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Research Paper

Modulation of oxidative and inflammatory cardiac response by nonselective 1- and 2-cyclooxygenase inhibitor and benznidazole in mice

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Keywords
cardiovascular pathology; Chagas disease; chemotherapy; oxidative stress

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Abstract

Objectives This study investigated the combined effects of benznidazole (BZ) and ibuprofen (IB) on the oxidative and inflammatory status of the cardiac tissue *in vivo*.

Methods Swiss mice were randomized in groups receiving BZ (100 mg/kg) and IB (400 mg/kg) alone or combined (BZ + IB 200 or 400 mg/kg). Control animals were concurrently treated with 1% carboxymethyl cellulose. All treatments were administered orally for 7 days.

Key findings BZ treatment increased cardiac production of nitrogen/oxygen-reactive species, malondialdeyde, carbonyl proteins, prostaglandins as well as the activities of catalase, superoxide dismutase and glutathione peroxidase. These parameters were attenuated by IB, with the best results at higher dose. Individually, BZ and IB significantly reduced the tissue levels of chemokine ligand 2, tumour necrosis factor-α and IL-10, but no reduction was observed when the treatments were combined.

Conclusions BZ triggers an oxidative and nitrosative route, which is associated with increased prostaglandin synthesis and marked damages to the lipids and proteins of the cardiac tissue. IB treatment attenuated reactive stresses triggered by BZ, which was an independent effects of this drug on the endogenous antioxidant enzymes. Individually, but not together, BZ and IB reduced the cardiac inflammatory status, indicating a beneficial and complex drug interaction.

Introduction

Since the late 1960s, two nitroheterocyclic drugs have been available for the clinical management of Chagas disease: a nitrofurane, Nifurtimox (Nfx) [3-methyl-4(nitrofurfurilideneamino)tetrahydro-4H-1,4-thiozine-1,1-dioxide] and a nitroimidazole, benznidazole (BZ) [N-benzyl-2-nitroimidazole acetamide].[1–3] Only BZ is commercially available in many endemic countries, especially in Central and Latin America. Despite its relative efficiency against parasitic infection, clinical studies have reported marked side effects of BZ associated with low specificity and systemic toxicity.[4–6]

Although the specific mode of action of BZ is not well understood,[5–7] it has been indicated that drug metabolism by nicotinamide adenine dinucleotide phosphate (NADPH) cytochrome P-450 system results in the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS),[3,6] which increases the toxicity induced by BZ against the protozoan parasite as well as the host.[1,3] Moreover, ROS and RNS trigger lipid peroxidation[8] and mobilization of the arachidonic acid, which suffers the action of the cyclooxygenase (COX) enzymatic system to produce prostaglandins (PG).[9] It has been proposed that these molecules can stimulate oxidative and nitrosative stress pathways, potentiating the toxic effects of BZ.[9,10]

Preclinical studies have suggested that chemotherapy based on the inhibition of PG biosynthesis can be

potentially relevant in the therapeutic management of Chagas' disease.[11,12] However, the parameters of cardiac toxicity induced by BZ[11,12] and the implications of chemotherapeutic interactions among BZ and COX inhibitors in the cardiac tissue are poorly understood. In the current manuscript, we reported the effect of ibuprofen (IB; a non-selective 1- and 2-COX inhibitor) in minimizing the cardiac toxicity caused by BZ-mediated oxidative and nitrosative tissue damages, with repercussions on the cardiomyocytes ultrastructure, production of proinflammatory cytokines, prooxidant mediators and PG biosynthesis *in vivo*.

Materials and Methods

Animals

Male Swiss mice (age: 12 weeks, weight: 33.5 ± 4.2 g) were obtained from the Central Animal Laboratory of the Center of Biosciences and Health of Federal University of Viçosa (Brazil) and maintained under conditions of controlled temperature at 21 ± 2°C, relative humidity of 60–70% and 12-h light/12-h dark cycle. The animals received food and water *ad libitum*. The research protocol was approved by the Ethics Committee of Animal Use of the Federal University of Viçosa, Brazil (CEUA/UFV) (protocol 77/2012).

Experimental protocol

The animals were randomized into five groups of eight animals each. G1 (Control): 1% carboxymethyl cellulose; G2 (BZ): 100 mg/kg BZ; G3 (IB2): 400 mg/kg IB; G4 (BZ/IB1): 100 mg/kg BZ + 200 mg/kg IB; and G5 (BZ/IB2): 100 mg/kg BZ + 400 mg/kg IB. All treatments were administered for 7 days, and the animals were euthanized 24 h after the last treatment by deep anaesthesia (ketamine 45 mg/kg and xylazine 5 mg/kg, i.p.), followed by cardiac puncture. BZ and IB were suspended in 1% carboxymethyl cellulose dissolved in distilled water and administered orally by gavage.

Tissue collection and histopathology

After thoracotomy, the hearts were removed and weighed. Fragments of the left ventricle from each animal were removed for morphological, biochemical, and molecular analysis. For histopathological analysis, fragments were immersed in histological fixative for 48 h (freshly prepared 10% w/v formaldehyde in 0.1 M phosphate buffer at pH 7.2) and embedded in paraffin.[13] Blocks were cut into 4-μm thick histological sections and stained with H&E for general histopathology. To avoid repeated analysis of the same histological area, the sections were evaluated in semiseries, using 1 out of every 20 sections. The slides were visualized, and the images were captured using a light microscope (Olympus BX-60, Olympus, Tokyo, Japan) equipped with a digital camera (Olympus QColor-3; Olympus, Tokyo, Japan).[13]

Cardiomyocytes ultrastructure

Left ventricle samples were immersed in a fixative solution (2.5% glutaraldehyde, 0.2% picric acid, 3% sucrose and 5 mM calcium chloride ($CaCl_2$) in 0.1 M sodium cacodylate buffer (pH 7.2)) for 24 h at 4°C for ultrastructural analysis.[14] The samples were postfixed in 1% osmium tetroxide for 2 h and embedding in LR White resin. Ultrathin sections were stained with 2% uranyl acetate and 0.2% lead citrate in 1 M sodium hydroxide and observed under the Zeiss EM 109 transmission electron microscope (Carl Zeiss, Jena, Germany). The quantitative parameters such as the number of mitochondria profiles per cytoplasmic area, mean mitochondrial area, sarcomere length and myofibril thickness were determined from the digitalized images by using an image analysis software (Image Pro-Plus 4.5; Media Cybernetics, Silver Spring, MD, USA).

