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## Mechanisms of vascular dysfunction in acute phase of *Trypanosoma cruzi* infection in mice

Josiane F. Silva<sup>a,1</sup>, Luciano S.A. Capettini<sup>a,b,1</sup>, José F.P. da Silva<sup>a</sup>, Policarpo Sales-Junior<sup>c</sup>, Jader Santos Cruz<sup>d</sup>, Steyner F. Cortes<sup>b</sup>, Virginia S. Lemos<sup>a,\*</sup>

<sup>a</sup> Department of Physiology and Biophysics, Universidade Federal de Minas Gerais, Belo Horizonte, MG 31270-901, Brazil

<sup>b</sup> Department of Pharmacology, Universidade Federal de Minas Gerais, Belo Horizonte, MG 31270-901, Brazil

<sup>c</sup> René Rachou Institute, FIOCRUZ, Belo Horizonte, MG, Brazil

<sup>d</sup> Department of Biochemistry and Immunology, Institute of Biological Sciences, Universidade Federal de Minas Gerais, Belo Horizonte, MG 31270-901, Brazil

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### ABSTRACT

Vascular disorders have a direct link to mortality in the acute phase of *Trypanosoma cruzi* infection. However, the underlying mechanisms of vascular dysfunction in this phase are largely unknown. We hypothesize that *T. cruzi* invades endothelial cells causing dysfunction in contractility and relaxation of the mouse aorta. Immunodetection of *T. cruzi* antigen TcRBP28 was observed in endothelial cells. There was a decreased endothelial nitric oxide synthase (eNOS)-derived NO-dependent vascular relaxation, and increased vascular contractility accompanied by augmented superoxide anions production. Endothelial removal, inhibition of cyclooxygenase 2 (COX-2), blockade of thromboxane A<sub>2</sub> (TXA<sub>2</sub>) TP receptors, and scavenger of superoxide normalized the contractile response. COX-2, thromboxane synthase, inducible nitric oxide synthase (iNOS), p65 NF-κB subunit and p22<sup>phox</sup> of NAD(P)H oxidase (NOX) subunit expressions were increased in vessels of chagasic animals. Serum TNF-α was augmented. Basal NO production, and nitrotyrosine residue expression were increased. It is concluded that *T. cruzi* invades mice aorta endothelial cells and increases TXA<sub>2</sub>/TP receptor/NOX-derived superoxide formation. Alongside, *T. cruzi* promotes systemic TNF-α increase, which stimulates iNOS expression in vessels and nitrosative stress. In light of the heart failure that develops in the chronic phase of the disease, to understand the mechanism involved in the increased contractility of the aorta is crucial.

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### 1. Introduction

The agent of Chagas' disease *Trypanosoma cruzi* affects millions of people in rural areas of Central and South America, with great economic burden [1]. Infecting parasites or trypomastigotes, by entering the bloodstream, infect a wide range of susceptible host cells such as macrophages [2,3], cardiomyocytes [4], skeletal [5] and smooth muscle cells [6,7], human endothelial cell lines such as HUVEC and EAhy926 [8–10].

The acute phase of Chagas' disease is characterized by high levels of parasitemia, fever, lymphadenopathy and hepatosplenomegaly [10]. Despite the absence of cardiac remodeling and heart failure in this phase, some studies showed the existence of myocarditis, increased platelet aggregation, fibrin microthrombi formation, spasm and vasculitis of coronary microcirculation [4,10,11].

In the chronic phase, there is activation of the acquired immunity leading to a gradual reduction in parasitemia [10,12,13]. In some patients the low-grade of infection masks the symptoms for many years (silent infection). Differently from the asymptomatic patients, about 30% of symptomatic patients show a progressive active chagasic cardiomyopathy associated with cardiomegaly and disturbance in heart rhythm [12,14], increasing morbidity and mortality.

While the cardiac manifestations of Chagas' disease are well known, the involvement of the vasculature in its pathogenesis is commonly neglected. Some studies suggested that inflammation of cardiac microvessels related to *T. cruzi* infection induces the synthesis of several pro-inflammatory cytokines, vascular adhesion molecules and some vasoactive molecules such as endothelin (ET-1) and thromboxane A<sub>2</sub> (TXA<sub>2</sub>) [15–17]. Moreover, vasculitis [18] and structural changes of the aorta endothelial layer were reported in the acute phase of Chagas' disease [19]. The vascular function of vessels removed from animals infected with *T. cruzi* is so far unknown.

Aortic pulse-wave velocity has been shown to be associated with cardiovascular risk [20]. Moreover, aortic stiffness has been shown as an independent predictor of all-cause and cardiovascular mortality [21]. Therefore, the study of aorta function of animals infected with

\* Corresponding author at: Department of Physiology and Biophysics, Federal University of Minas Gerais, Av. Antônio Carlos 6627, CEP – 31970-901 Belo Horizonte, MG, Brazil.

E-mail address: [vslemos@icb.ufmg.br](mailto:vslemos@icb.ufmg.br) (V.S. Lemos).

<sup>1</sup> Co-first authors.

*T. cruzi* is crucial. Thus, the aim of the present study was to investigate the reactivity of the aorta during the acute phase of Chagas' disease and the underlying mechanism.

## 2. Methods

### 2.1. Animals

All animal procedures were performed according to the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health (United States), as well as, the guidelines for the humane use of laboratory animals at our Institute. These studies were approved by the ethics committee of the Federal University of Minas Gerais (UFMG; protocol # 72/2010). Male C57bl/6 mice were used. All animals were obtained from Rene Rachou Research Center (CPqRR-Fiocruz; Belo Horizonte, MG, Brazil) and maintained in the animal facilities of the Cellular and Molecular Parasitology Laboratory. Free access was allowed to standard diet and filtered water was supplied ad libitum. All mice were maintained at five per cage and in a constant temperature ( $24 \pm 2^\circ\text{C}$ ), with a 12-h dark/light cycle.

### 2.2. Protocol of infection

Animals (eight-week-old) were infected intraperitoneally with 50 bloodstream trypomastigote forms of Colombian *T. cruzi* strain [22], which was maintained by serial passages. Animals were sacrificed 30 days post-infection, and the aorta and serum were collected.

### 2.3. Vascular function studies

Experiments to assay vascular function were performed in an organ bath system, as previously described [23]. Briefly, thoracic aortic rings (3–4 mm length) from infected and control mice were obtained, mounted in an organ bath system containing Krebs–Henseleit solution (in  $\text{mmol}\cdot\text{L}^{-1}$ : 110.8 NaCl, 5.9 KCl, 25.0  $\text{NaHCO}_3$ , 1.07  $\text{MgSO}_4$ , 2.49  $\text{CaCl}_2$ , 2.33  $\text{NaH}_2\text{PO}_4$  and 11.51 glucose, pH 7.4), for 60 min. Concentration–response curves to acetylcholine (ACh) were constructed in vessels pre-contracted to the same tension level (approximately  $2.5 \text{ mN}\cdot\text{mm}^{-1}$ ) with submaximal concentrations of phenylephrine ( $0.03$ – $0.1 \mu\text{mol}\cdot\text{L}^{-1}$ ). Vascular contractions were evaluated by concentration–response curves to phenylephrine. When necessary, some vessels were incubated for 20 min with the indicated drugs before the construction of curves. Mechanical activity was recorded isometrically by a force transducer (World Precision Instruments, Inc.) connected to an amplifier-recorder (Model TBM-4; World Precision Instruments, Inc.) and to a personal computer equipped with an analog-to-digital converter board (DI-720; Dataq Instruments, Inc.), using Windaq data acquisition/recording software (Dataq Instruments, Inc.).

