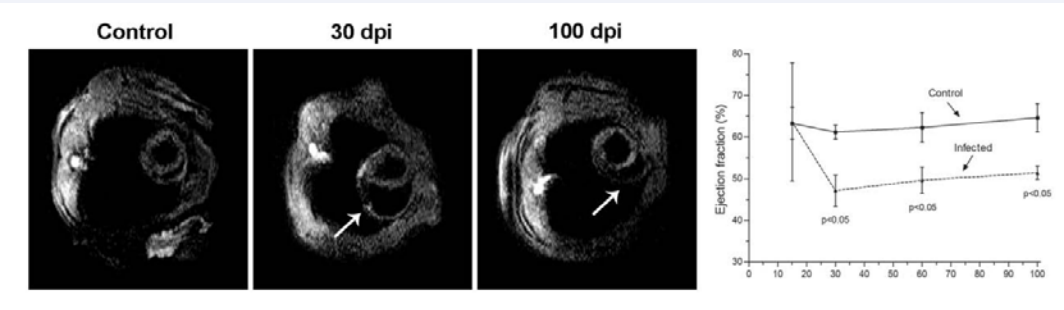

Figure 4 Transverse magnectic resonance imaging (MRI) images of mice showing the short axis of the heart. The inner dimension of the right ventricle was significantly dilated from 30 to 100 dpi. Arrows = right ventricle.

and fibrosis had a faster progression to heart failure and death in comparison with Duchenne patients with evidence of healed myocarditis [57]. These observations indicate that inflammatory mechanisms could also be involved in the dystrophin changes observed in our studies. In addition, it has been suggested that inflammation plays a key role in the activation of proteases, mainly calpains. Many of the proteins linking the cytoskeleton to the plasma membrane are cleaved rapidly by the calpains, especially dystrophin [58,59]. To this end, tumor necrosis factor alpha (TNF-α), nuclear factor-kappa B (NF-kB) and calpain-1 levels were investigated in the peak of mortality at the acute phase.

The amount of TNF-α, quantified by Western blotting, was significantly increased, representing an increase of 110% in comparison with control mice. High TNF-α fluorescence expression was observed (Figure 5). Similarly, the amount of NF-KB increased 130% when compared with control animals. The immunofluorescence revealed that NF-kB peak was associated with increased number of inflammatory cells (Figure 5). Proinflammatory cytokines have been demonstrated to exert their actions on NF-kB, contributing to the dystrophic damage progression through activation of intracellular calcium dependent proteases, mainly calpain [43,60].

Calpains are calcium-activated neutral cysteine proteases with two major isoforms besides tissue specific forms: (a) calpain-1 or μ that requires micromolar Ca^{2+} concentrations for activity and (b) calpain-2 or m that requires millimolar Ca^{2+} concentrations [59]. The amount of calpain-1 was significantly increased, representing an increase of 70% compared to control mice. The fluorescent signal for calpain-1 was associated with an increased number of inflammatory cells (Figure 5). The detection of significantly increased expression of intracellular calpain implicates this protease, activated by increased intracellular calcium concentration, in the mechanism of dystrophin loss and proteolysis. Previous study demonstrated that calpains digest dystrophin very rapidly when the calcium concentration is compatible with their activation [61]. Increased calpain activity has often been reported as an aggravating factor in cardiovascular diseases and other pathophysiological conditions [62]. The activation of calpains following the elevated intracellular Ca^{2+} and proteolysis of dystrophin have been shown in different experimental models of cardiomyopathies such as myocardial infarction [46], isoproterenol [44,47] and doxorubicin administration [48] and sepsis [63].

Since proinflammatory cytokine response is associated with Chagas disease, the evaluation of the association among the high mortality rate observed in the acute infection, inflammation and loss/reduction of dystrophin was done. The levels of TNF-α and NF-kB were increased in mice infected with *T. cruzi*, but increased mortality was observed only when cardiac dystrophin expression was significantly decreased, highlighting the correlation among inflammation, dystrophin loss and mortality (Figure 6).

