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ORIGINAL PAPER

Protection of vascular endothelium by aspirin in a murine model of chronic Chagas' disease

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Abstract Chronic Chagas' disease affects 10–30 % of patients infected with *Trypanosoma cruzi*, and it mainly manifests as cardiomyopathy. Important pathophysiological mechanisms involved in the cardiac lesions include activation of the endothelium and induced microvascular alterations. These processes involve the production of endothelial adhesion molecules and thromboxane A_2, which are involved in inflammatory cell recruitment and platelet aggregation, respectively. Cyclooxygenase inhibitors such as aspirin decrease thromboxane production and alter the course of Chagas' disease, both in the acute and chronic phases. We studied the effects of the administration of low and high doses of aspirin during the early phase of *T. cruzi* infection, following microvascular damage in the context of a chronic murine model of Chagas' disease. The effects of both schedules were assessed at 24 and 90 days postinfection by evaluating parasitemia, mortality, and cardiac histopathological changes as well as the expression of ICAM, VCAM, and E-selectin in cardiac tissue. Thromboxane A_2, soluble ICAM, and E-selectin blood levels were also measured. While aspirin did not affect parasitemia or mortality in the infected mice, it decreased both cardiac inflammatory infiltrates and thromboxane levels. Additionally, at 90 days postinfection, aspirin normalized sICAM and sE-selectin levels. Considering the improved endothelial function induced by aspirin, we propose the possibility of including this drug in clinical therapy to treat chronic Chagas' disease.

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Introduction

Ten to 30 % of patients infected with *Trypanosoma cruzi* develop chronic chagasic cardiomyopathy. At this end-stage of the chronic phase of the disease, circulating parasites are absent, and clinical manifestations of biventricular cardiac failure are evident (Rassi et al. 2010). Diagnosis relies on clinical evaluation, electrocardiogram findings, and serological tests (Ribeiro et al. 2012). Nevertheless, heart failure, thromboembolism, complex arrhythmias, and sudden death are typical outcomes of this cardiomyopathy (Ribeiro et al. 2012). One of the pathophysiological mechanisms involved in chronic cardiomyopathy is the activation or dysfunction of the endothelium, which could lead to focal ischemic events related to microvascular abnormalities (Constans and Conri 2006; Keller et al. 2003; Marin-Neto et al.

2007). Indeed, there is agreement that a vasoconstrictive, procoagulant, platelet-activating, and antifibrinolytic status is evident in endothelial cells from murine chagasic hearts (Herrera et al. 2011; Pinazo et al. 2011; Rossi et al. 2010). Microvascular alterations in the small intracardiac and epicardiac vessels are evident, as platelet aggregates forming occlusive thrombi can be observed. In addition, the production of vasoconstrictor mediators such as platelet activating factor by macrophages causes transitory ischemia and myocardial necrosis (Prado et al. 2011; Rossi et al. 2010; Scharfstein and Andrade 2011). Thus, inflamed endothelium and platelet activation are chronic Chagas' disease-related phenomena (Danese et al. 2007; Factor et al. 1985; Ramos and Rossi 1999; Tanowitz et al. 1990). Furthermore, platelet aggregation is augmented in endothelial cells infected with *T. cruzi*, which might stimulate thrombotic processes (Rossi et al. 2010).

Upon activation due to an inflammatory stimulus, the vascular endothelium increases the expression of several adhesion molecules on its surface, including intercellular adhesion molecule-1 (ICAM-1 or CD54), vascular cell adhesion molecule-1 (VCAM-1 or CD106), and E-selectin (Burger and Touyz 2012; Kobayashi et al. 2007). These molecules are involved in the recruitment of monocytes and other inflammatory cells, which are found in the vessels of hypertensive, atherosclerotic, and diabetic individuals. Furthermore, these adhesion molecules are increased in *T. cruzi*-infected myocardium (Soares et al. 2010), and it is proposed that they participate in the pathogenesis of chagasic myocarditis by facilitating lymphocyte adhesion to the activated endothelium of cardiac blood vessels (Lannes-Vieira et al. 2009b). In addition, it has been postulated that these adhesion molecules may participate in the establishment of chagasic infection through interaction with blood *T. cruzi* trypomastigotes (Andrade et al. 2012).