### 2.4. Superoxide and nitric oxide detection

Superoxide anions and nitric oxide (NO) production were measured by fluorescence microscopy, in aortic rings [24]. For superoxide measurements, the rings were incubated for 20 min at  $37^\circ\text{C}$  in Krebs–Henseleit solution containing or not  $10 \mu\text{mol}\cdot\text{L}^{-1}$  tiron, a superoxide scavenger (Merck-Millipore, USA). Then, the superoxide sensitive dye dihydroethidium (DHE;  $10 \mu\text{mol}\cdot\text{L}^{-1}$ ; Invitrogen, USA), was added for 30 min, protected from light. After that, the excess of dye was washed out, the rings were embedded in Tissue-Tek® O.C.T.™ freezing medium (Sakura®, USA) and quickly frozen in liquid nitrogen. For NO measurements, aortic rings were incubated for 20 min at  $37^\circ\text{C}$  in Krebs–Henseleit solution containing or not  $10 \mu\text{mol}\cdot\text{L}^{-1}$  of the iNOS inhibitor N(6)-(1-*iminoethyl*)-l-lysine dihydrochloride (L-NIL; Merck-Millipore, USA). The intracellular NO sensitive dye 4-amino-5-methylamino-2',7'-difluorescein diacetate (DAF-FM;  $10 \mu\text{mol}\cdot\text{L}^{-1}$ ; Invitrogen, USA) was added for 30 min, protected from light. After

washing, rings were frozen, as described above. Frozen rings were sliced in a cryostat ( $10 \mu\text{m}$ ; Leica 1850, Leica, USA) and mounted in slides with DAPI/antifade-containing medium (Santa Cruz Biotechnology, USA). Slides were imaged with an inverted fluorescence microscope (Eclipse Ti, Nikon, USA). Fluorescence intensity (Ex/Em: 518/605 and 495/515, to DHE and DAF-FM, respectively) was calculated from at least 8 fields from 4 different experiments using ImageJ software (NIH, USA).

### 2.5. Determination of tumoral necrosis factor alpha (TNF- $\alpha$ ) in serum by ELISA

Blood was collected during the sacrifice and the serum was separated by centrifugation ( $1500 \times g$  for 10 min). Quantification of TNF- $\alpha$  level was performed by the use of colorimetric kits (R&D Systems). The concentration of TNF- $\alpha$  in each sample was determined by a standard curve with known concentrations of TNF- $\alpha$ .

### 2.6. Western-blot analysis

Western blot was performed as previously described [25] with some modifications. Briefly, the frozen aorta segments ( $\sim 50 \text{ mg}$ ) were homogenized in lysis buffer (in  $\text{mmol}\cdot\text{L}^{-1}$ ): 150 NaCl, 50 Tris-HCl, 5 EDTA.2Na, and 1  $\text{MgCl}_2$  containing 1% Triton X-100 and 0.5% SDS plus protease inhibitors (SigmaFAST; Sigma-Aldrich, MO, USA). Equal amounts of proteins ( $30 \mu\text{g}$ ) were denatured and separated in denaturing SDS/7.5% polyacrylamide gel. Proteins were transferred onto a polyvinylidene fluoride membrane (Immobilon-P; Millipore, MA). Blots were blocked at room temperature with 5% BSA in TBS enriched with 0.1% Tween 20 before incubation with rabbit polyclonal anti-eNOS (1:1000; Sigma-Aldrich), goat polyclonal anti-phospho-eNOS<sup>Ser1177</sup> (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat polyclonal anti-phospho-eNOS<sup>Thr495</sup> (1:1000; Santa Cruz Biotechnology), mouse monoclonal anti-iNOS (1:2000; Santa Cruz Biotechnology), mouse monoclonal anti-nitrotyrosine (1:2000; Santa Cruz Biotechnology), mouse polyclonal anti-COX-2 (1:1000; Cayman Chemical), mouse monoclonal anti-NADPH p-22<sup>phox</sup> (1:1000; Santa Cruz Biotechnology), rabbit polyclonal anti-thromboxane synthase (1:500; Abcam, Cambridge, UK), rabbit polyclonal anti-NF- $\kappa\text{B}$  p65 (1:1000; Santa Cruz Biotechnology), mouse monoclonal anti- $\beta$ -actin (1:8000; Santa Cruz Biotechnology) or mouse monoclonal anti-GAPDH (1:8000; Santa Cruz Biotechnology), at room temperature. The immunocomplexes were detected by chemiluminescent reaction (ECL Plus kit; Amersham, Les Ulis, France) followed by densitometric analyzes with software ImageJ.

### 2.7. *T. cruzi* immunostaining

*T. cruzi* localization was performed in aortic rings (3–4 mm length) by immunofluorescence. Aortic rings were collected as described above, washed in Krebs–Henseleit solution, imbibed in Tissue-Tek® O.C.T.™ freezing medium (Sakura®, USA) and quickly frozen in liquid nitrogen. Frozen rings were sliced in a cryostat ( $10 \mu\text{m}$ ; Leica 1850, Leica, USA), fixed and permeabilized with cold acetone and 0.5% Triton X-100 (in PBS, pH 7.4). After block procedure (5% bovine serum albumin in PBS), slices were incubated with mouse polyclonal antibody against *T. cruzi* antigen TcRBP28 [26], followed by Alexa Fluor 647 donkey anti-mouse (Invitrogen, USA, OR). Slides were mounted with DAPI/antifade-containing medium (Santa Cruz Biotechnology) and imaged in an inverted fluorescence microscope (Eclipse Ti, Nikon, USA) at Ex/Em: 495/515 and 647/665, to elastin and TcRBP28, respectively.

### 2.8. Data analysis

Results are expressed as mean  $\pm$  SEM. Two-way ANOVA was used to compare concentration–response curves. Student's *t*-test was used in the other experiments. All statistical analyzes were considered significant when  $p < 0.05$ .

### 3. Results

Investigation of vascular function showed a reduced endothelium-dependent vasodilation evoked by ACh in aortas from mice acutely infected with *T. cruzi* (Fig. 1A). Analysis of NO/NOS signaling pathway showed that non-selective NOS inhibition with L-NAME ( $300 \mu\text{mol}\cdot\text{L}^{-1}$ ), inhibited ACh-induced vasodilation in both groups (Fig. 1B). This result suggests that NO is the main endothelial-derived relaxing factor in the mice aorta from control and chagasic mice. However, the area under the curve (A.U.C.) is reduced in vessels from infected mice, and indicates an impairment of ACh-stimulated NO production in aorta from chagasic mice (Fig. 1C). Evaluation of the relative contribution of NOS to ACh-dependent relaxation in both groups was calculated by the difference of the A.U.C. ( $\Delta$  A.U.C.) in the presence and the absence of L-NAME (Fig. 1D). This result suggests a reduction of eNOS expression and/or functioning in *T. cruzi*-infected vessels. Western blot experiments showed that total eNOS expression was reduced in the aorta from chagasic mice (Fig. 2A). Moreover, *T. cruzi* infection induced a reduction in eNOS functioning characterized by reduced phosphorylation of the activation site of the enzyme eNOS<sup>Ser1177</sup> (Fig. 2B) and increased phosphorylation in the inactivation site of eNOS<sup>Thr495</sup> (Fig. 2C).