Lipid and protein oxidation

For analysis of tissue malondialdehyde (MDA), an end-product of lipid peroxidation, 100 mg of frozen heart was homogenized in phosphate buffer (pH 7.0) by centrifugation at 10 000*g* for 10 min; the homogenate was reacted with thiobarbituric acid solution (trichloacetic acid 15%, thiobarbituric acid 0.375%, and 0.25 N HCl) for 15 min. The formation of thiobarbituric acid-reactive substances was monitored at 535 nm as described previously.[15] The total protein levels in the heart tissues were determined by using the Bradford method.[16] Protein carbonyl content was measured in the cardiac tissue pellets by adding 0.5 ml of 10 mM of 2,4-dinitrophenylhydrazine (DNPH).[17] The reaction involved derivatization of the carbonyl group with DNPH, leading to the formation of a stable 2,4-dinitrophenyl hydrazone product. The optical density was measured spectrophotometrically at 370 nm.[18]

Nitric oxide analysis

Nitric oxide (NO) production was indirectly quantified through nitrite content in the supernatants of the heart homogenate by the standard Griess reaction. Briefly, 50 μl of heart homogenate supernatants were incubated with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylene diamine dihydrochloride and 2.5% H_3PO_4) at room temperature for 10 min. The absorbance was measured at 550 nm in a microplate scanning spectrophotometer (Power Wave X, BioTek, Winooski, VT, USA). The nitrite concentration was calculated with reference to the sodium nitrite ($NaNO_2$) standard curve.[19]

In-situ assessment of reactive mediators

Dihydroethidium (DHE), a fluorescent dye, was used to evaluate in-situ ROS/RNS production.[20] Briefly, left ventricle samples were frozen in liquid nitrogen and cut in serial 5-μm sections. Unfixed cryosections were incubated at room temperature, in the dark, with 30 μl of DHE (10 μmol/l in 0.01% dimethyl sulfoxide) for 30 min. Sections were examined by fluorescence microscopy (Olympus BX-60, Olympus, Tokyo, Japan) and the image was captured at 400× magnification. Red fluorescence produced by DHE oxidation to hydroxyethidium was evaluated by using Image Pro-Plus 4.5 software (Media Cybernetics).

Antioxidant enzymes assay

Activity assay of catalase (CAT), glutathione s-transferase (GST), and superoxide dismutase (SOD) were performed with the supernatant obtained from the following: an aliquot of frozen cardiac muscle (100 mg) was homogenized in ice-cold phosphate buffer (pH 7.0) and centrifuged at 4000 *g* (5°C) for 10 min. CAT activity was evaluated according to the method described by Aebi[21] by measuring the kinetic of the decomposition of hydrogen peroxide (H_2O_2). GST activity was followed spectrophotometrically at 340 nm as described by Habig *et al.*[22] and calculated from the rate of NADPH oxidation. SOD activity was estimated by pyrogallol method based on

Table 1 Biometric parameters of mice treated with BZ and IB

Parameter/Groups	Control	BZ	IB2	BZ/IB1	BZ/IB2
BW (g)	25.88 ± 1.44	24.70 ± 3.86	23.00 ± 3.67	25.84 ± 1.00	24.52 ± 1.61
HW (g)	0.12 ± 0.01	0.15 ± 0.03	0.12 ± 0.02	0.18 ± 0.01	0.11 ± 0.01
HSI	0.48 ± 0.05	0.64 ± 1.18	0.55 ± 0.12	0.68 ± 0.10	0.45 ± 0.07

BW, body weight; BZ, benznidazole; HSI, heart somatic index; HW, heart weight; IB, ibuprofen. Data are represented as means and standard deviation (mean ± SD). All parameters were similar among the groups ($P > 0.05$).

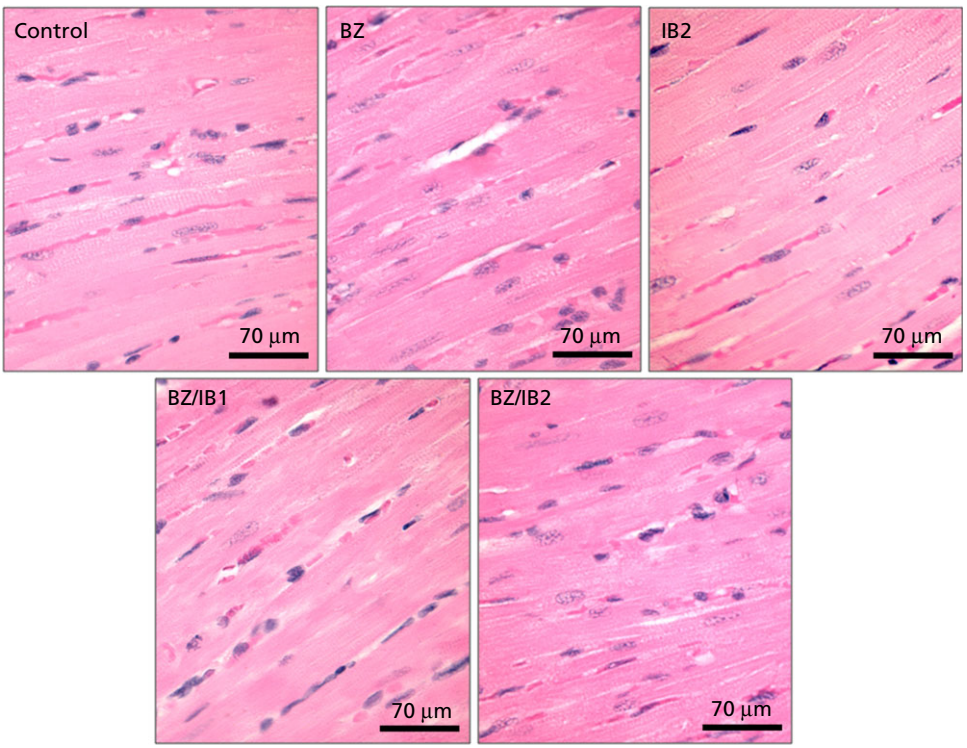

Figure 1 Representative photomicrography's of the cardiac tissue from mice treated with benznidazole and ibuprofen (H&E staining, bar = 70 μm). Similar microscopic structure can be observed in all groups. Control: 1% carboxymethyl cellulose solution, benznidazole: 100 mg/kg benznidazole, ibuprofen 2: 400 mg/kg ibuprofen, benznidazole/ibuprofen 1: 100 mg/kg benznidazole + 200 mg/kg ibuprofen, benznidazole/ibuprofen 2: 100 mg/kg benznidazole + 400 mg/kg ibuprofen.