Conversely, thromboxane A_2 (TXA_2) participates in the pathogenesis of endothelial dysfunction in Chagas' disease (Nagajyothi et al. 2012). In chronic cardiomyopathy, the observed microvascular damage is aggravated by platelet aggregation, which is triggered by TXA_2. Additionally, *T. cruzi* itself produces TXA_2, and thus endothelial activation involves the participation of thromboxane produced by both the parasite and the host (Ashton et al. 2007; Rossi et al. 2010). Moreover, activation of the thromboxane receptor increases the expression of adhesion molecules (Tanowitz et al. 2011). In light of these data, it is reasonable to postulate that acetylsalicylic acid (ASA, aspirin) and other cyclooxygenase (COX) inhibitors could help to prevent the microvascular damage in chronic Chagas' by decreasing TXA_2 levels. In addition, salicylates modulate the expression of adhesion molecules by affecting the NF-kB pathway (Pierce et al. 1996). Therefore, ASA might protect the endothelium in the context of *T. cruzi* infection. Our results suggest that ASA improves cardiac histopathology and adhesion molecule production. Consequently, ASA could potentially be included in clinical protocols using conventional antichagasic drugs to ameliorate the microvascular damage observed in chronic Chagas' cardiomyopathy.

Methods

Animals

Adult male BALB/c mice (20–25 g) were obtained from the animal facility at the Faculty of Medicine, University of Chile. All animal handling protocols were approved by the institutional ethical committee at the Faculty of Medicine, University of Chile, according to the "guide for the care and use of laboratory animals" from the National Institutes of Health, USA (National Research Council (US). Committee for the update of the guide for the care and use of laboratory animals, Institute for Laboratory Animal Research (US), National Academies Press (US) (2011)).

Parasites and infection model

Mice were intraperitoneally inoculated with 500 blood trypomastigotes of the Dm28c *T. cruzi* strain. After randomization, animals were distributed in groups of eight individuals, each of which received a different treatment. After 5 days postinfection (dpi), direct microscopic visualization of circulating trypomastigotes in peripheral blood was used to confirm *T. cruzi* infection (Bustamante et al. 2007; Huang et al. 2002). At 24 and 90 dpi, animals were euthanized with 150 mg/kg ketamine (Drag Pharma Invetec, Santiago, Chile) and 30 mg/kg of xylazine (Laboratorios Alfasan, Buenos Aires, Argentina) to confirm acute and chronic cardiac alterations by histopathological analysis (Bustamante et al. 2007; Huang et al. 2002).

Treatments

Mice were treated with ASA for 20 days starting at 2 dpi. Drugs were suspended in aqueous 1 % methylcellulose and administered orally by gavage. The ASA doses, which were intended to inhibit thromboxane and prostaglandin synthesis, were 2 and 40 mg/kg/day (Bulckaen et al. 2008; Cyrus et al. 2002; Hideko Tatakihara et al. 2008).

Cardiac tissue preparation

Heart samples from euthanized mice were fixed in 10 % formaldehyde in 0.1 M phosphate buffer (pH 7.3) for 24 h and were then dehydrated in alcohol, clarified in xylene, embedded in paraffin, and sectioned at 5 μm. Paraffin

histological sections were stained with hematoxylin–eosin for routine histological analysis and to microscopically evaluate the presence of *T. cruzi* amastigote nests and inflammation in the myocardium (Duaso et al. 2010).

Expression of endothelial adhesion molecules

Standard immunoperoxidase techniques were used to assay for the expression of endothelial adhesion molecules. For this purpose, ICAM antibodies (rat-anti-mouse 1:1,000 dilution v/v, Santa Cruz Biotechnology) were used. Primary antibodies were applied to each section overnight at 4 °C. Biotinylated anti-rat IgG diluted 1:50 v/v (Vector Laboratories) was used as a secondary antibody. Immunostaining was performed by applying a horseradish peroxidase-labeled streptavidin biotin kit (RTU-Vectastain kit) following the manufacturer's directions and using diaminobenzidine as the chromogen. Sections were counterstained with Mayer's hematoxylin (DAKO) and mounted with Entellan (Merck). Immunohistochemical controls were performed by replacing the primary antibodies with phosphate buffered saline. All controls were negative. Sections were examined by light microscopy (Leitz Orthoplan), and images were captured with a Canon 1256 camera.

