Parasitology

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Experimental combination therapy using low doses of benznidazole and allopurinol in mouse models of *Trypanosoma cruzi* chronic infection

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Research Article

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Abstract

This study evaluated the effectiveness of low doses of benznidazole (BNZ) on continuous administration (BNZc), combined with allopurinol (ALO), in C57BL/6J and C3H/HeN mice infected with *Trypanosoma cruzi* Nicaragua strain and *T. cruzi* Sylvio-X10/4 clone. *Tc*N-C57BL/6J was also treated with intermittent doses of BNZ (BNZit). The drug therapy started 3 months post infection (pi) in the chronic phase of mice with heart disease progression, followed-up at 6 months pi. *Tc*N-C57BL/6J treated with BNZc was also monitored up to 12 months pi by serology and electrocardiogram. These mice showed severe electrical abnormalities, which were not observed after BNZc or BNZit. ALO only showed positive interaction with the lowest dose of BNZ. A clear parasitic effect, with significant reductions in antibody titres and parasitic loads, was achieved in all models with low doses of BNZ, and a 25% reduction of the conventional dose showed more efficacy to inhibit the development of the pathology. However, BNZ 75 showed partial efficacy in the *Tc*Sylvio-X10/4-C3H/HeN model. In our experimental designs, C57BL/6J allowed to clearly define a chronic phase, and through reproducible efficacy indicators, it can be considered a good preclinical model.

Introduction

Chagas disease is the most common cause of congestive heart failure and related deaths among young adults in the endemic areas of South and Central America and Mexico (WHO, 2017). It has also become an important health issue in the USA and Europe due to large-scale migration of Latin Americans over the last few decades (Moncayo and Silveira, 2009). Its aetiological agent is the protozoan parasite *Trypanosoma cruzi*. One of drugs that is typically used for acute and chronic phases in children (Sosa Estani *et al*., 1998) and adult patients is benznidazole (BNZ), despite some adverse effects (Viotti *et al*., 2009).

Recently, the BENEFIT study has shown a trypanocidal effect of BNZ, with decreased parasitic loads in patients with severe chronic disease, and no association with clinical outcomes (Morillo *et al*., 2015). Another working group (the TRAENA trial) has shown negative levels of specific IgG in serum, and undetectable parasite loads in chronic patients treated with BNZ, with no impact in clinical events (Riarte *et al*., 2016). It is unknown whether a mono-therapy or combined therapies can have an impact on the cure of Chagas disease; in fact, there is uncertainty due to controversial results between randomized clinical trials and observational studies. Within this framework, preclinical studies can shed some light on the use of variations in dosage, treatment schedules and interactions of known and unknown drugs. In this sense, we have studied experimental treatments with low doses of BNZ in acute (Grosso *et al*., 2013; Scalise *et al*., 2016; Rial *et al*., 2017*a*, 2017*b*) and chronic infection in mice (Rial *et al*., 2016, 2017*a*, 2017*b*), which is in line with pharmacokinetic studies in Chagas disease patients, supporting the hypothesis that treatment with lower BNZ doses may be effective (Fernández *et al*., 2016; Wiens *et al*., 2016), with an impact on the potential reduction of adverse effects (Bustamante *et al*., 2014).

The combination of BNZ with allopurinol (ALO) to control *T. cruzi* infection and to generate less tissue damage was previously observed in an experimental acute mouse model (Grosso *et al*., 2013), and in a pilot trial in chronic patients (Perez-Mazliah *et al*., 2013). Other groups have also proposed trypanocidal treatments with continuous and intermittent BNZ administration (Bustamante *et al*., 2014; Álvarez *et al*., 2016).

Here we present different trypanocidal treatment schemes with low doses of BNZ in different chronic models of mice infected with DTU I parasites, *Tc* Nicaragua (*Tc*N) isolate (Grosso *et al*., 2010), and *TcSylvio*-X10/4 clone, in line with recommendations from several workshops focusing on trypanocidal drug action preclinical model studies. Our paper shows a comparison