the ability of this enzyme to catalyse the reaction of the superoxide (O^{-2}) and hydrogen peroxide.[23]

Immunoassay for prostaglandins

The amounts of individual PG were measured by enzyme immunoassay kits according to manufacturer's instructions (Cayman Chemical, Ann Arbor, MI, USA). Briefly, fresh fragments of the left ventricle (100 mg) were homogenized in 1 ml of 0.1 M phosphate buffer (pH 7.4) containing 1 mM EDTA and 10-μl indomethacin and centrifuged at 8000*g* (4°C) for 10 min. Aliquots of the supernatant (10 μl) were incubated in a 96-wells microplate with specific antibodies against $PGF_{2\alpha}$ and PGE_2. To determine tissue levels of these PGs, a microplate reader was used at 412 nm. PG levels were corrected by protein concentration in the supernatant, determined according the Bradford method.[16]

Cardiomyocyte damage and ELISA for cytokines

Cardiomyocyte damage was investigated by spectrophotometric quantification of creatine kinase izoenzyme MB (CK-MB) serum levels by using a commercial diagnostic kit (Bioclin, Belo Horizonte, Minas Gerais, Brazil).

Fragments of cardiac tissues were homogenized with a protease inhibitor (Protease Inhibitor Cocktails, Sigma-Aldrich, St. Louis, MO, USA) in portable tissue homogenizer (LabGEN YO 0427-09, Cole-Parmer, Vernon Hills, IL, USA) and centrifuged at 3000*g* for 10 min; the supernatant was collected for the cytokine assay. The concentrations of cytokines were measured by sandwich ELISA. The chemokine ligand 2 (CCL2)/Monocyte chemoattractant protein-1 (MCP-1), tumour necrosis factor-α (TNF-α) and IL-10 were measured by using commercial kits as per the

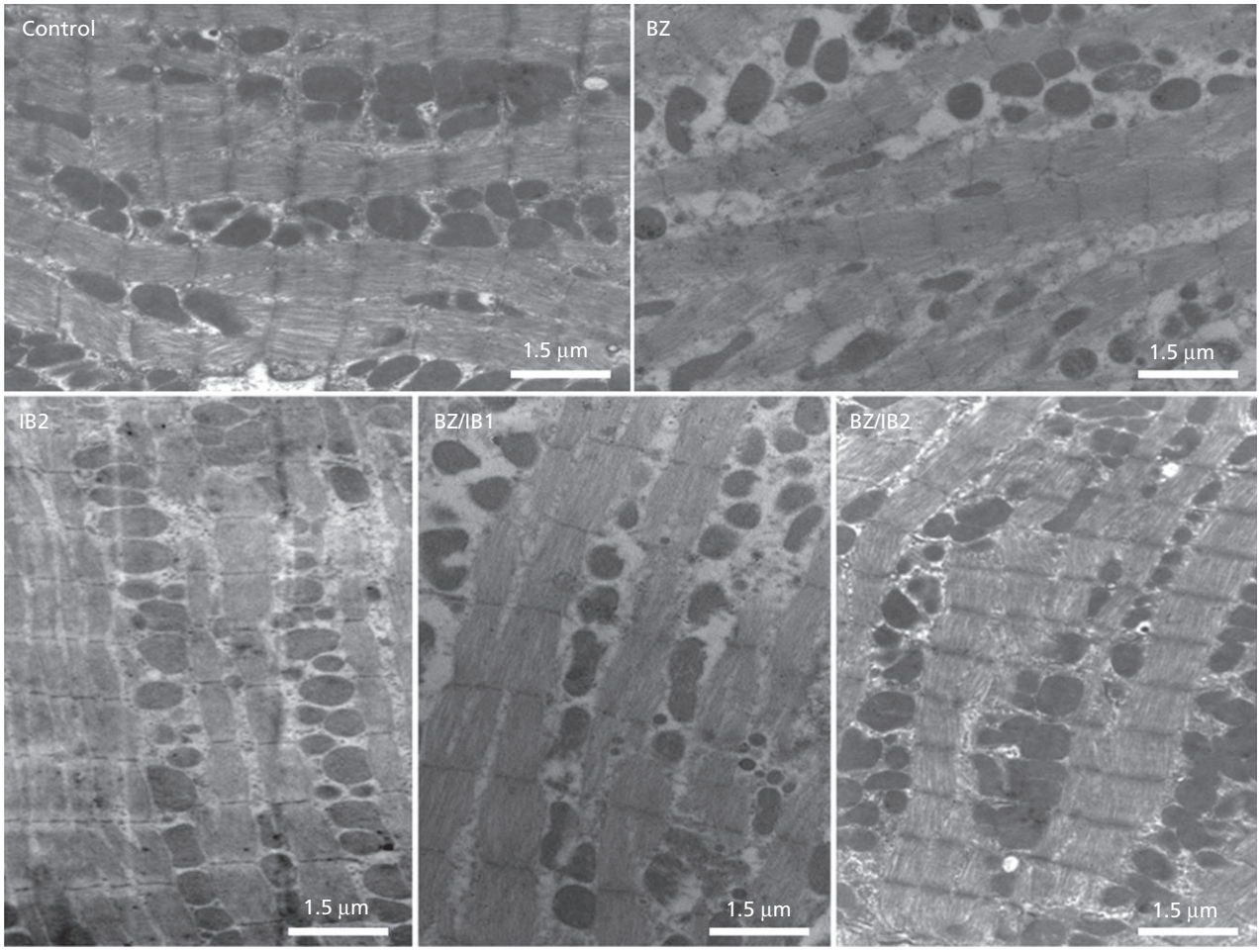

Figure 2 Transmission electron micrographs of cardiomyocytes from mice treated with benznidazole and ibuprofen (bar = 1.5 μm). In benznidazole and benznidazole/ibuprofen 1 treatment groups, mild mitochondrial and myofibril cytoplasm dispersion were noted. Control: 1% carboxymethyl cellulose solution, benznidazole: 100 mg/kg benznidazole, ibuprofen 2: 400 mg/kg ibuprofen, benznidazole/ibuprofen 1: 100 mg/kg benznidazole + 200 mg/kg ibuprofen, benznidazole/ibuprofen 2: 100 mg/kg benznidazole + 400 mg/kg ibuprofen.