***In vitro* studies:** In order to confirm our *in vivo* results, *in vitro* experiments were performed using cultured neonatal mouse cardiomyocytes. Cardiomyocytes were isolated from neonatal hearts, routinerally processed and when cardiomyocytes showed spontaneous contractility, serum (free of parasites) from either *T. cruzi*-infected or control mice were added to the cells. This serum was collected in the peak of cytokine production [64,65].

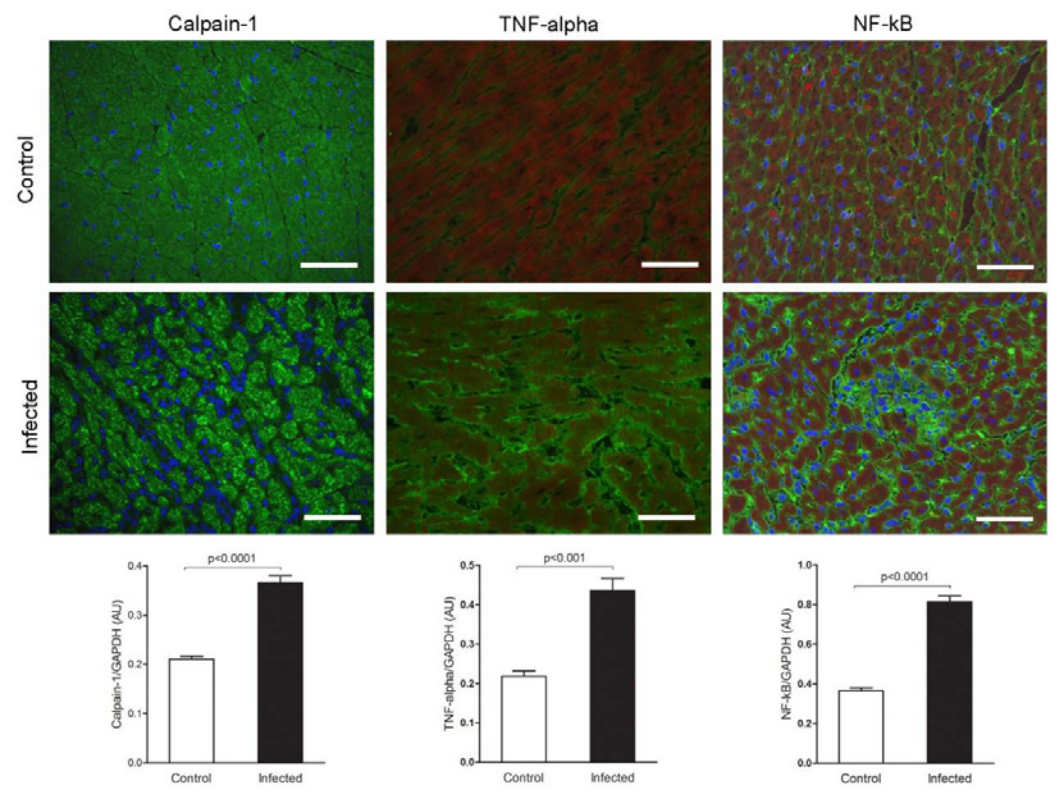

Figure 5 Representative images of IF for calpain-1, TNF-alpha and NF-kB in control and T. cruzi-infected mice and immunoblotting quantification. The IF signals for calpain-1 (green fluorescence) was significantly increased and associated with an increased number of inflammatory cells revealed by blue fluorescence of DAPI. Graph shows the quantification of the calpain-1 by Western blotting. The TNF-alpha IF signal (green fluorescence) was quite weak in control mice as compared to infected mice. F-actin is showed in red. The immunoblotting quantification corroborates the IF findings. The same phenomenon was observed in the IF signal for NF-kB (green fluorescence). GAPDH signal was used to normalize loading differences between lanes in the WB quantification. Bars=80 microns.