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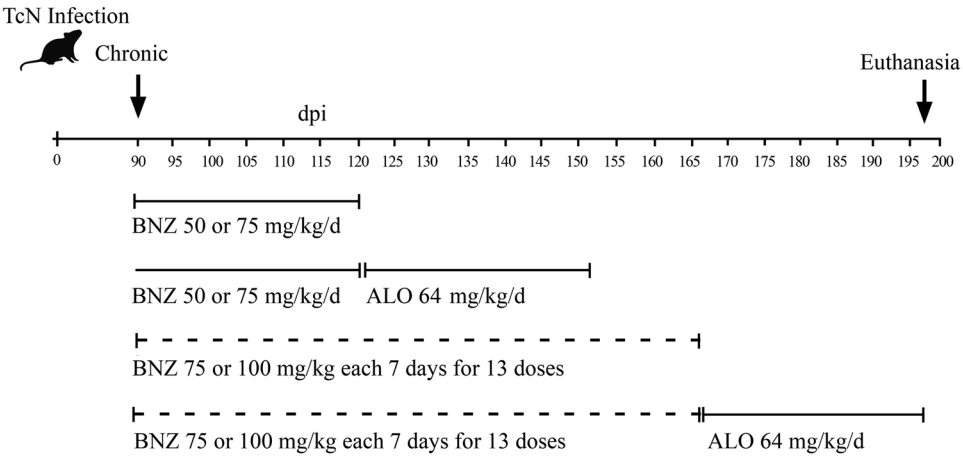

Fig. 1. Schedules of treatment with low doses of BNZ alone or combined with ALO in TcNicaragua experimental chronic models, at 3 months pi. BNZ continuous treatments with 50 or 75 mg kg^{-1} day^{-1} for 30 days (full line). Intermittent treatment with 75 or 100 mg kg^{-1}, one dose every 7 days for 13 doses (dotted line). Treatments with ALO 64 mg kg^{-1} day^{-1} were daily administered for 30 days, immediately after BNZ in both experimental schedules. Each group included 8–10 animals.

of serological, parasitological, histopathological and electrocardiographic (ECG) studies in response to different BNZ and ALO treatment regimens.

Materials and methods

Chemical compounds

The following compounds were used in this study: BNZ [(VN-benzyl-2-nitro-1-imidazole-acetamide) (®Abarax ELEA Lab, Buenos Aires, Argentina)], ALO [(4-hydroxypyrazol (3, 4-d) pyrimidine) (Gador Lab, Buenos Aires, Argentina)], bovine fetal serum (Gibco, Rockville, MD, USA), horse serum (Internegocios SA, Córdoba, Argentina), tryptose (Difco, Detroit, MI, USA), 10% formaldehyde solution, haematoxylin–eosin (H&E) and collagen Masson's trichrome stains, guanidine (Sigma Chemical Co., St Louis, MO, USA).

Parasites

Culture trypomastigotes of *Tc*N and *Tc*Sylvio-X10/4 were obtained from passage through Vero cells (kidney epithelial cells from African green monkeys) (ABAC, Pergamino, Argentina).

Mice infection

Four-week-old, similar weight (media 20.8 g ± 1.5) female mice, C57BL/6J and C3H/HeN, were obtained from the National Institute of Parasitology Dr Mario Fatala Chaben, ANLIS Malbrán, Buenos Aires, Argentina bioterium, under specific pathogen-free conditions. Mice were in a controlled room with water and food *ad libitum* and were randomly selected prior to infection and assignment to the treatment groups. C57BL/6J and C3H/HeN mice were infected intra-peritoneally with 3×10^3 and 10 *Tc*N trypomastigotes, respectively, and another group of C3H/HeN were infected with 1×10^6 *Tc*Sylvio-X10/4 trypomastigotes.

In *Tc*N and clone *Tc*Sylvio-X10/4 mice models, the chronic phase was entered around 3 months post infection (pi). At this time, a group of mice were euthanized for the analysis of serology and histopathological studies (data not shown). BNZ treatments were then initiated. Mice received 30 doses of continuous BNZ (BNZc) (50 or 75 mg kg^{-1} day^{-1}); another scheme of intermittent doses of BNZ (BNZit) (75 and 100 mg kg^{-1}) was supplied in one dose every 7 days for 13 times (Bustamante *et al.*, 2014). Half of the mice that were treated with BNZc and BNZit received 30 daily doses of ALO 64 mg kg^{-1} day^{-1}, as shown in Fig. 1. ALO was administered immediately following BNZ treatment. Uninfected, untreated *T. cruzi*-infected mice, and BNZ alone or plus ALO-treated mice were the study groups. Drugs were given directly into the mouth of each mouse by using a top cut tip, BNZ was suspended in oil and ALO in distilled water. Prior to being euthanized – after 6 months – uninfected, infected, untreated and treated mice were studied for ECG alterations. After euthanasia, all mice were coded and blinded for analysis. Parasitaemia by qPCR, IgG levels by ELISA, heart inflammation and fibrosis by histopathological studies and ECG abnormalities were evaluated. A group of C57BL/6J mice from each BNZc administration were followed-up for 12 months to observe the evolution of serology and ECGs.