manufacturer's instructions (USCN Life Science Inc., Wuhan, China).

Statistical analysis

Results are expressed as means and standard deviations (mean ± SD). The data were submitted to unifactorial one-way analysis of variance (ANOVA), followed by the Student–Newman–Keuls post-hoc test for multiple comparisons. Statistical significance was established at $P < 0.05$.

Results

The body and heart weights (absolute and relative) of the animals were similar ($P > 0.01$) among all groups (Table 1).

In all groups, the histopathology under bright field microcopy analysis indicated a well-structured microscopic organization of the myocardial tissue with parallel distribution of cardiomyocytes, typical cross-striation, defined blood vessels and low density of interstitial cells (Figure 1). Transmission electron microscopy showed cytosol expansion and myofibril separation in BZ and BZ/IB1 as compared with those in the other groups (Figure 2). Morphometric analysis indicated reduced number of mitochondrial profiles by cytoplasm area in BZ and BZ/IB1 as compared with that in the other groups. The mean area of organelles, myofibril thickness and sarcomere length were similar among all groups (Figure 3).

The animals exposed to BZ alone or in combination with lower dose of IB presented significant increase in the tissue levels of NO_2^-/NO_3^- (nitrite/nitrate), malondialdeyde and carbonyl protein than CG and IB2, with no statistical difference between them (Figure 4). In-situ analysis revealed increased amount of reactive metabolites (ROS/RNS, Figure 5a) and PGs (PGE_2 and $PGF_{2\alpha}$, Figure 5b) in cardiac tissue from BZ and BZ/IB1 animals compared with the other groups.

The activities of antioxidants enzymes in the cardiac tissue are shown in Figure 6. Animals exposed to BZ alone or in combination with IB in all doses presented significantly increased CAT activity than animals exposed to CG and IB2, results of which were similar. The GST tissue level was significant higher in animals exposed to BZ alone or in

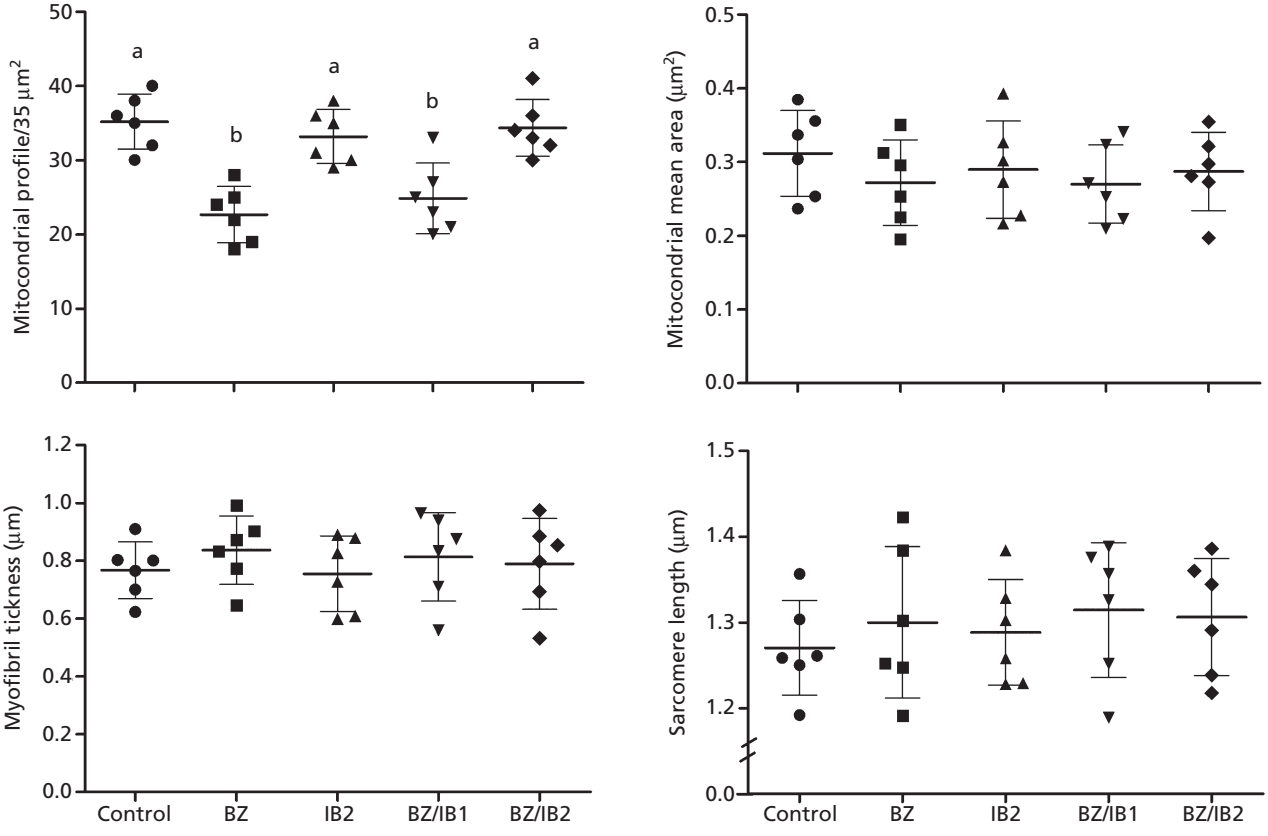

Figure 3 Quantitative ultrastructural analysis of cardiomyocytes from mice treated with benznidazole and ibuprofen. Control: 1% carboxymethyl cellulose solution, benznidazole: 100 mg/kg benznidazole, ibuprofen 2: 400 mg/kg ibuprofen, benznidazole/ibuprofen 1: 100 mg/kg benznidazole + 200 mg/kg ibuprofen, benznidazole/ibuprofen 2: 100 mg/kg benznidazole + 400 mg/kg ibuprofen. Different letters in the columns (a and b) denote statistical difference among the groups ($P < 0.05$).