Plasma levels of endothelial dysfunction markers

Plasma levels of soluble ICAM-1 (sICAM-1), soluble E-selectin (sE-selectin), and thromboxane B_2 (the stable hydrolytic metabolite of TXA_2) were determined by ELISA kits according to manufacturer's protocols (R&D Systems, Cayman Chemical). The samples were obtained at 24 and 90 dpi

Statistical analysis

Results represent mean ± SD from at least three independent experiments. One- and two-way ANOVA analysis with Tukey's post hoc test were performed when required. For survival analysis, the log-rank test was performed.

Results

Parasitemia and survival rates were determined in *T. cruzi*-infected BALB/c mice to assess the effect of the ASA treatments (Figs. 1 and 2). Contrary to previous reports (Mukherjee et al. 2011), the ASA doses used had no effect on parasitemia levels or survival rates when compared with infected controls, with the exception of a decrease in the second peak of parasitemia (see Fig. 1a). In this report, a chronic model of infection was used; consequently, the mortality rate was low due to the amount of parasite inoculum employed (Garcia et al. 2005). In contrast, in acute models where the parasite inocula were higher than that used here, 100 % mortality was achieved at 30 dpi (Faundez et al. 2008). However, parasitemia behaves similarly in both the acute and chronic models. The double peak observed in the parasitemia curve (Fig. 1) appear to be characteristic for BALB/c mice (Bryan et al. 2010).

At 24 dpi, the heart samples obtained from the infected mice showed intense inflammatory infiltration, several amastigote nests, edema, and cardiac disorganization (Fig. 3b). Compared to the infected control at 24 dpi, treatment with 2 mg/kg ASA decreased the amount of inflammatory infiltrate (Fig. 3c), whereas 40 mg/kg ASA decreased cardiac fiber disarrangement, while the inflammatory infiltrates appeared more focalized (Fig. 3d). However, ASA treatment had no effect on the number of amastigote nests (not shown). At the chronic phase of infection (90 dpi), cardiac muscle fiber disarrangement was still present, and the inflammatory infiltrates were focalized in some areas, indicating chronic cardiomyopathy (Fig. 3f). It should be noted that these lesions were scarce when compared with the generalized damage seen in samples from infected

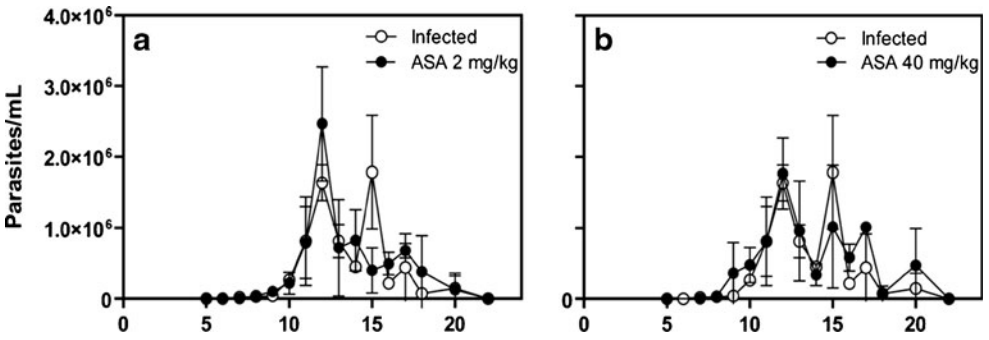

Fig. 1 Effect of aspirin (*ASA*) treatment on parasitemia levels in *T. cruzi*-infected mice. Male BALB/c mice were infected intraperitoneally with 500 Dm28c *T. cruzi* trypomastigotes. Parasitemia was assessed as described in the methods starting at 5 days postinfection in mice treated with 1 % methylcellulose vehicle (*open circles*) or with ASA (*closed circles*) with doses of **a** 2 mg/kg/day or **b** 40 mg/kg/day, until parasites were no longer detected. All measurements were made in triplicate (n= 8 mice per group)