ECGs

They were performed to assess mice cardiac electrical alterations on uninfected, infected untreated and infected treated mice at 6 and 12 months pi. We evaluated heart rate (HR), atrio-ventricular node conduction time (PR interval), ventricle depolarization (QRS) and interval as the measure of the time between the beginning of the Q wave and the end of the T wave (QT interval). The mice were evaluated under anaesthesia (Avertin, Sigma Chemical Co., St Louis, MO, USA) with a Cardimax FX-2111 electrocardiograph. The measurements were analysed with the ImageJ program.

Measurement of antibody response

Blood from infected untreated and treated mice ($n = 5$–9 animals per treatment) was collected from the orbital venous sinus (500 μL) at 6 months pi. Duplicate sera samples were taken, and the final result was the average of the duplicates. Samples were analysed for IgG antibodies by use of an enzyme-linked immunosorbent assay (ELISA). A lysate preparation derived from epimastigotes of the *T. cruzi* Tulahuen strain (20 μg mL^{-1}) was used as the antigen source. Briefly, flat-bottomed plates (96-well) were coated overnight at 4 °C with 50 μL $well^{-1}$ of antigen diluted in carbonate buffer pH 9.6. Plates were blocked for 1 h at RT with 100 μL $well^{-1}$ of 5% skimmed milk in PBS. After being washed three times with PBS-0.05% Tween 20 (PBS-T), plates

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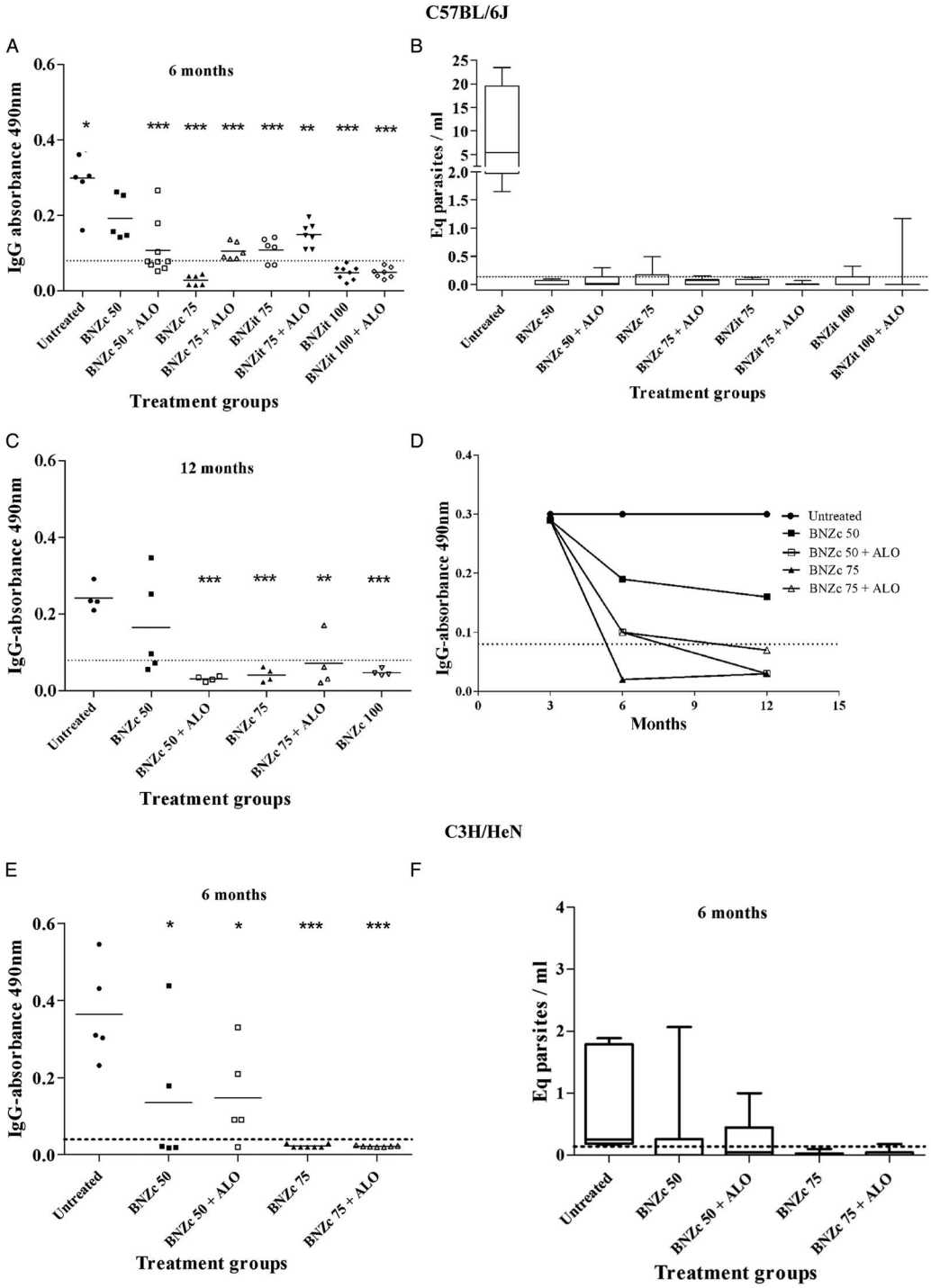