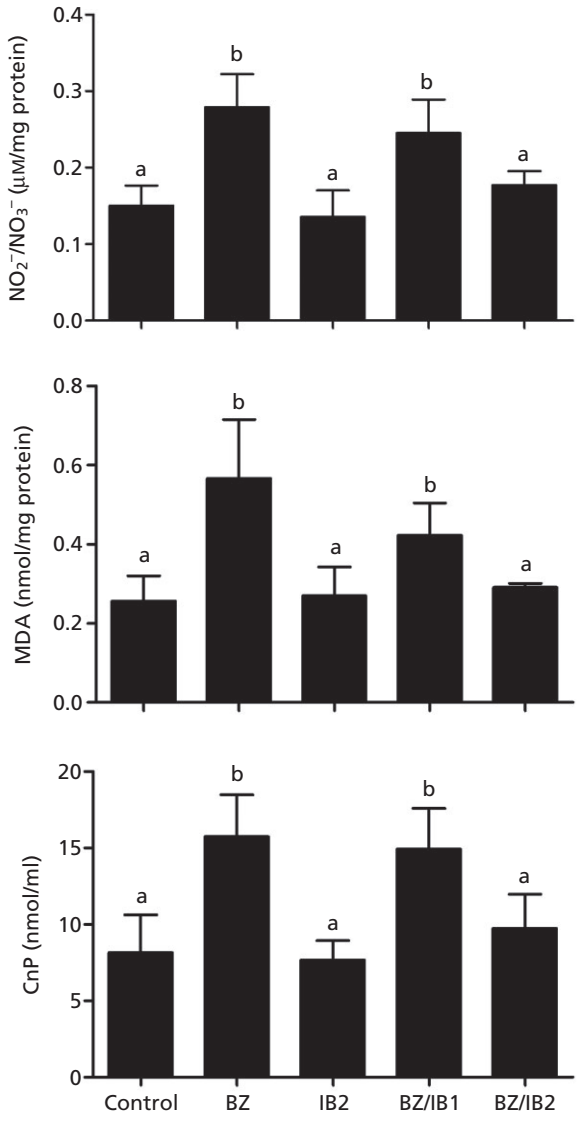

Figure 4 Markers of reactive stress in the cardiac tissue from mice treated with benznidazole and ibuprofen. Control: 1% carboxymethyl cellulose solution, benznidazole: 100 mg/kg benznidazole ibuprofen 2: 400 mg/kg ibuprofen, benznidazole/ibuprofen 1: 100 mg/kg benznidazole + 200 mg/kg ibuprofen, benznidazole/ibuprofen 2: 100 mg/kg benznidazole + 400 mg/kg ibuprofen. Data are represented as mean ± standard deviation. Different letters in the columns (a, b and c) denote statistical difference among the groups ($P < 0.05$).

combination with low dose of IB as compared with that in animals exposed to CG and IB2. This parameter presented no statistical difference in all groups exposed to higher dose of IB as compared with CG. The SOD activity was significantly increased in the animals exposed to BZ alone as compared with that in the animals exposed to CG, IB2 and BZ/IB2. Except for BZ alone, the SOD activity was similar in the other groups.

The levels of creatine kinase-MB in the serum and the myocardial content of chemokines and cytokines are shown in Figure 7. The CK-MB levels were similar in all groups. The CCL2, TNF-α and IL-10 tissue levels were significantly reduced in animals treated with BZ and IB2 alone as compared with those treated with CG and its combination groups, results of which were similar.

Discussion

Clinical studies have shown therapeutic efficacy of BZ in control acute phase of *Trypanosoma cruzi* infection, with a significant potential for parasitological cure.[24] However, organic manifestations indicative of systemic toxicity such as fever, vomiting, gastrointestinal syndromes, depression of bone marrow and polyneuropathy are evident in the first days of treatment and represent a major threat of BZ in clinical use with frequent therapy suspension.[1,25–28] Considering the early toxicity induced by BZ, the short-time experimental protocol used here was potentially applicable to investigate the acute effects of BZ exposition on cardiac tissue. The dosage of 100 mg/kg per day of BZ was adopted because it is the therapeutic dose used in the management of Chagas' disease.[7,12,29] The dosages of 200 and 400 mg/kg/day of IB was based on its pharmacokinetic and metabolic profile that favours the low toxic potential and effective anti-inflammatory activity mediated by COX-1 and COX-2 inhibition.[30,31]

In this study, only ultrastructural analysis indicated morphological changes induced by BZ alone or in association with low doses of IB. The implication of these morphological changes in cardiomyocytes cannot be underestimated, especially the distribution and organization of the contractile filaments, which are preponderant to cell contractility and cardiac function.[13] Although it has been indicated in a preclinical study that BZ can cause pathological changes in organometric and functional parameters of the heart,[32] this effect has not been consistently investigated and remains as a neglected issue. Apparently, reports of cardiotoxicity are accompanied by inconsistencies such as absence of biometric, structural, ultrastructural[32] or mitochondrial[33] deleterious effects after BZ exposition.