The vascular contractility was also analyzed. The phenylephrine-induced contraction in endothelium-intact aortic rings from chagasic mice was augmented when compared to controls (Fig. 3A). Endothelium removal restored the contractile response in chagasic group to the same level measured in control vessels (Fig. 3A). These data suggest that increased contractility in infected mice was probably caused by an endothelial factor.

A common intracellular signaling pathway activated by *T. cruzi* infection is mediated by activation of ET-1 receptors [17]. To test

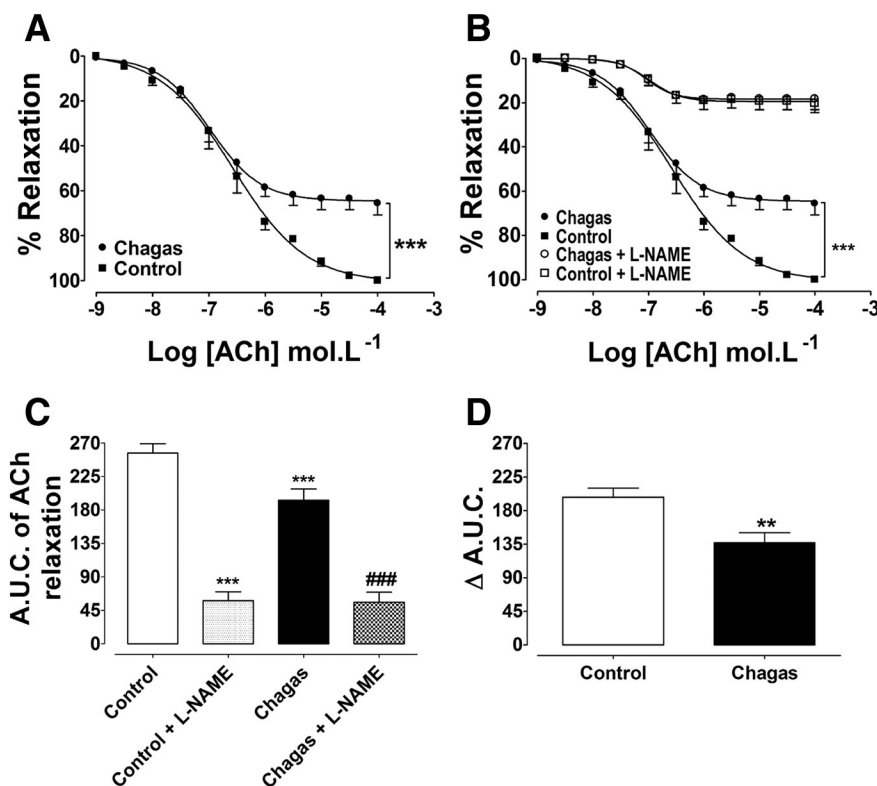
the possible participation of ET-1 on the increased vascular contractility, we used a non-selective antagonist of ET-1 receptors, bosentan ( $10 \mu\text{mol}\cdot\text{L}^{-1}$ ). Bosentan did not modify the contractile response in both groups (Fig. 3B).

Thromboxane A<sub>2</sub> (TXA<sub>2</sub>) production by *T. cruzi* and/or host cell has been implicated in cardiovascular dysfunction in Chagas' disease [15]. To evaluate the role of TXA<sub>2</sub> in endothelial dysfunction in chagasic mice aorta, the TXA<sub>2</sub> receptor antagonist SQ29548 ( $1 \mu\text{mol}\cdot\text{L}^{-1}$ ), the non-selective cyclooxygenase (COX) inhibitor ibuprofen ( $1 \mu\text{mol}\cdot\text{L}^{-1}$ ) and a selective inhibitor of cyclooxygenase 2 (COX-2) etoricoxib ( $1 \mu\text{mol}\cdot\text{L}^{-1}$ ), were used. Pre-incubation of vessels with SQ29548, ibuprofen or etoricoxib normalized the contractile response to phenylephrine in chagasic vessels to the level of controls (Fig. 3C, D and E).

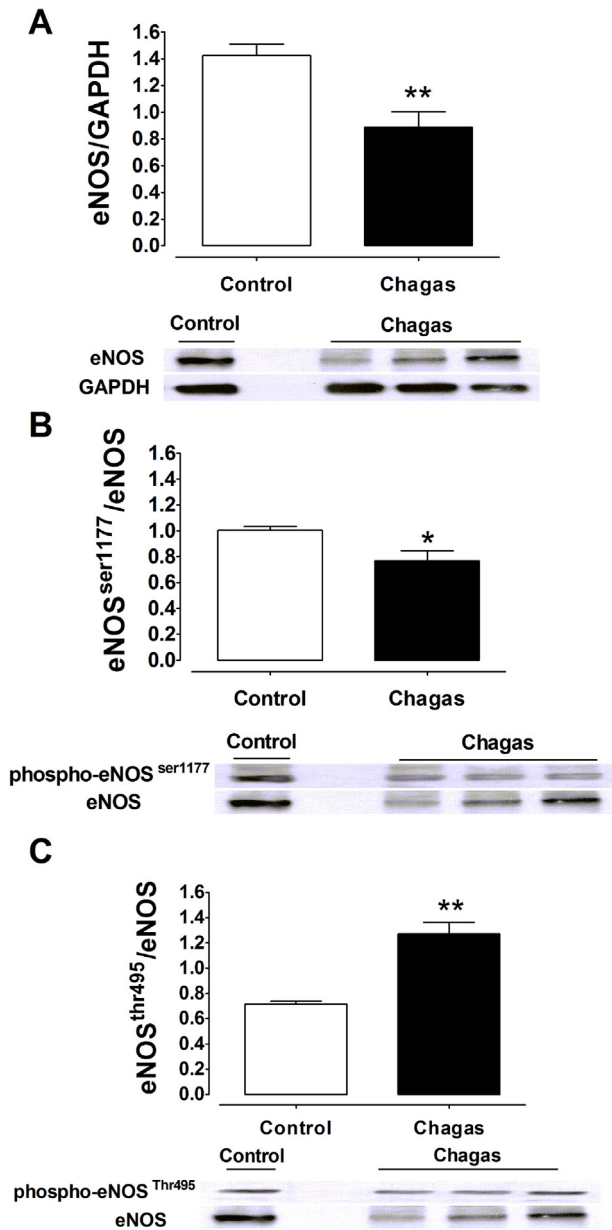
To evaluate the possibility of a direct infection of endothelial cells by *T. cruzi*, we performed immunostaining experiments to localize the parasite in the arterial wall. The immunodetection of *T. cruzi* antigen TcRBP28 showed that endothelial cells but not smooth muscle cells were infected with the parasite (Fig. 4).