Fig. 2. *Trypanosoma cruzi*-specific antibody levels in serum samples from TcNicaragua-infected untreated mice or infected treated mice, at 6 months pi. (A) and 12 months pi. (C and D) in C57BL/6J; and in CH3/HeN at 6 months pi. (E). The cut-off value for negative antibody levels, as described in the Materials and methods section, is represented by the horizontal dotted line. Parasitaemia by quantitative PCR amplification of a *T. cruzi* satellite DNA from blood of C57BL/6J (B) and C3H/HeN (F) mice infected with TcNicaragua isolate untreated and treated subjects. Boxes represent the inter-quartile interval with the median line. The cut-off value of undetectable equivalent parasites mL^{-1} (0.14) is represented by the horizontal dotted line. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

were incubated with serum samples (1:50–1: 400 dilution, 50 μL $well^{-1}$) for 30 min at 37 °C. After washing with PBS-T, 50 μL $well^{-1}$ of horseradish peroxidase-labelled goat anti-mouse IgG (The Jackson Laboratory, ME, USA) was added for 30 min at RT. The reaction was developed with 50 μL $well^{-1}$ of o-phenylenediamine dihydrochloride, and stopped with 2N sulphuric acid. Optical density (OD) was read at 490 nm with an ELISA microplate reader (MINDRAY ME-96A). The mean absorbance for 10 negative control samples, plus three standard deviations was used as the cut-off point to discriminate positive and negative results (OD = 0.08).

Parasitaemia detected by DNA amplification

One volume of blood, collected from euthanized uninfected, infected untreated and treated mice at 6 months pi ($n = 5$ samples per treatment), was mixed with an equal volume of guanidine-HCl 6 M, EDTA 0.1 M, pH 8, kept at room temperature for 1 week and then at 4 °C until use. DNA was isolated from 0.2 mL of guanidine-EDTA buffer B mixture using a commercial High Pure PCR template Preparation kit (Roche, Basel, Switzerland), and eluted in 0.2 mL, according to the manufacturer's protocol. A bacterial commercial plasmid – pQE (Qiagen, Hilden, Germany)

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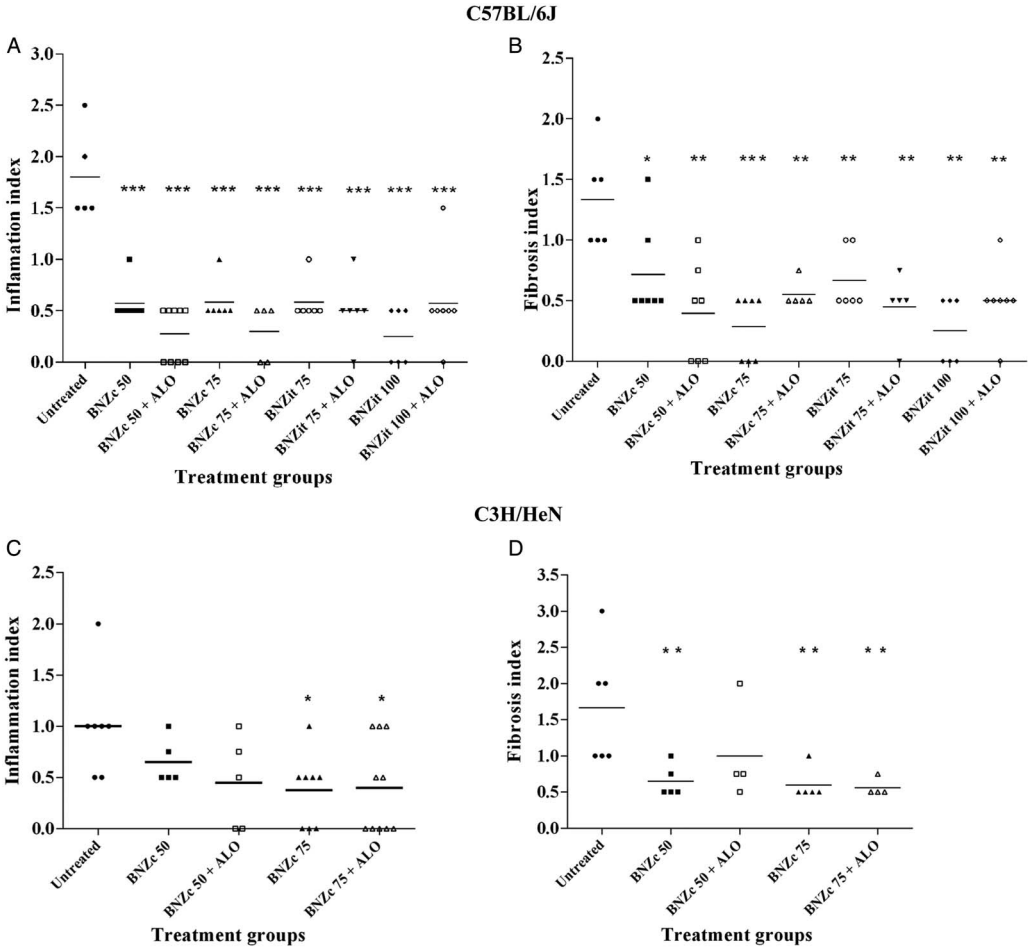