Despite limited microscopic evidence of cardiotoxicity, BZ induced oxidative and nitrosative tissue damage. Although the mechanisms that explain the efficacy of BZ against *T. cruzi* are not completely understood, it is believed that the production of reactive species such as O^-, OH^-, NO^- and H_2O_2 are involved in both damage to parasite and host cells.[1,3,4] This aspect was reinforced in the present study by in-situ myocardial production of reactive metabolites (ROS/RNS), indicating that cardiac tissue is a direct target of pro-oxidant events triggered by BZ. Thus, a relevant question in BZ toxicity is the direct NO production

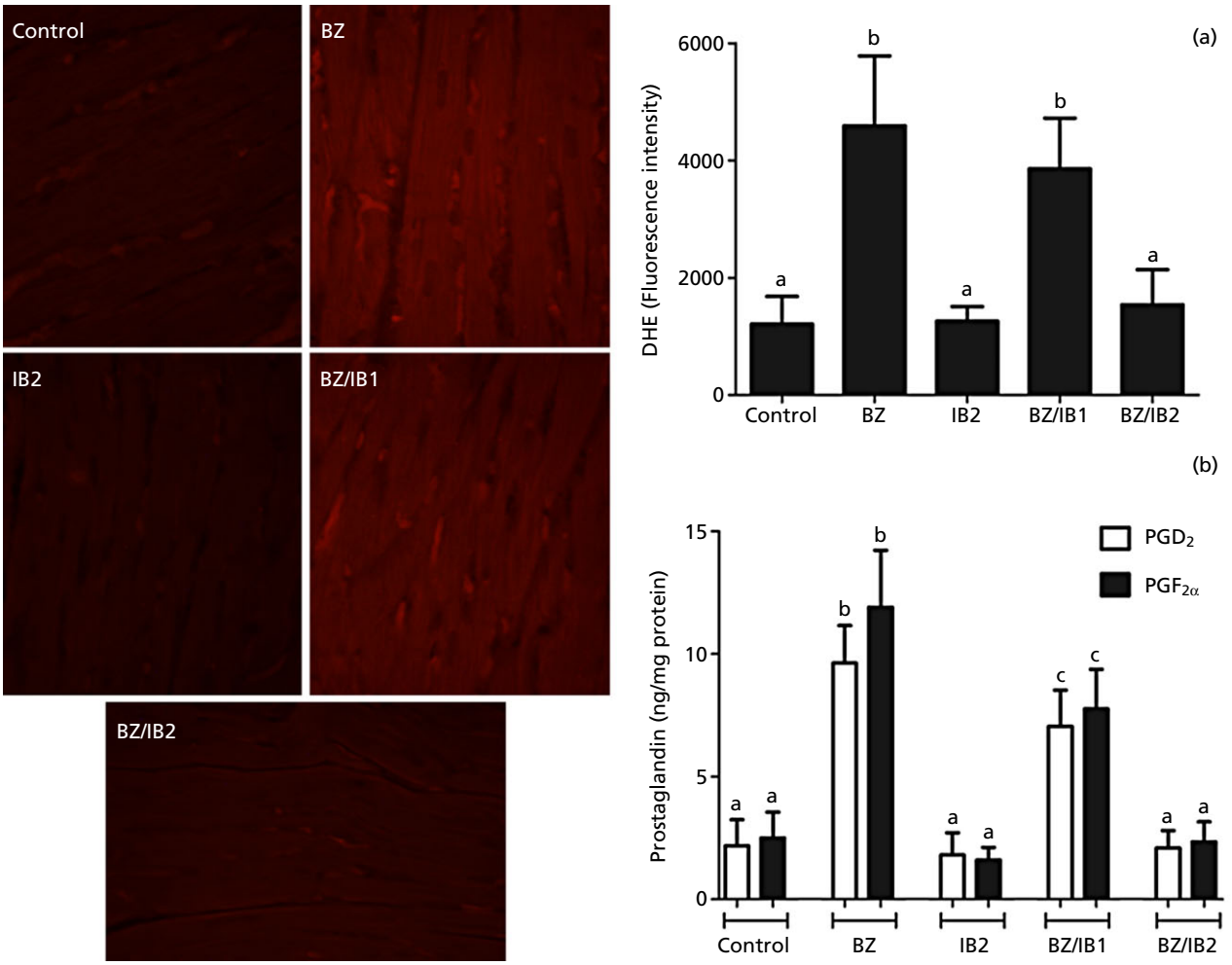

Figure 5 In-situ production of (a) reactive metabolites (oxygen/nitrogen-reactive species, photomicrography's and graphic) and (b) prostaglandin levels in the left ventricle from mice treated with benznidazole and ibuprofen. Tissue levels of reactive species were estimated by red fluorescence intensity (dihydroethidium oxidation). Control: 1% carboxymethyl cellulose solution, benznidazole: 100 mg/kg benznidazole, ibuprofen 2: 400 mg/kg ibuprofen, benznidazole/ibuprofen 1: 100 mg/kg benznidazole + 200 mg/kg ibuprofen, benznidazole/ ibuprofen 2: 100 mg/kg benznidazole + 400 mg/kg ibuprofen. Data are represented as mean ± standard deviation. Different letters (a and b) in the columns denote statistical difference among the groups ($P < 0.05$).

during the metabolism of this drug.[3,32] Although BZ inhibits gene transcription and inducible nitric oxide synthase (iNOS) expression,[34–36] there is evidence that BZ triggers NO synthesis independently of the conventional enzymatic mechanisms associated with NO synthase.[1,3] Hall and Wilkinson[5] described that nitroheterocyclic compounds such as BZ act as prodrugs and must undergo activation, specifically through reduction of the nitro group by nitroreductase enzymes, to mediate their toxic effects responsible for oxidative and nitrosative tissue damages.[1] This mechanism is preponderant to ensure NO production, which acts in host defences against parasitic infections.[3,4,9]

In this study, BZ also stimulated CAT and SOD activities. There is sufficient evidence that the increased activity of endogenous antioxidant enzymes occurs as a natural response to attenuates tissue damage mediated by ROS/RNS.[8,37] Here, the increased myocardial production of reactive metabolites and the evidence of protein (i.e. carbonyl proteins) and lipid oxidation (i.e. MDA) in animals exposed to BZ indicated that this adaptive enzymatic response contributed to maintain cardiomyocytes morphological integrity (i.e. similar CK-MB levels). However, this response was insufficient to neutralize reactive molecular damage in cardiac tissue, reinforcing a potential applicability of complementary therapeutic agents to restrict BZ cytotoxicity. The highest dose of IB was effective in reducing NO_2^-/NO_3^- production and oxidative damage triggered by BZ, preventing the reduction of antioxidant enzymes. These