TNF $\alpha$  is a proinflammatory cytokine produced systemically during the acute phase of Chagas' disease [10]. TNF $\alpha$  is an important inducer of NF- $\kappa$ B consequently leading to the transcription of genes including COX-2 [27] and thromboxane synthase [28]. ELISA assay using serum showed a large increase in TNF- $\alpha$  concentration in infected mice (Fig. 5A). Moreover, our Western blot experiments corroborate our functional data. As shown in Fig. 5B–D there were increases in expression of p65 subunit of NF- $\kappa$ B, COX-2 and thromboxane synthase in aortas from chagasic mice. Expression of COX-1 was not modified (Fig. 5E).

NF- $\kappa$ B also activates transcription of iNOS gene [29], which produces massive amounts of NO [30]. Therefore, we next evaluated if iNOS interferes with contractile function during acute infection by *T. cruzi*. Selective inhibition of iNOS with L-NIL ( $10 \mu\text{mol}\cdot\text{L}^{-1}$ ) did not change



**Fig. 1.** Vascular relaxation induced by acetylcholine (ACh) (A) in the presence and in the absence of L-NAME ( $300 \mu\text{mol}\cdot\text{L}^{-1}$ ) in control and chagasic aortas (B). Data are expressed as mean  $\pm$  S.E.M.  $n = 5$ ; \*\*\* $p < 0.001$  by two-way ANOVA. Area under the curve (A.U.C.) of the effect of ACh (C). Difference of A.U.C. ( $\Delta$  A.U.C.) between the curves in the absence and presence of L-NAME (D). Data are expressed as mean  $\pm$  S.E.M.  $n = 5$ ; \*\* $p < 0.01$  and \*\*\* $p < 0.001$  chagasic vs control or control + L-NAME vs control. ### $p < 0.001$  chagasic + L-NAME vs chagasic, one-way ANOVA with Newman-Keuls post hoc test.



**Fig. 2.** Western blot analysis of total eNOS (A) and acetylcholine-induced changes in the phosphorylation status of eNOS<sup>Ser1177</sup> (B) and eNOS<sup>Thr495</sup> (C) in the aortas from control and infected mice stimulated with acetylcholine. The vessels were stimulated with ACh (100  $\mu\text{mol}\cdot\text{L}^{-1}$ ). Images are representative blots from three separate experiments. Bar graphs represent mean  $\pm$  S.E.M. \* $p < 0.05$  and \*\* $p < 0.01$  by Student's *t*-test.

contractile response to phenylephrine in control vessels. However, L-NIL increased vasoconstrictor response in infected mice (Fig. 6A). In addition, as seen in Fig. 6B, there was a strong increase in iNOS expression in vessels from chagasic mice. Moreover, measurements of basal NO production by fluorescence microscopy using DAF confirmed that smooth muscle cells from chagasic mice produce large amounts of NO (Fig. 6C). Inhibition of iNOS with L-NIL (10  $\mu\text{mol}\cdot\text{L}^{-1}$ ) restored NO levels to the same values of control vessels (Fig. 6C). Corroborating with this result, chagasic mice presented high levels of nitrotyrosine, a marker of protein nitrosylation by NO or peroxynitrite (Fig. 6D).

Inflammatory diseases have been associated with vascular superoxide production and induction of oxidative stress [31,32]. Pre-incubation of vessels with tiron (10  $\mu\text{mol}\cdot\text{L}^{-1}$ ; Fig. 7A) a superoxide scavenger, and superoxide dismutase (SOD; 300 IU $\cdot\text{mL}^{-1}$ ; Fig. 7B), normalized the

contraction of chagasic mice to the same level of controls. Tiron and SOD did not modify the contraction in the control mice (Fig. 7A and B). Analysis of superoxide production by fluorescence microscopy using DHE staining confirmed that aorta from chagasic mice presented higher levels of superoxide compared to those of controls that were normalized by tiron treatment (Fig. 8). Finally, the subunit p22<sup>phox</sup> of NAD(P)H oxidase (NOX) expression was increased (Fig. 7C).

#### 4. Discussion

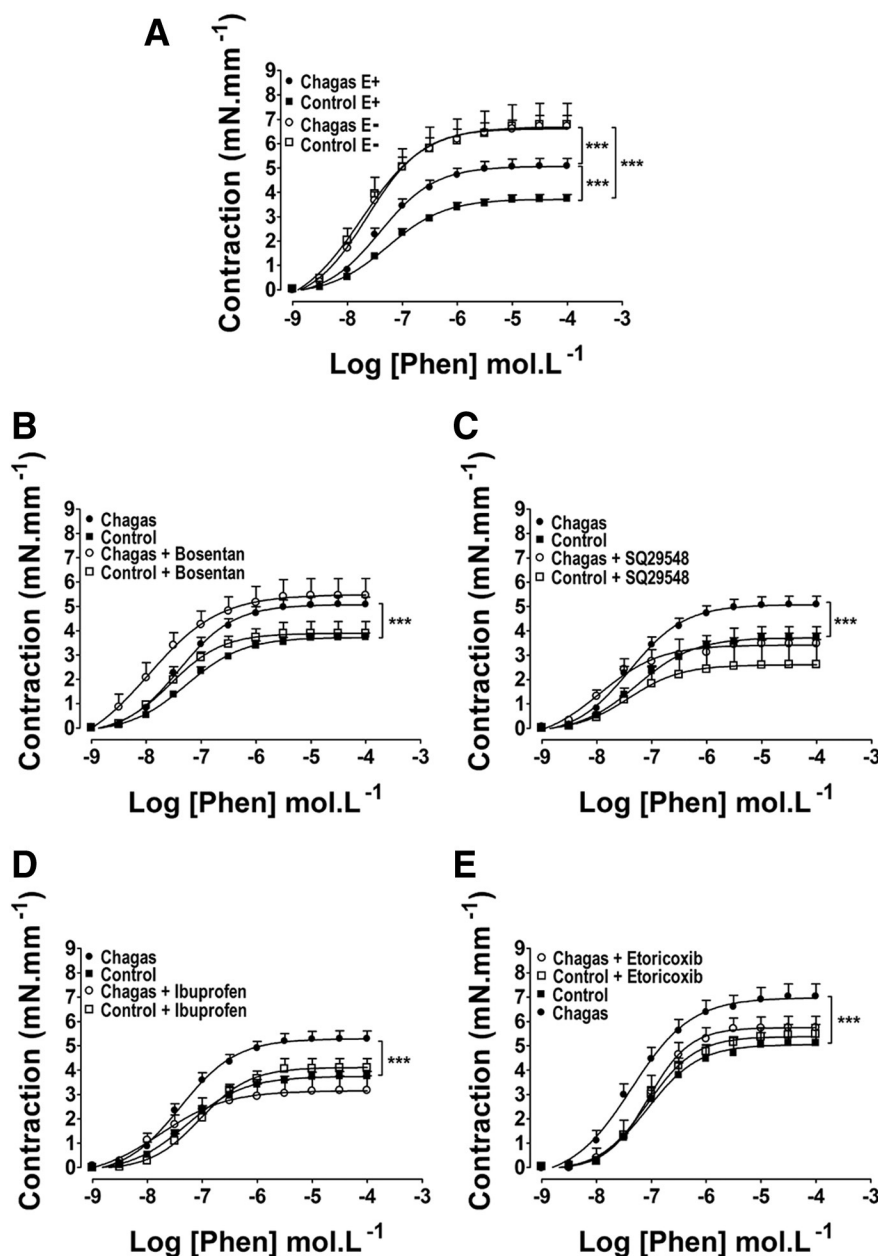
Cardiovascular studies on Chagas' disease are commonly related to cardiac adaptations during the chronification process. Although the involvement of vasculature is recognized to contribute to the pathogenesis of chagasic disease, the mechanisms involved in vascular dysfunction, mainly in the acute phase, is largely unknown. Moreover, most studies have focused on heart microvasculature. In view of the acute myocarditis and chronic cardiomyopathy caused by infection with *T. cruzi*, investigation of modifications of aorta function might be relevant to a better understanding of this fatal disease. In this study, we provide a detailed characterization of the underlying mechanisms of aorta dysfunction associated with Chagas' disease.