Fig. 3. Evaluation of inflammation (A and C); fibrosis (B and D) in TcN-C57BL/6J and TcN-C3H/HeN and infected treated mice. Data represent morphometric quantification in heart tissue of inflammatory cells stained with haematoxylin and eosin and fibrosis with collagen Masson's trichrome. Animals were treated with BNZ in concentrations at 100, 75 or 50 mg kg^{-1} day^{-1}, alone or combined with ALO (64 mg kg^{-1} day^{-1}) for a period of 30 days (BNZc), or treated intermittently (BNZit) with 13 doses, once every 7 days. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

– was used as internal standard for DNA extraction (Bua *et al.*, 2012). An ABI 7500 thermo-cycler (Applied Biosystems, Carlsbad, CA, USA) was used to amplify a *T. cruzi* satellite DNA flanked by the highly conserved Sat Fw and Sat Rv oligonucleotides in the parasite genome (Duffy *et al.*, 2009). Duplicated samples were run with a commercial kit, SYBR® GreenER® qPCRSuperMix Universal (Invitrogen, Life Technologies, CA, USA) as previously described (Bua *et al.*, 2013). Epimastigotes of the *Tc*N isolate, DTU *Tc*I, were used as a standard in artificially spiked mouse blood. The parasite curve, negative samples and non-template DNA were included in each determination. The cut-off value was determined to be = 0.14 Equivalent parasites mL^{-1} (EqP mL^{-1}).

Histopathological studies

Uninfected, infected untreated and treated mice were euthanized at 6 months pi after completing the treatment ($n = 8$–10 mice per treatment). Hearts were removed, fixed in 10% formaldehyde solution and embedded in paraffin. Five-micron tissue sections were stained with H&E and collagen Masson's trichrome stains and evaluated by light microscopy, recording the extent of mononuclear infiltrates and fibrosis. The extent of lesions was evaluated according to a modified classification (Gupta and Garg, 2010). Briefly, eight different areas of the heart (left and right atria, upper and lower halves of each ventricular wall and septum) were scored according to the extension of inflammation as: (0) absent/none; (0.5) isolated focal one foci; (1) mild myocarditis, with at least two inflammatory foci; (2) moderate, with multiple inflammatory foci; (3) extensive, with inflammatory foci or disseminated inflammation with necrosis and preservation of tissue integrity; and (4) severe, with diffused inflammation, interstitial oedema and loss of tissue integrity. Fibrosis was scored on a scale of 0–3 according to the damage recorded by microscopy: (0) absent/mild; (1) short and less than four isolated foci of fibrosis; (2) moderate and diffuse connective tissue that partially compromises the wall; (3) severe and diffuse connective tissue that compromises the whole wall. A numeric sum for each heart section represents the inflammation or fibrosis index.

Statistical analysis

Data were expressed as mean, median and 95% CI, as appropriate, and were analysed by the Student's *t*-test. qPCR was evaluated using the Mann–Whitney test due to asymmetric distribution of the data and unmatched groups. Statistical significance was considered at $P < 0.05$. Data and graph were analysed and performed with GraphPad Prism5.0 software.