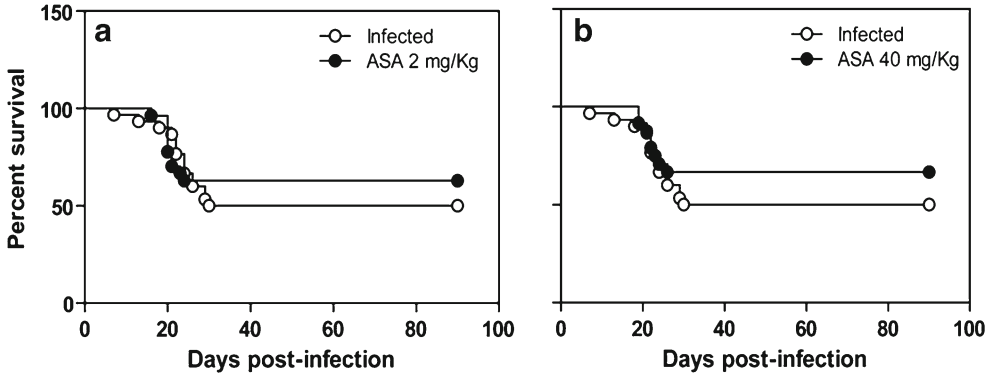

Fig. 2 Effect of aspirin (*ASA*) treatment on *T. cruzi*-infected mouse survival. Mice were infected, and survival was assessed in vehicle-treated mice (*open circles*) or (*closed circles*) mice treated with ASA at 2 mg/kg/day (**a**) or 40 mg/kg/day (**b**) until 90 days postinfection. The graphs summarize four independent experiments ($n=6$ mice per group). *$P<0.05$ when compared with the infected group with the log-rank test

controls at 24 dpi (Fig. 3b), which is consistent with persistent low-level inflammation. Importantly, when ASA-treated hearts were compared with infected hearts at 90 dpi (Fig. 3g, h), a significant improvement in cardiac histology was observed. Although ASA is not a trypanocidal agent, it decreased signs of chronic inflammation in mouse hearts. Thus, ASA is able to improve cardiac histology, and treatment with ASA, beginning as early as 2 dpi, can decrease chronic cardiac damage.

To study the effects of ASA on endothelial function, the expression of ICAM-1 was assayed in cardiac tissue from infected control and treated mice. Additionally, several endothelial dysfunction markers, such as thromboxane A_2 and the soluble forms of ICAM and E-selectin, were measured in plasma from infected, treated, and untreated mice.

Cardiac tissue from *T. cruzi*-infected mice showed elevated ICAM-1 expression in the acute (Fig. 4b) and chronic (Fig. 4f) phases of infection compared with healthy controls (Fig. 4a, e). At the two experimental doses assayed, ASA decreased tissue expression of ICAM-1 at 24 dpi (Fig. 4c, d). Similarly, there was a clear decrease in immunoreactivity in hearts from mice that were chronically infected, although ICAM-1 expression was not abolished entirely (Fig. 4g, h). Thus, both ASA doses are effective in the long-term prevention of the expression of this adhesion molecule.