We showed here that aorta contractility was importantly increased in chagasic mice. Cellular invasion by *T. cruzi* represents an important event in the pro-inflammatory process involved in the progression of Chagas' disease [33]. Cultured endothelial and smooth muscle cells are known to be infected by *T. cruzi* [6–10]. In the present study, we show that the parasite invades endothelial but not smooth muscle cells from mouse aorta. TcRBP28 is an antigen present in the cytoplasm of cells infected with *T. cruzi* [26]. The immunostaining of TcRBR28 in the endothelial layer of the aortas of infected mice suggests that this event may be involved in the induction of the vascular dysfunction in the acute phase of the disease. It is known that after cell invasion the rate of TXA<sub>2</sub> production greatly increases [15]. TXA<sub>2</sub>, the most potent vasoconstrictor known, is the predominant eicosanoid present in all life stages of *T. cruzi*, suggesting that TXA<sub>2</sub>-TP signaling plays an important role in Chagas' disease [34]. Here we have shown that endothelial removal or pre-treatment of the vessels with ibuprofen (a non-selective COX inhibitor), etoricoxib (a selective COX-2 inhibitor) and SQ29548 (a TXA<sub>2</sub> receptor antagonist) restored the contractile response to the level found in controls. Moreover, p65 NF- $\kappa$ B subunit, COX-2 and thromboxane synthase expression levels were increased in infected vessels. Taken together, these data are in line with the hypothesis that *T. cruzi* infection induces the expression of NF- $\kappa$ B, which prompts to the expression of COX-2 [27] and thromboxane synthase [28], finally increasing the endothelial production of TXA<sub>2</sub> and vascular contraction.

ET-1 is a powerful vasoconstrictor secreted by endothelial cells, which is known to be increased after *T. cruzi* infection as well [35]. Despite the deleterious effects of large amounts of ET-1, especially inducing contraction in coronary arteries, ET-1 contributes to the early control of parasitemia [36]. Our results showed that ET-1 is not involved in the increased vasoconstriction in aortas from chagasic mice, suggesting that the mechanisms involved in vascular dysfunction in the acute phase of Chagas' disease may be different depending on the type of vessel studied.

Acute Chagas' disease has been associated with reactive oxygen species (ROS) formation [37]. To investigate whether the aorta of infected mice had increased production of ROS and whether they interfere with vascular function, a series of experimental protocols were performed. Our fluorescent experiments using the fluorescent dye DHE showed an important increase in superoxide anions formation in aortic rings sections from infected mice. In addition, Western blot experiments showed an increase in nitrotyrosine residues in vessels from infected mice. Finally, pre-incubation of aortic rings with tiron (a superoxide anion scavenger) or SOD, which dismutates superoxide into H<sub>2</sub>O<sub>2</sub> and water, normalized contractile response of infected vessels to the level of controls, similarly to COX-2 and TXA<sub>2</sub>-receptor blockade. NOX





**Fig. 3.** The contraction induced by phenylephrine (Phen) is increased in chagasic vessels with endothelium (E+). Endothelium removal (E−) restored contraction to the same level of control without endothelium (A). Blockade of endothelin receptors with bosentan ( $10 \mu\text{mol}\cdot\text{L}^{-1}$ ) did not change Phen-induced contraction in both groups (B). Blockade of thromboxane  $A_2$  receptors with SQ29548 ( $1 \mu\text{mol}\cdot\text{L}^{-1}$ ) (C), cyclooxygenase inhibition with ibuprofen ( $1 \mu\text{mol}\cdot\text{L}^{-1}$ ) (D) and type 2 cyclooxygenase selective inhibition with etoricoxib ( $1 \mu\text{mol}\cdot\text{L}^{-1}$ ) (E) normalized the vascular contraction elicited by Phen in chagasic vessels. Data are expressed as mean  $\pm$  S.E.M.  $n = 5$ ; \*\*\* $p < 0.001$  by two-way ANOVA.

importantly contributes to the production of ROS in vessels [38,39]. Recently, it was shown that TP receptors stimulation by  $\text{TXA}_2$  increased NOX-dependent superoxide and peroxynitrite production in aortic endothelial cells [40]. Here we showed that the aortas of infected mice have an increase in the  $p22^{\text{phox}}$  regulator of NOX1, an important NOX subunit expressed in vessels [41]. Therefore, together these results indicate that in aorta,  $\text{TXA}_2$ /TP receptor-derived ROS account for the increased contractility found in vessels of infected mice.

Besides the increase in ROS, acute Chagas' disease is also associated with systemic inflammation [42] and increase in nitric oxide (NO) formation [43]. It is a consensus in the literature that, under physiological conditions, eNOS is the major source of NO in vessels [44,45]. However, in some pathological conditions, especially inflammation, iNOS may be expressed and produces large amounts of NO [46,47]. The cytokine TNF- $\alpha$  has been shown to play a major role in driving the expression

of iNOS in inflammatory states. In line with this proposal, the level of TNF- $\alpha$  in the serum of our infected mice was extremely high compared to controls, which is consistent with the occurrence of systemic inflammation. Moreover, there was increased iNOS expression and enhanced basal NO production in infected mouse aorta. The increase in NO and superoxide production are in agreement with the nitrosative stress found in this work.