Results

Course of infection in chronic T. cruzi *Nicaragua mice*

C57BL/6J and C3H/HeN mice were infected with 3×10^3 and 10 *Tc*N trypomastigotes, respectively, with a survival rate of 45 and 35% after the acute phase. At 3 months pi, the chronic phase of

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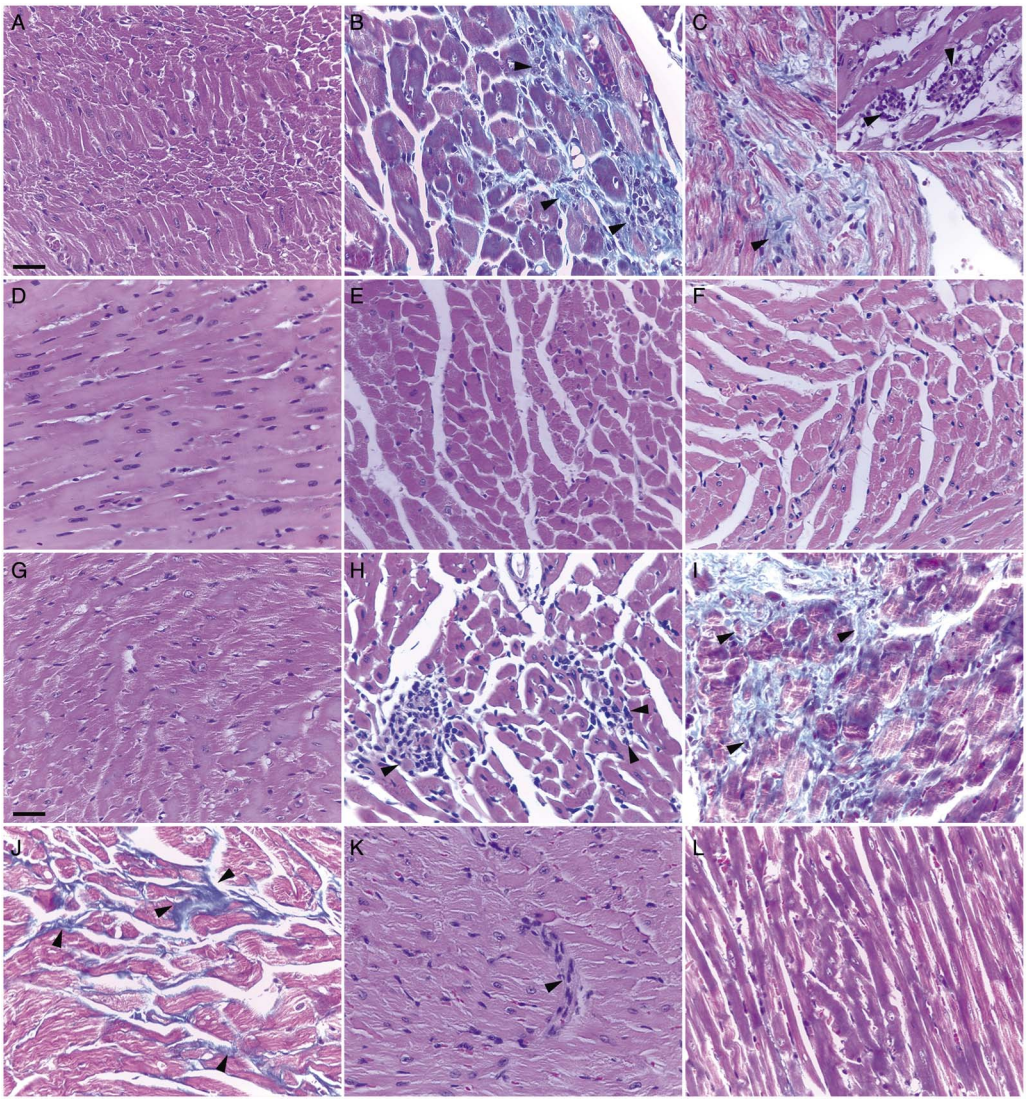

Fig. 4. Histopathological images of heart tissues in chronically C57BL/6J (A–F) and C3H/HeN (G–L); normal myocardium of C57BL/6J-uninfected mice: (A) inflammatory lesions and fibrosis (arrows) in the chronic infected mice; (B) interstitial and in patches fibrosis (arrows) with BNZc 50 treatment; (C) insert: perivascular inflammatory mononuclear cells (arrows). Recovered normal myocardium after BNZc 75; (D) and BNZ it 75; (E) and residual isolated mononuclear cells with BNZit 100 treatment; (F) normal myocardium of C3H/HeN, uninfected mice; (G) inflammatory cell foci (arrows) and isolated mononuclear cells (arrows), and patches of fibrosis in the chronic infected mice; (H and I) interstitial and in patches fibrosis (arrows) after BNZc 50 treatment; (J) residual isolated mononuclear cells (arrows) with BNZc 75; (K) absence of fibrosis and recovered normal myocardium; (L) haematoxylin and eosin stain; (A, D, E–H, K) collagen Masson trichrome stain; (B, C, I, J, L) bars in A and G correspond to 25 μM.