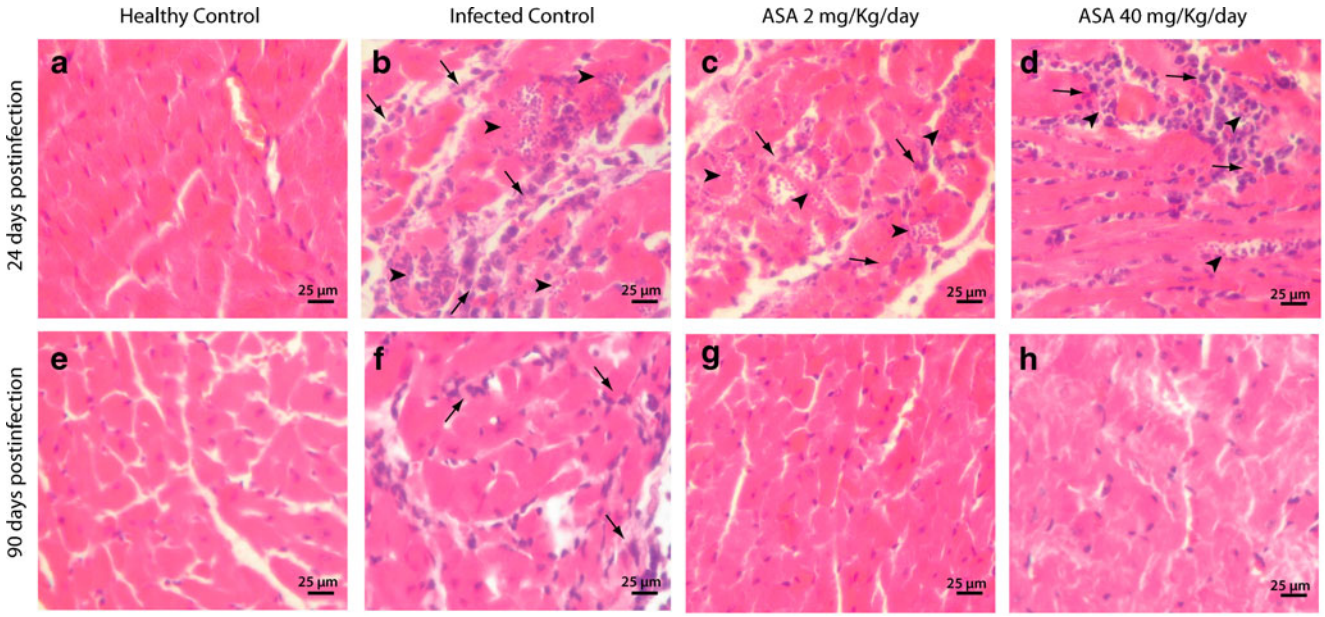

Fig. 3 Effect of aspirin (*ASA*) on cardiac histology in the acute and chronic phases of infection. Histopathology of infected BALB/c mice with or without aspirin treatment at 24 dpi (*upper panel*) and 90 dpi (*lower panel*). Sections were stained with hematoxylin and eosin as described in the methods section. **a**, **e** Healthy controls, **b**, **f** infected controls, **c**, **g** ASA 2 mg/kg/day, **d**, **h** 40 mg/kg/day. *Arrows* indicate the presence of inflammatory infiltrate. *Arrowheads* indicate amastigote nests. Images are representative of at least five mice in each group