It is important to note that although NO production by infected vessels under basal conditions is considerable high in infected mice, there was a reduced NO-dependent vascular relaxation in chagasic mice. Our Western blot experiments that showed decreased eNOS expression and functioning corroborate to the above results. TNF- $\alpha$  is known to reduce eNOS expression [48]. Moreover, it is well established that ROS uncouple eNOS [21,49,50]. Of note, it was reported that activation of NOX by  $\text{TXA}_2$  TP receptor uncouples eNOS [40]. Finally,

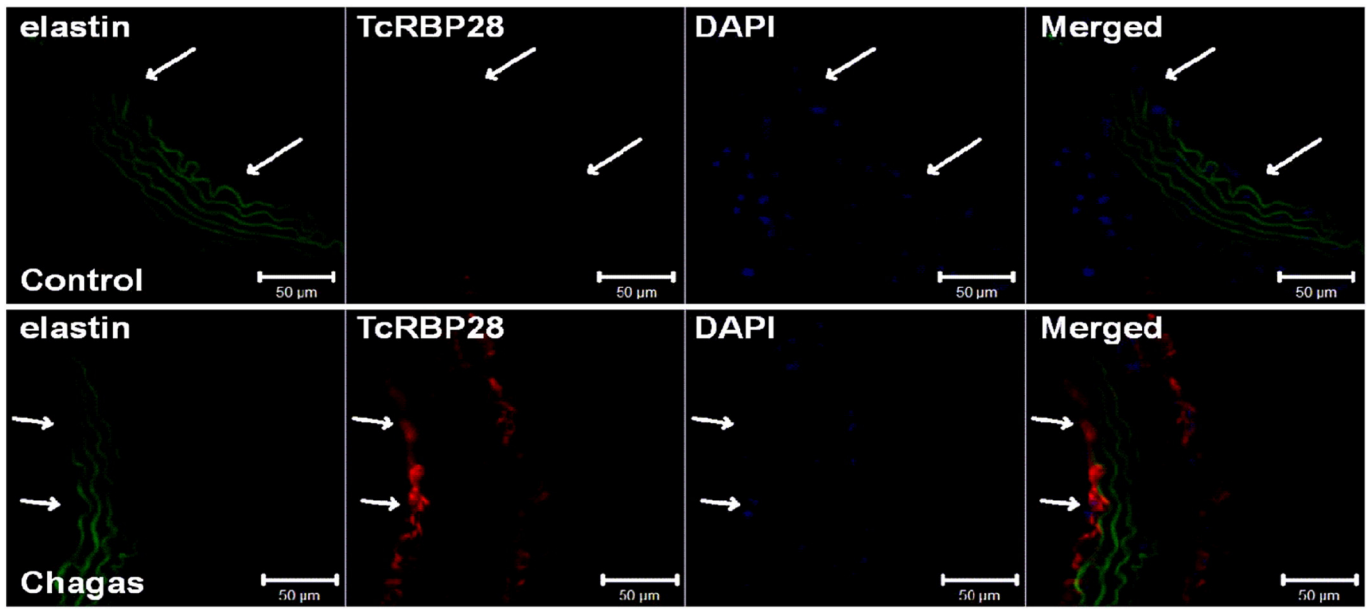


Fig. 4. Representative immunostaining against the parasite antigen TcRBP28 ( $n = 4$ ). Green: elastin; red: *T. cruzi* antigen TcRBP28; blue: nuclei staining with DAPI. Arrows indicate endothelial layer. Bar scale = 50  $\mu\text{m}$ .

up-regulation in NO/iNOS production can also contribute to uncouple eNOS via activation of arginase-1 consequently lowering L-arginine concentration [51]. Hence, it is likely that increased serum TNF- $\alpha$  production

in association with TXA<sub>2</sub> and iNOS-mediated oxidative and nitrosative stress found in the present study might account for eNOS-related decreased vascular relaxation in *T. cruzi*-infected mice.