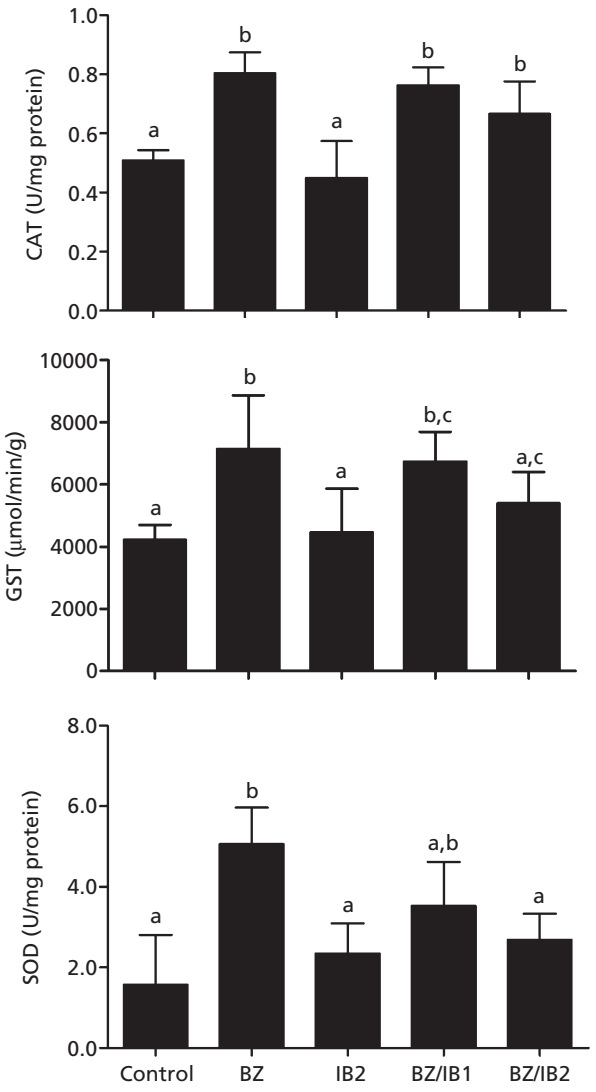

Figure 6 Activity of antioxidant enzymes in the cardiac tissue from mice treated with benznidazole and ibuprofen. Control: 1% carboxymethyl cellulose solution, benznidazole: 100 mg/kg benznidazole, ibuprofen 2: 400 mg/kg ibuprofen, benznidazole/ ibuprofen 1: 100 mg/kg benznidazole + 200 mg/kg ibuprofen, benznidazole/ ibuprofen 2: 100 mg/kg benznidazole + 400 mg/kg ibuprofen. Data are represented as mean ± standard deviation. Different letters in the columns (a, b and c) denote statistical difference among the groups ($P < 0.05$).

responses suggest that IB could modulate metabolic pathways associated with tissue damage mediated by reactive species. Apparently, this effect was independent of the influence of IB on the endogenous antioxidant enzymes, a finding potentially related to COX inhibition and attenuation of PG synthesis, a lipid proinflammatory mediator.[38,39]

The relationship among ROS/RNS and COX enzymatic system remain poorly understood.[40] According to Mollace *et al*.,[41] this interaction can occur at multiple levels, in which reactive metabolites interferes directly with COX activity, stimulating PG biosynthesis.[8,42] In fact, the findings of this study indicated that increased ROS/RNS production induced by BZ is potentially related with upregulation of PG biosynthesis. There is evidence that ROS/RNS-mediated PG production occurs by COX activation and is proportional to ROS/RNS tissue levels and lipid peroxidation, reflecting the extent of reactive injury *in vivo*.[42,43] Reciprocally, previous studies proved that PGs are potent inducers of intracellular reactive stress that mediates cell degeneration. Thus, $PGF_{2\alpha}$ and PGD_2 have been identified as pivotal molecules that potentiate ROS/RNS biosynthesis in cardiac tissue.[42,44] This effect can contribute to increase BZ cytotoxicity by triggering the 'Ping-Pong' effect, a self-powered pathological mechanism in which reactive metabolites produced during drug metabolism stimulate PG biosynthesis and additional reactive metabolites, contributing to amplify tissue damage.[40,41] In this context, it is reasonable to assume that COX inhibitors, such as IB could significantly inhibit the PG-induced ROS production, thereby preventing myocardial cytotoxicity.[42,44]

Although ROS/RNS are frequently associated with proinflammatory processes, IL-10, CCL-2 and TNF-α tissue levels were similarly reduced in the groups BZ and IB compared with CG. Interestingly, IB also contributed to attenuate NO and oxidative markers, which did not occur in the BZ group. The anti-inflammatory profile of BZ was described in previous studies.[34–36] There is evidence that by inhibiting nuclear factor-κB (NF-κB) (a key regulator of the inflammatory response), BZ exerts immunomodulatory effects besides its antiparasitic activity, more specifically, downregulating the pro-inflammatory activity of macrophages.[34,35] Immunomodulatory effects of BZ were also evidenced by its ability to increase survival and decrease serum levels of IL-6 and TNF-α in C57BL/6 mice treated with lipopolysaccharide (LPS).[34,36] Apparently, this activity is not restricted to the LPS-mediated macrophage activation, as it is also observed when employing other stimuli such as pro-inflammatory cytokines (IL-1 and TNF-α) and H_2O_2, a nonradical molecule involved in reactive tissue damage.[34] Corroborating the findings of this study, although BZ present anti-inflammatory effect, the inhibitory mechanism that restricts cytokines production does not interfere in ROS synthesis (i.e. H_2O_2 and O_2^-), essential molecules that increase host resistance against infectious agents.[34]

The effect of nonsteroidal anti-inflammatory drugs (NSAIDs) on the immune system is better understood and reinforces the findings of cytokine downregulation observed in this study in the IB group, irrespective of the NO biosynthesis. Evidence suggests that NSAIDs can modulate COX activity independent of the inflammatory