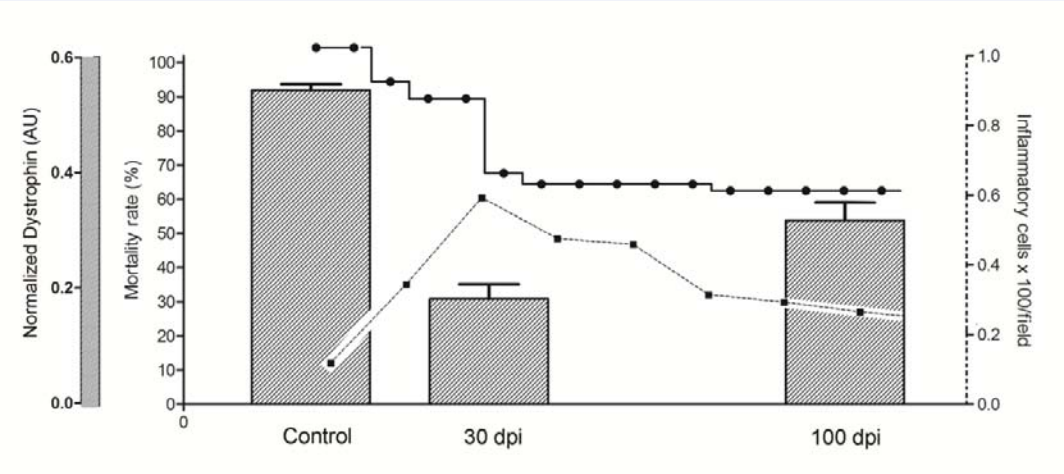

Figure 6 Mortality rate,quantification of inflammatory cells and dystrophin expression. Peaks of mortality, dystrophin loss and inflammation are coincident, highlighting the association among inflammation, dystrophin loss and mortality.