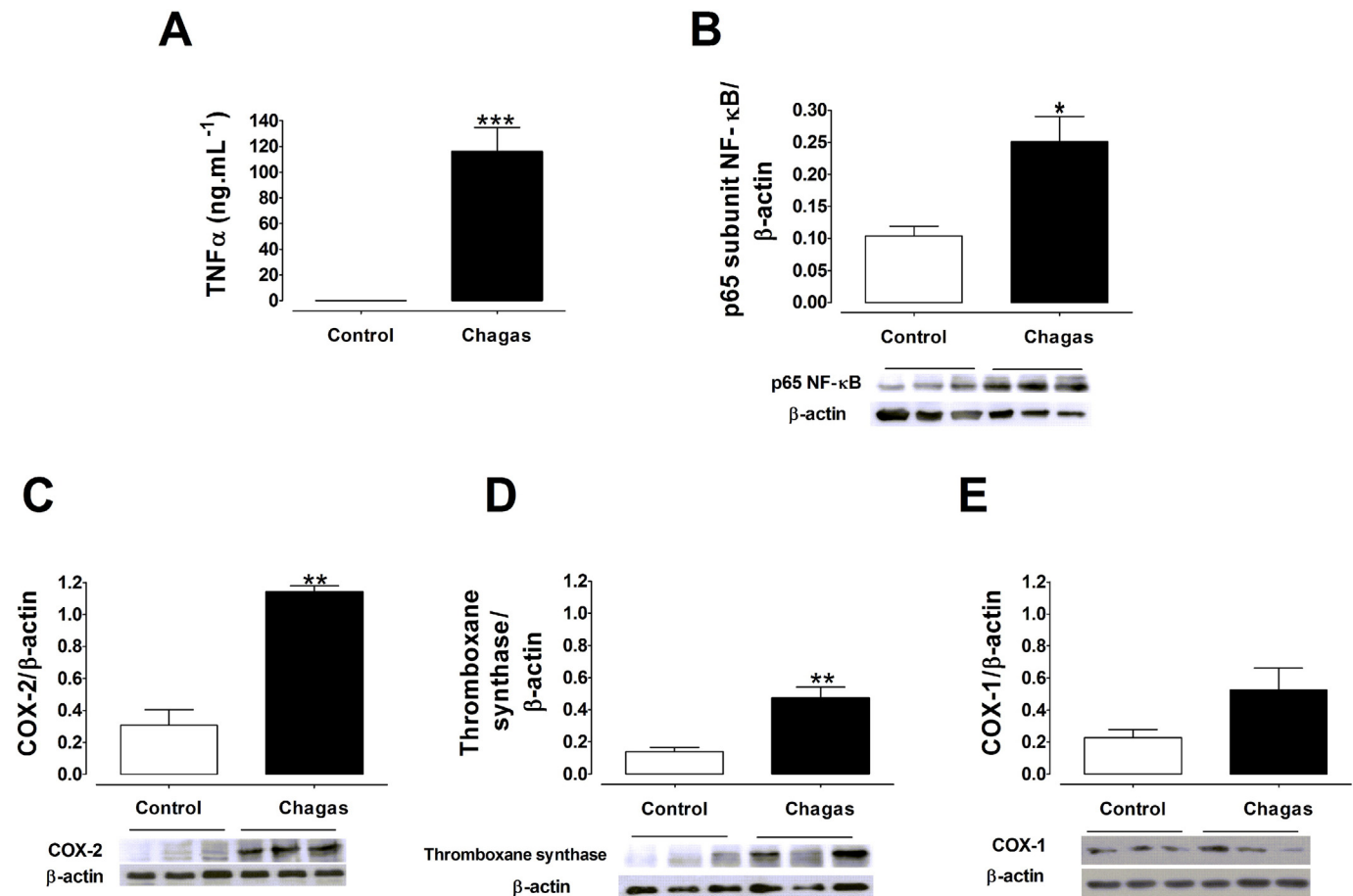
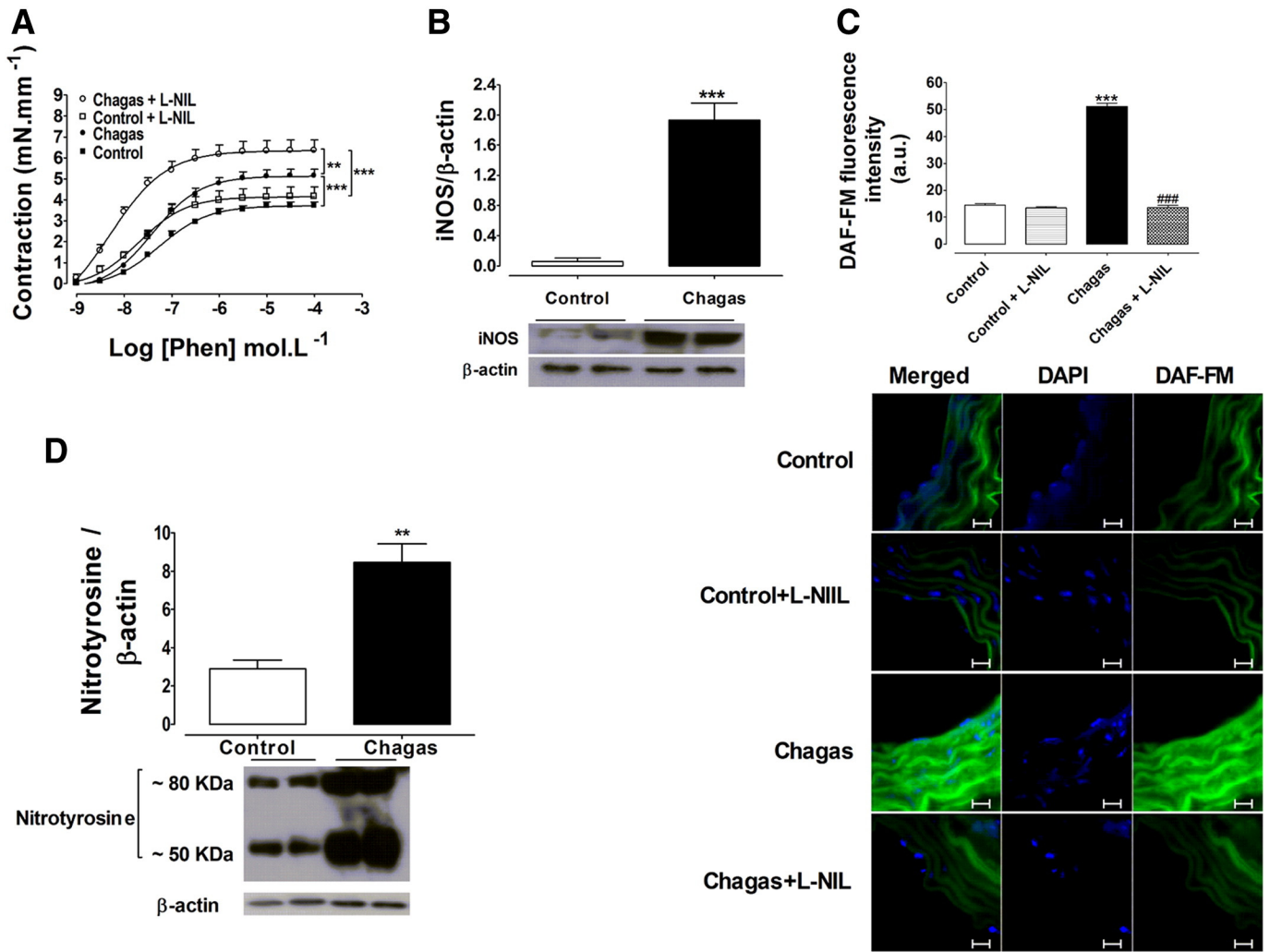
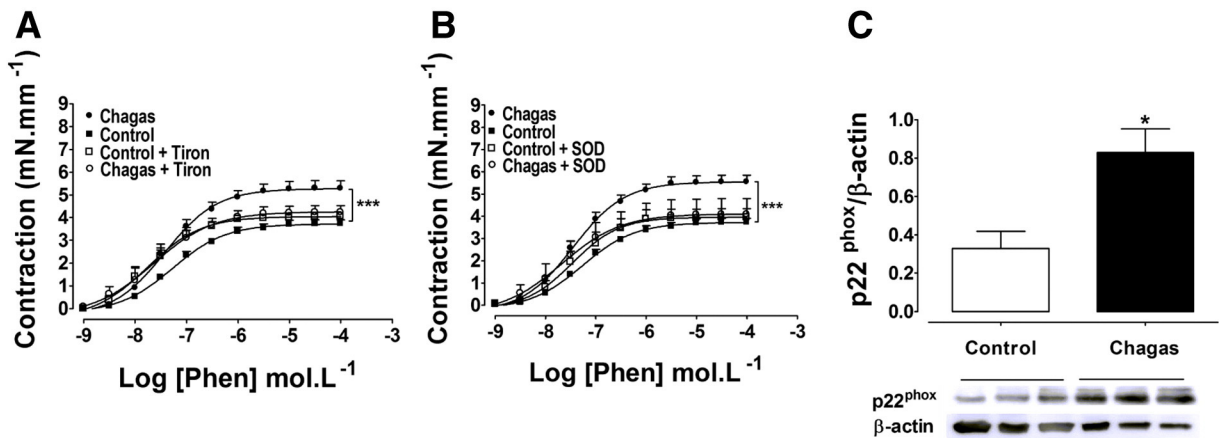