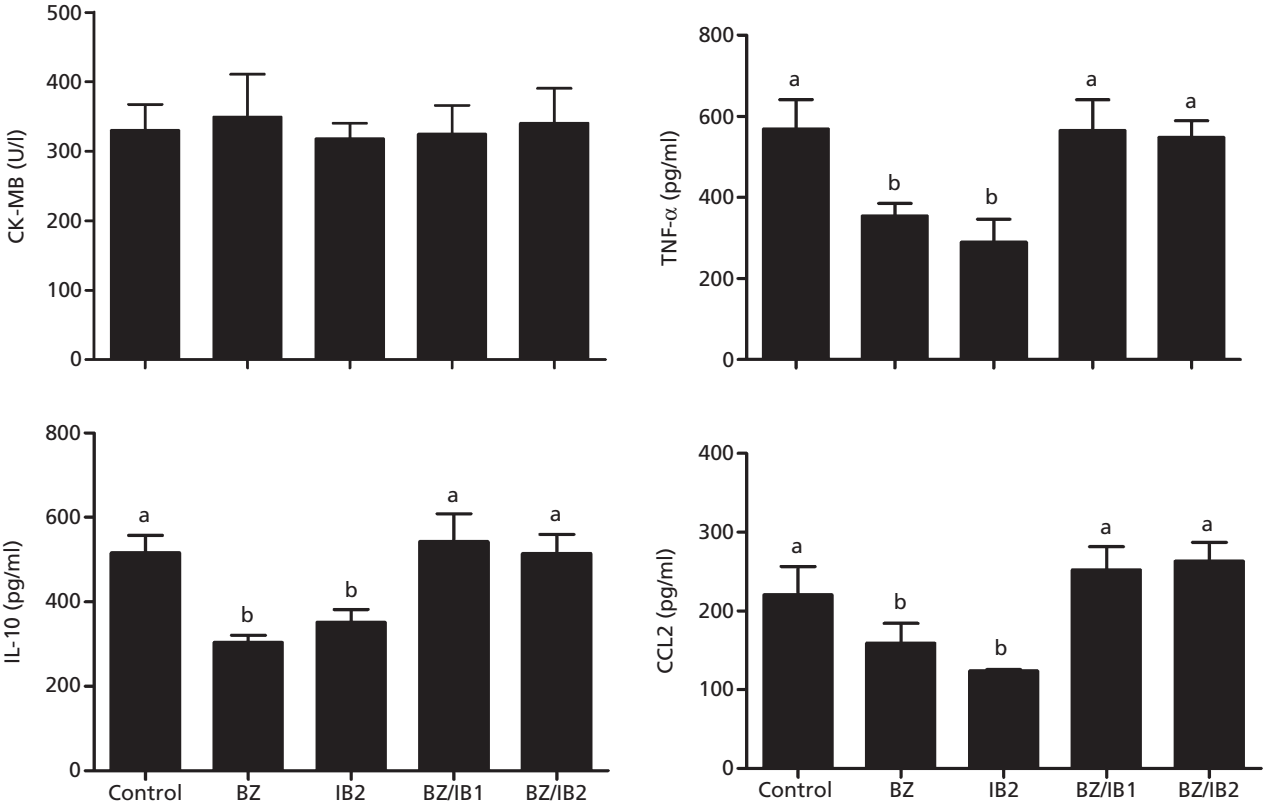

Figure 7 Concentration of creatine kinase-MB in the serum and inflammatory molecules in the cardiac tissue from mice treated with benznidazole and ibuprofen. Control: 1% carboxymethyl cellulose solution, benznidazole: 100 mg/kg benznidazole, ibuprofen 2: 400 mg/kg ibuprofen, benznidazole/ibuprofen 1: 100 mg/kg benznidazole + 200 mg/kg ibuprofen, benznidazole/ibuprofen 2: 100 mg/kg benznidazole + 400 mg/kg ibuprofen. Data are represented as mean ± standard deviation. Different letters in the columns (a,b and c) denote statistical difference among the groups ($P < 0.05$).

signals transduction pathways.[39,45] Thus, IB can interact directly with the transcription factor NF-κB inhibiting its activation directly. This effect is potentially dependent on the alterations in the activity of IκB kinase (IKK).[46] In-vitro and in-vivo studies with other NSAIDs have shown their ability to bind and inhibit directly the IKK-β activity, reducing its ability to bind to adenosine triphosphate (ATP). There seems to be a competitive inhibition between NSAIDs and ATP cleavage, which prevents IKK-β phosphorylation and activation. These events block the activation of the transcription factor NF-κB and, consequently, its proinflammatory activity.[39–41,46]

As expected, the therapeutic association of IB revealed a protective effect in attenuating cardiac nitrosative and oxidative damage induced by BZ. However, interestingly, the levels of IL-10, CCL-2 and TNF-α were maintained similar to those in the CG group. Thus, these drugs interacted in a complex manner capable of modulating the expression cytokines by an alternative pathway, which needs to be elucidated. Despite the hypothesis proposed, the association between BZ and IB can be considered to be potentially beneficial, because the levels of proinflammatory markers were maintained. This pattern of response may be useful in immunological readiness against *T. cruz*i infection, preventing the installation of an anti-inflammatory status during an infection, a condition that is potentially dangerous to the host. However, this issue is extremely complex and poorly understood, requiring further studies to determine the applicability and potential benefits of BZ and IB association in infectious conditions.

Conclusions

This preclinical study indicated that BZ triggers an oxidative and nitrosative route that is associated with marked damages to the lipids and proteins and increased PG biosynthesis in the cardiac tissue. Despite upregulation of antioxidant enzymes, this mechanism is not sufficient to counteract the cardiac oxidative damage induced by BZ. The chemotherapeutic association with IB restored the

cytokines to the basal levels and abolished any harmful effects induced by BZ, in a process potentially mediated by inhibition of PGD_2, $PGF_{2\alpha}$ and ROS/RNS biosynthesis. The beneficial outcome of this association in attenuating the pro-oxidant status of the heart was an independent effect of IB on the endogenous antioxidant enzymatic pathway. Individually, but not in association, BZ and IB significantly reduced the cardiac levels of CCL2, TNF-α and IL-10, indicating a complex drug interaction. Although the association between BZ and IB has been beneficial, the applicability of this association as a therapeutic strategy to restrict BZ toxicity in Chagas disease requires further investigation.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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