both mouse models was characterized showing disappearance of parasitaemia by direct methods, which was only detectable by qPCR, a clear serological response and typical histopathological lesions, such as active chronic myocarditis with sclerotic sequelae being other lesions intra-myocardial perivasculitis. In addition, usual ECG abnormalities of chronic human Chagas disease were diagnosed mainly in C57BL/6J, at 6 months pi. Despite the different inocula in both chronic infected C57BL/6J and C3H/HeN models, the serology was similar: IgG levels in C57BL/6J were 0.300 OD (95% CI 0.220–0.378) and C3H/HeN 0.364 OD (95% CI 0.210–0.518), with no significant differences. All chronically infected and treated mice were followed-up until 6 months pi. Another group of C57BL/6J was followed-up for serological and ECG studies until 12 months pi.

Humoral immune responses specific for T. cruzi *following chemotherapy*

Sera from both models, C57BL/6J and C3H/HeN, *Tc*N infected and treated mice had significantly decreased levels of antibodies against the specific *T. cruzi* antigen ($P < 0.001$) compared with untreated mice (Fig. 2A and E). In C57BL/6J, BNZc 75, BNZit 100 and BNZit 100 plus ALO produced negative titres at 6 months pi. The addition of ALO to BNZc 50 induced 66% of negative sera, boosting the effect of the lower dose of BNZ, while BNZc 50 alone induced 40% of negative titres at 1 year pi (Fig. 2C). At this time, treated mice with low doses of BNZc plus ALO (50 and 75) had a higher percentage of negative serum (Fig. 2C and D). Moreover, no significant differences were found between these doses and BNZc 100. In the *Tc*N-C3H/HeN mouse model, treatment with BNZc 50 showed a greater variability in the serological response, while BNZc 75 induced negative titres in 100% of these mice. The addition of ALO did not modify this response in either case.

Parasitaemia by qPCR drug treated in TcN-infected mice

Quantification of parasitaemia at basal times had different medians: 5.44 (95% CI 1.65–23.5) and 0.250 (95% CI 0.12–1.6) EqP mL^{-1} in both C57BL/6J and C3H/HeN mice, respectively

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Table 1. Electrocardiogram parameters

(*a*)

Parameters ECGs	Uninfected mice	Untreated chronic mice	At 6 months						
			BNZ continuous doses				BNZ intermittent doses		
			50	50 + ALO	75	75 + ALO	75	75 + ALO	100	100 + ALO
HR (beats min^{-1})	476 ± 16.73	393.3 ± 24.22	515** ± 55.08	510* ** ± 38.3	455** ± 25.17	484*** ± 21.91	510** ± 62.18	484** ± 58.99	476** ± 47.75	493.3** ± 41.63
PR interval (s)	0.07 ± 0.02	0.12 ± 0.01	0.08** ± 0.008	0.08** ± 0.007	0.07** ± 0.008	0.08** ± 0.008	0.07** ± 0.02	0.06** ± 0.02	0.08* ± 0.01	0.07** ± 0.01

(*b*)

Parameters ECGs	Uninfected mice	Untreated chronic mice	At 12 months						
			BNZ continuous doses						
			50	50 + ALO	75	75 + ALO	100		
HR (beats min^{-1})	513 ± 37.5	430 ± 52.7	525* ± 47.7	532** ± 36.33	480 ± 60.0	483 ± 28.87	506 ± 23.09		
PR interval (s)	0.062 ± 0.017	0.113 ± 0.017	0.07* ± 0.01	0.072** ± 0.013	0.07* ± 0.01	0.063** ± 0.011	0.063** ± 0.005		

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

($P = 0.032$). In both models, treatments decreased parasite load to undetectable levels (Fig. 2B and F) at 6 months pi. No significant differences were found in parasitaemia levels, between the different administrations of BNZc and BNZit in C57BL/6J mice (Fig. 2B).

Inflammatory lesions and fibrosis in hearts of treated chronic TcN mice

In the *Tc*N-C57BL/6J model, all treatments reduced the inflammatory lesions in the chronic infected mice ($P = 0.0005$); the ALO addition reduced the inflammation only with BNZc 50 ($P = 0.03$), while in the other treatments, the efficacy of ALO had greater variability. The inhibition of fibrosis was greater with BNZc 75, BNZit 100 *vs* BNZc 50, with some heterogeneity in the pathological, inflammation and fibrosis responses (Fig. 3A and B; Fig. 4A–F). The inflammation in the hearts of *Tc*N-C3H/HeN animals treated with BNZc 50 was not different from that observed in the chronic infected mice. However, there was a variability in the inflammatory response in this group. The reduction of inflammatory lesions in treated mice with BNZc 75 was different from the infected control [$P = 0.01$ (Fig. 3C) and Fig. 4H, I and K]. The addition of ALO to BNZc 75 did not modify this response. Likewise, the reduction of inflammation to any degree *per se* seems to be a sufficient condition to inhibit fibrosis with BNZc 50 and 75 (Fig. 3C and D; Fig. 4G–L).