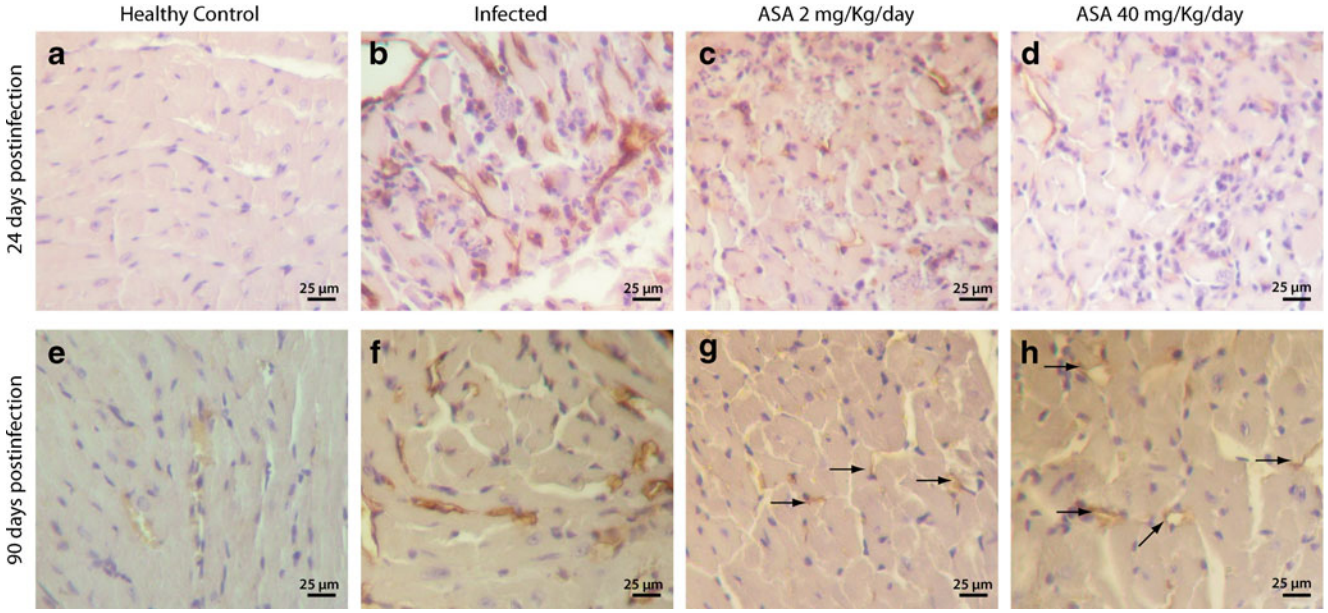

Fig. 4 Effect of aspirin (*ASA*) on ICAM-1 cardiac expression in the acute and chronic phases of infection. Immunohistochemistry of hearts from infected BALB/c mice with or without ASA treatment at 24 and 90 days postinfection. Sections were stained with DAB-immunoperoxidase and hematoxylin as described in the methods section. **a**, **e** Healthy control, **b**, **f** infected control, **c**, **g** ASA 2 mg/kg, **d**, **h** ASA 40 mg/kg. *Arrows* show scarce immunoreactivity. Images are representative of at least five mice in each group

In *T. cruzi*-infected BALB/c mice, TXA_2 levels were significantly elevated at 24 dpi (Fig. 5a); this increase was even higher at 90 dpi (Fig. 5b). These findings are in agreement with previous reports (Ashton et al. 2007). During the acute (Fig. 5a) and chronic (Fig. 5b) phases, both doses of ASA decreased TXA_2 levels in control, noninfected mice ($p>0.005$, Tukey's post hoc test following ANOVA analysis).

Importantly, sICAM-1 plasma levels increased vertically during the initial phase of the disease. ASA, at the two doses assayed, did not modify this burst of sICAM expression (Fig. 5c). On the contrary, sICAM plasma levels showed a very modest increase during the chronic phase of the disease that returned to control levels after each treatment (Fig. 5d). Finally, sE-selectin levels increased in infected mice, both at the acute (Fig. 5e) and the chronic (Fig. 5f) phases of the disease. Aspirin at 2 mg/kg was able to return sE-selectin to control plasma levels in the experimental acute phase, while ASA at 40 mg/kg decreased sE-selectin nearly to control levels (Fig. 5e). Similar results were observed when 2 mg/kg ASA was administered to chronically infected mice; however, 40 mg/kg ASA decreased sE-selectin levels but not to the levels of the healthy controls (Fig. 5f). These results are most likely related to the fact that sE-selectin is an inflammatory and endothelial dysfunction marker (Bjerre et al. 2010; Shechter et al. 2009). The mechanism by which a low dose of ASA is effective in normalizing sE-selectin levels remains to be elucidated.

Discussion

Chronic Chagas' disease is one of the most devastating causes of cardiac failure (Barbosa et al. 2011; Vilas Boas et al. 2012). Although extensive public health measures to control vectors and to prevent blood transmission have decreased the incidence and prevalence of this disease, current therapies have not reached levels of high efficacy. In addition, human migration has facilitated the expansion of this disease to nonendemic countries, increasing risk in regions where systematic blood testing is not performed. Thus, understanding the pathophysiological basis of current therapy and searching for other therapeutic strategies will help to improve Chagas' disease treatment, particularly in the chronic phase of the disease.

The microvascular aspects of chronic cardiac Chagas' disease are a well-documented issue (Lannes-Vieira et al. 2009a; Rossi and Ramos 1996; Tanowitz et al. 2005; Tanowitz et al. 2009). However, its impact on the pathogenesis of chagasic cardiomyopathy is just starting to be elucidated. The roles of endothelin and platelet aggregation associated with ischemic lesions have been described (Andrade et al. 2012; Petkova et al. 2001). Moreover, immunopathological analysis has shown that endothelin-1 expression is increased in chronic Chagas' disease, which, together with an elevated production of thromboxane A2, explains at least in part the molecular events involved in chagasic vasculopathy (Ashton et al. 2007; Petkova et al. 2001; Tanowitz et al. 2005).