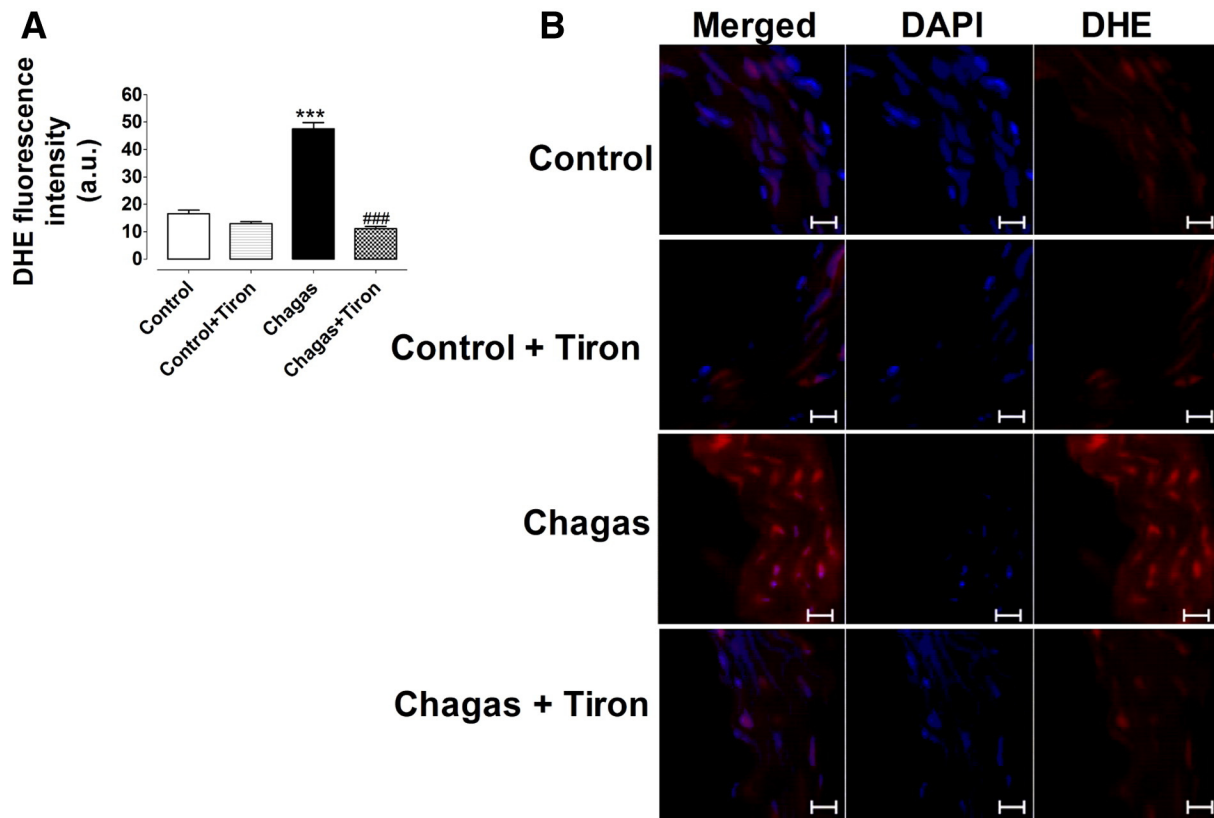
Fig. 5. TNF- $\alpha$  concentration is increased in the serum of infected mice (A). Western blot analysis of total p65 subunit of NF- $\kappa$ B (B), cyclooxygenase 2 (COX-2; C), thromboxane synthase (D) and cyclooxygenase 1 (COX-1; E) in aortas from control and infected mice. Images are representative blots from four separate experiments. Bar graphs represent mean  $\pm$  S.E.M. \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  by Student's *t*-test.



**Fig. 6.** Contractile effect of phenylephrine in vessels from the control and infected mice in the absence and the presence of L-NIL ( $10 \mu\text{mol}\cdot\text{L}^{-1}$ ) (A). Western blot analysis of total iNOS in aortas from control and infected mice (B). Fluorescence detection of NO in aortic rings sections from control and infected mice in the presence and in the absence of L-NIL ( $10 \mu\text{mol}\cdot\text{L}^{-1}$ ) (C). Western blot analysis of nitrotyrosine residues in vessels from the control and infected mice (D). Data from contraction curve are expressed as mean  $\pm$  S.E.M.  $n = 5$ ;  $**p < 0.01$  and  $***p < 0.001$  by two-way ANOVA. Western blot images are representative blots from four separate experiments. Bar graphs represent mean  $\pm$  S.E.M.  $**p < 0.01$  and  $***p < 0.001$  by Student's  $t$  test. Fluorescence images are representative of five animals for each group. Bar graph shows the mean  $\pm$  S.E.M of the fluorescence intensity. Green: DAF; blue: nuclei staining with DAPI.  $***p < 0.001$  chagasic vs control;  $###p < 0.001$  chagasic vs chagasic + L-NIL by one-way ANOVA with Newman-Keuls post hoc test.



**Fig. 7.** The superoxide scavenger Tiron ( $10 \mu\text{mol}\cdot\text{L}^{-1}$ ) (A) and superoxide dismutase (SOD;  $300 \text{IU}\cdot\text{mL}^{-1}$ ) (B) normalized phenylephrine-induced contraction from chagasic mice aortas. Data were expressed as mean  $\pm$  S.E.M.  $n = 5$ ;  $***p < 0.001$  by two-way ANOVA. Western blot analysis of p22<sup>phox</sup> in the aortas from control and infected mice. Bar graphs represent mean  $\pm$  S.E.M.  $*p < 0.05$  by Student's  $t$ -test.



**Fig. 8.** Fluorescence detection of superoxide anions in aortic rings sections from control and infected mice in the presence and the absence of tiron ( $10 \mu\text{mol} \cdot \text{L}^{-1}$ ). Bars graph shows the mean  $\pm$  S.E.M of the fluorescence intensity (A). Fluorescence images are representative of five animals for each group (B). Red: DHE; blue: nuclei staining with DAPI. <sup>\*\*\*</sup> $p < 0.001$  chagasic vs control; <sup>###</sup> $p < 0.001$  chagasic vs chagasic + tiron by one-way ANOVA with Newman–Keuls post hoc test.

Clearly our data show a dysfunction in contractility and relaxation of the mouse aorta. Vascular dysfunction is a common problem associated with hypertension [52]. However, the relationship between Chagas' disease and high blood pressure remains controversial. Although several reports mentioned that the frequency of hypertension in patients with Chagas' disease is similar to that described in general populations [53, 54], a study performed by Vicco et al. (2014), showed that the incidence of high blood pressure was higher in the patients with Chagas' disease compared with the control group [55].

The increase in NO bioavailability, ROS production and generation of proinflammatory cytokines represent a common host defense mechanism against the proliferation of several pathogens. Therefore, this defense mechanism is useful to the immune response in several infectious diseases such as Chagas [43], dengue [56], leishmaniasis [57] and malaria [58]. In addition, it has been reported that, *Leishmania donovani*, the etiological agent of visceral leishmaniasis disease, also invades cultured endothelial cells. However, contrary to the mechanism described in this work, *L. donovani* increases the synthesis of ceramide, which leads to a decrease in endothelial-derived NO production and activation of NADPH oxidase [57]. Because the literature is scarce, further studies are necessary to clarify the mechanisms involved in vascular dysfunction in infectious diseases. However, so far, it is likely that although some aspects of the host defense in several infectious disorders are similar, the mechanism proposed in this work involving COX-2/TXA<sub>2</sub>/receptor TP in the mouse aorta, seems to be particular for Chagas' disease.

In conclusion, in this paper we present consistent evidence showing that acute *T. cruzi* infection induces systemic and vascular inflammations that change vascular function. The parasite enters the vascular endothelial cell, causing an increase in COX-2/TXA<sub>2</sub>/TP receptor/superoxide local signaling. Systemically the parasite provokes an increase in the levels of TNF- $\alpha$ , which induces iNOS expression in the vessels of infected mice.

iNOS produces large amounts of NO that along with TXA<sub>2</sub>/TP receptor-mediated superoxide formation, provoke oxidative stress, eNOS uncoupling and consequent decrease in aorta relaxation and increase in contractility. The rise in serum TNF- $\alpha$  might also exacerbate the endothelial increase in COX-2/TXA<sub>2</sub>/TP/superoxide signaling. The comprehension of the mechanism involved in the aorta dysfunction is crucial in light of the augmentation of cardiac afterload, which could participate in the onset of heart failure and aggravate its consequences, and contribute to increase morbidity and mortality during the progression of Chagas' disease.

#### Conflicts of interest

The authors declare no conflicts of interest.

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