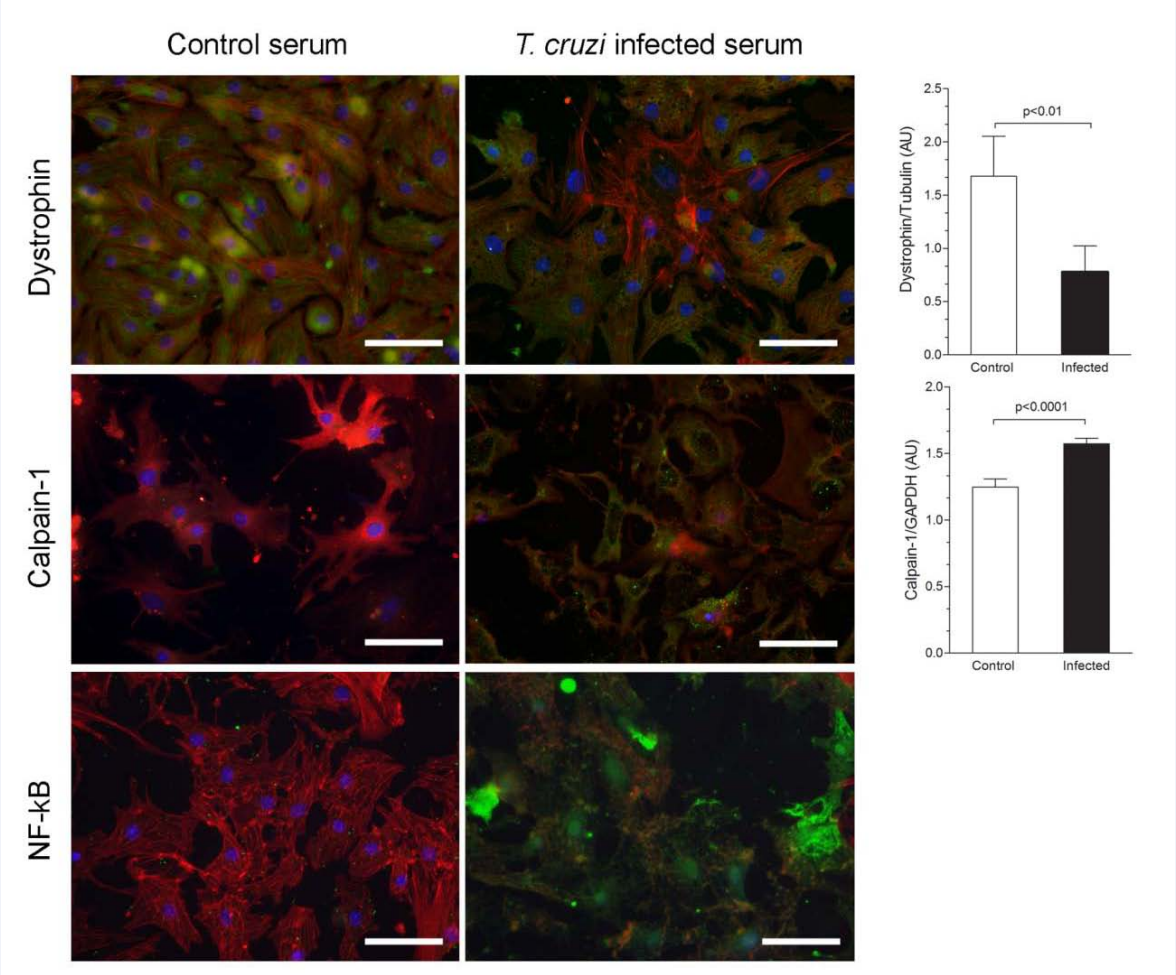

Figure 7 Representative images of IF for dystrophin (green fluorescence), calpain-1 (green fluorescence) and NF-kB (green fluorescence) and immunoblotting quantification in cultured newborn cardiomyocytes incubated with control and T. cruzi-infected sera. F-actin is stained with Alexa fluor 594 (red fluorescence) and nuclei with DAPI (blue fluorescence). Cardiomyocytes incubated with sera obtained from infected mice presented bleb formation and decreased expression of dystrophin. Immunoblotting quantification demonstrated that the amount of dystrophin was markedly decreased. The IF for calpain-1 showed increased expression of calpain-1 as compared to expression in control cardiomyocytes. Western blotting quantification confirms the IF findings. The IF for NF-kB showed a marked increase of fluorescence signal. Bars=50 microns.

Following the experimental periods, the cardiac cells were processed for Western blotting and immunofluorescence labeling for dystrophin, calpain-1 and NF-kB.

The quantification of dystrophin by immunoblotting showed a reduction of 50% in cultured cardiomyocytes incubated with sera obtained from infected mice in comparison to those incubated with control sera. The IF analysis clearly showed decreased expression of dystrophin associated with formation of multiple blebs dispersed in the cytoplasm of cardiomyocytes. F-actin fluorescence staining revealed disruption and rearrangement of the filaments (Figure 7). Studies using renal tubule cells of rabbit and hepatocytes of rats exposed to highly toxic agents that act by increasing cytosolic calcium levels have shown that intracellular concentrations of calcium precede bleb formation. Pretreatment of these cells with protease inhibitor prevented bleb formation and decreased proteolysis rate [66-68]. The activation of calcium-dependent proteases could be responsible for the rearrangement of F-actin filaments inducing the formation of bubbles [69]. These findings emphasize the role of proinflammatory cytokines present in the serum of animals experimentally infected with *T. cruzi* in the activation of calpain-1 observed in our studies.

Immunoblotting results demonstrated that the amount of calpain-1 was increased by 33% in cardiomyocytes incubated with sera obtained from infected mice. This increase was clearly observed in the immunofluorescence (Figure 7).

Cultured cardiomyocytes incubated with sera obtained from infected mice displayed a marked increased fluorescence of NF-kB in comparison with cardiomyocytes incubated with control sera. In addition, disruption and rearrangement of F-actin associated with reduced size of cardiomyocytes were observed after the addition of sera obtained from infected mice (Figure 7).

DISCUSSION AND CONCLUSION

Chagas disease is one of the neglected tropical diseases now found in non-endemic areas of the world. In the past 100 years since the discovery of this disease by Carlos Chagas, the understanding of the pathology and pathogenesis of this disease has grown. There are many developments that are exciting and require further investigation. Among these, the long delay after the initial infection to the chronic cardiac manifestations deserves attention. Based on the evidences presented in the present review, these processes involving the decrease of dystrophin expression could be initiated in the acute stage of the disease and perpetuated to the chronic stage contributing to the late development of Chagas cardiomyopathy. Further studies with the use of specific calpain inhibitors could provide novel therapeutic targets to minimize cardiac damage during Chagas disease. This is a continuing interest of our laboratories.

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