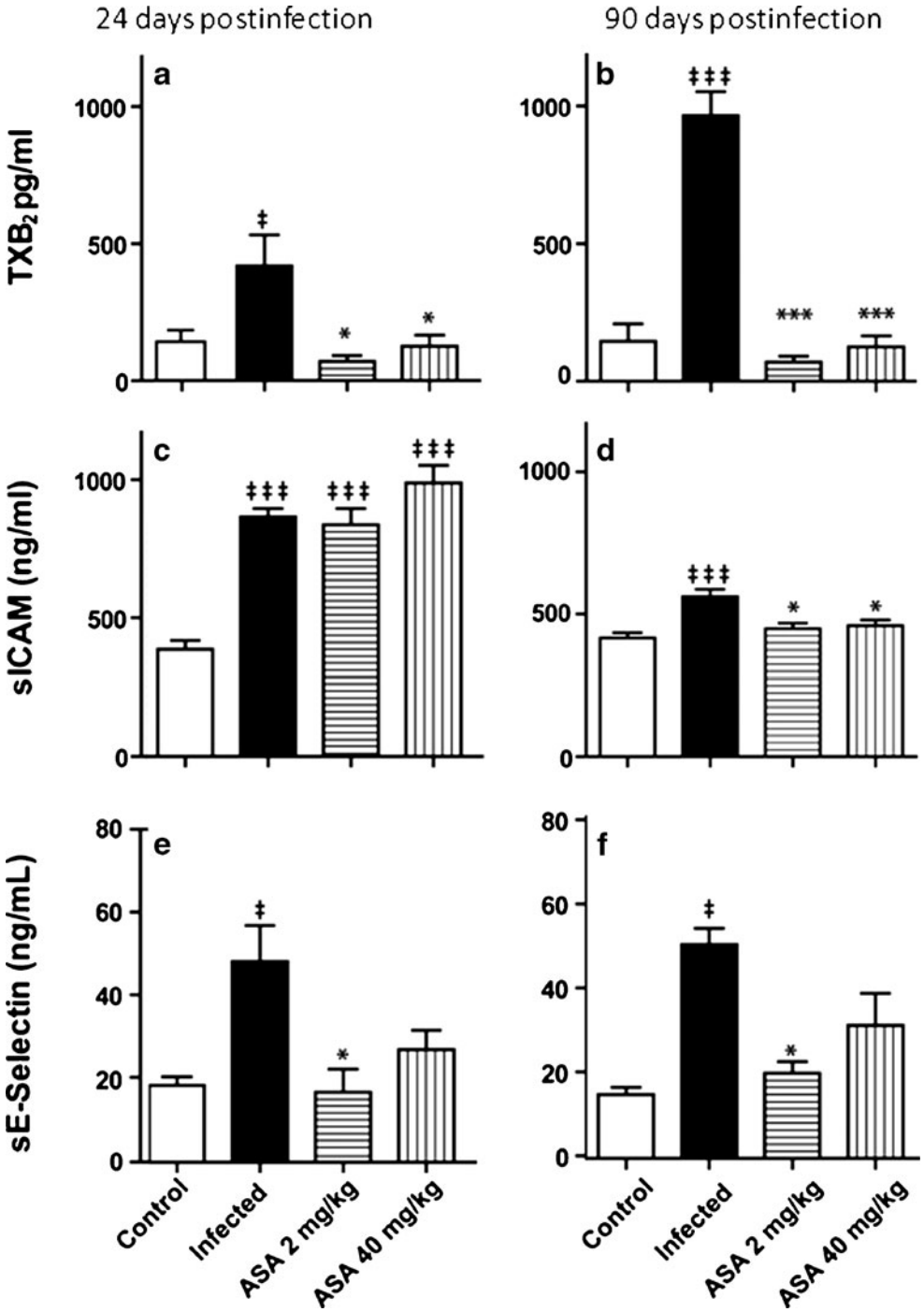

Fig. 5 Effects of benznidazole (*Bz*) or aspirin (*ASA*) on endothelial dysfunction markers. Plasma levels of thromboxane B_2 (**a**, **b**), sICAM-1 (**c**, **d**), and sE-selectin (**e**, **f**) in the acute and chronic phases of infection (24 and 90 days postinfection, respectively). *Symbols* indicate significance with respect to the infected group (*$p<0.05$, **$p<0.01$, ***$p<0.001$) or the healthy controls (‡$p<0.05$, ‡‡‡$p<0.001$) based on Tukey's posttest analysis. The *bars* represent the mean ± SD of at least three independent experiments ($n=5$)

Previous reports indicated that ASA could increase mortality in *T. cruzi*-infected mice in an acute model of the disease (Hideko Tatakihara et al. 2008; Mukherjee et al. 2011). This finding was explained by the apparent immunosuppression caused by PGE2 during acute infection (Abdalla et al. 2008), as well as by the need for TXA2 to establish infection, control parasite burden and facilitate the progression of the disease towards chronicity (Ashton et al. 2007; Michelin et al. 2005). During the acute phase, COX inhibitors, especially COX-2 inhibitors, might act to blunt evasion of the innate immune response (Hideko Tatakihara et al. 2008; Michelin et al. 2005). Thus, a fragile balance exists between the functions of TXA2 and PGE2 during the acute phase that might determine the fate of the chronic phase. However, in our chronic model of the disease, mortality and parasitemia were unaffected by ASA.

Indeed, ASA did not have any effect on cardiac involvement, although there was a decrease in heart inflammation and fiber disarrangement that was observed both at 24 and

90 dpi (Fig. 3). This is in agreement with the suggestion that, during the chronic phase, ASA can improve cardiac function (Mukherjee et al. 2011).

Endothelial activation as a consequence of thromboxane production can worsen Chagas' disease, and the parasite also produces this eicosanoid (Ashton et al. 2007). TXA2 production by *T. cruzi* is not affected by ASA, although it is apparently dependent on precursors provided by the host (Mukherjee et al. 2011). ASA modulates endothelial activation through thromboxane, but it also exerts this action via prostaglandin-independent pathways. Several studies have reported high serum levels of soluble vascular CAM-1 (sVCAM-1 or CD106), ICAM-1 or CD54, and CD44 (a fibronectin and hyaluronic acid ligand) during the acute phase of infection, whereas soluble P-selectin (sP-selectin or CD62P) was associated with the severity of the chronic disease (Lannes-Vieira et al. 2009b; Laucella et al. 1996; Laucella et al. 1999). The regulation of adhesion molecule expression, promoting endothelial activation, leukocyte migration, and other inflammatory processes (Morris et al. 2009) can be exerted through NF-κB via complex mechanisms. During endothelial activation in response to inflammatory inputs, the endothelium increases the expression of ICAM, VCAM, and E-selectin among other molecules. These molecules have in common the involvement of the IKK-beta/I-κB/NF-κB axis.

When administered during the early stages of the disease, ASA normalized thromboxane, sICAM, and sE-selectin levels, and this effect persisted even 60 days after the administration of these drugs. This effect was evident at the two doses of aspirin assayed. This finding is consistent with that reported for the same range of aspirin doses; however, at higher doses (75 and 100 mg/Kg for 7 days), the effect on mortality, parasitemia, and heart damage disappeared (Molina-Berrios et al. 2013). The effect of ASA on thromboxane production by COX is well-known. However, the antiinflammatory effects of salicylates through COX inhibition have been questioned due to their weak effects on COX (Kenneth 2003); thus, the pathways mentioned above are relevant when explaining the effects of ASA on endothelial adhesion molecules. It has been proposed that the modulatory effects of ASA could occur by blocking the IκB kinase (IKK) complex and p38 MAPK (Pierce et al. 1996), two pathways that are involved in regulating the expression of adhesion molecules such as ICAM and VCAM (Al-Mutairi et al. 2010; Huang et al. 2004; Wang and Dong 2012). Thus, in chronic chagasic cardiomyopathy, aspirin treatment could promote a protective endothelial environment. It has been proposed that at these low doses, the effect of aspirin is related to the production of a COX derivative named 15-epi-lipoxin A4, which has antiinflammatory properties (Molina-Berrios et al. 2013).

In conclusion, ASA induced a decrease in the expression of endothelial adhesion molecules in an experimental model of Chagas' disease, most likely acting through alternative pathways to COX. However, the decrease in endothelial activation might be aided by diminished thromboxane levels. As a result, ASA decreased the aggravating factors for the acute (thromboxane and sICAM) and chronic (thromboxane and sE-selectin) phases of the disease. Thus, the efficacy of conventional treatments for Chagas' disease might be increased with the incorporation of ASA into therapeutic protocols for chagasic patients because ASA contributes to the amelioration of vascular damage.

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Conflict of interest The authors declare that they have no conflict of interest.

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