ECGs in chronically infected mice

Healthy, infected and treated mice were examined according to ECG parameters, such as HR, PR, QRS and QT, at 6 and 12 months pi. In the *Tc*N-C57BL/6J model, a significant decrease in HR and an increase in the PR interval, compared with healthy mice, were reversed with both continuous and intermittent schedule of BNZ treatments at 6 months pi (Table 1*a*). At 12 months pi, severe abnormalities, such as sustained ventricular tachycardia (Fig. 5, panel 3), atrial fibrillation (Fig. 5, panel 4) and high-grade AV block (Fig. 5, panels 5, 6) in infected mice with *Tc*N were observed (Table 1*b*). These abnormalities were absent in uninfected (Fig. 5, panels 1, 2) and infected treated mice both with administration regimen BNZc and BNZit (Fig. 5, panels 7–10) at 6 and 12 months pi. Conversely, the *Tc*N-C3H/HeN model did not show ECG changes, while the *TcSylvio-X10/4*-C3H/HeN model showed an increase in PR, which did not revert by continuous treatment (data not shown).

Course of infection and treatment in T. cruzi *Sylvio-X10/4-infected mice*

The infection with clone *Tc*Sylvio-X10/4 DTU I in C3H/HeN mice showed the development of a severe chronic myocarditis from the third month pi as previously described (Postan *et al.*, 1986). Sera from all treated mice had decreased levels of antibodies against the *T. cruzi* antigen, compared with sera from untreated infected mice at 6 months pi ($P < 0.001$). At this point, only 20–30% of those treated mice were serologically negative with the highest doses (Fig. 6A). Parasite load in these chronic mice had a median of 5.18 EqP mL^{-1} (95% CI 1.30–7.22) and decreased to 0.29 EqP mL^{-1} (95% CI 0.0–0.91) with BNZ 50, to undetectable levels (95% CI 0.0–0.50) with BNZ 75 (Fig. 6B).The addition of ALO did not modify any results compared with BNZ 50 and 75 alone, although there was a greater variability in the response. Chronic myocarditis and fibrosis showed a significant decrease with all treatments, being BNZ 75

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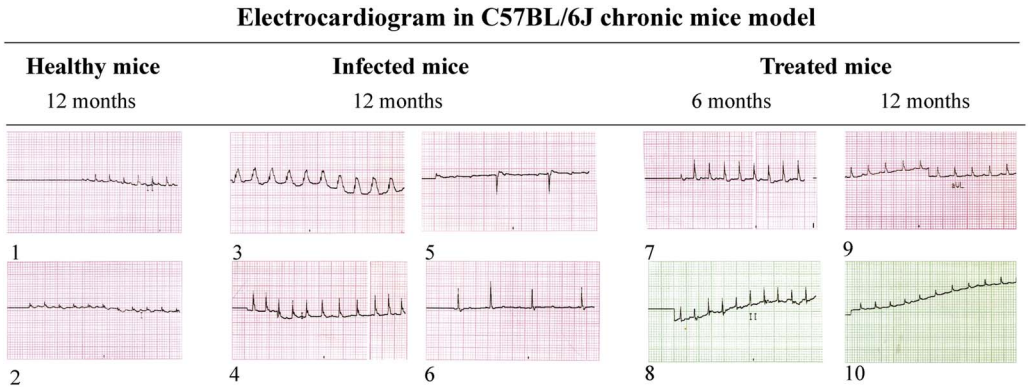

Fig. 5. Comparison of ECGs in healthy, TcNicaragua-infected untreated C57BL/6J mice at 12 months, and treated mice at 6 and 12 months pi. Healthy mice: panels 1 and 2; severe abnormalities such as sustained ventricular tachycardia: panel 3; atrial fibrillation: panel 4; high-grade AV block: panels 5 and 6; no abnormalities in treated mice: panels 7–10; BNZ it 75: panel 7; BNZc 50 + ALO: panel 8; BNZc 75: panel 9 and BNZc 50: panel 10. Panel 1 in lead II, panel 2 in lead I, panel 3 in lead III, panel 4 in lead I, panel 5 in lead aVR, panel 6 in lead II, panel 7 in lead I, panel 8 in lead II, panel 9 in lead aVL and panel 10 in lead aVL.

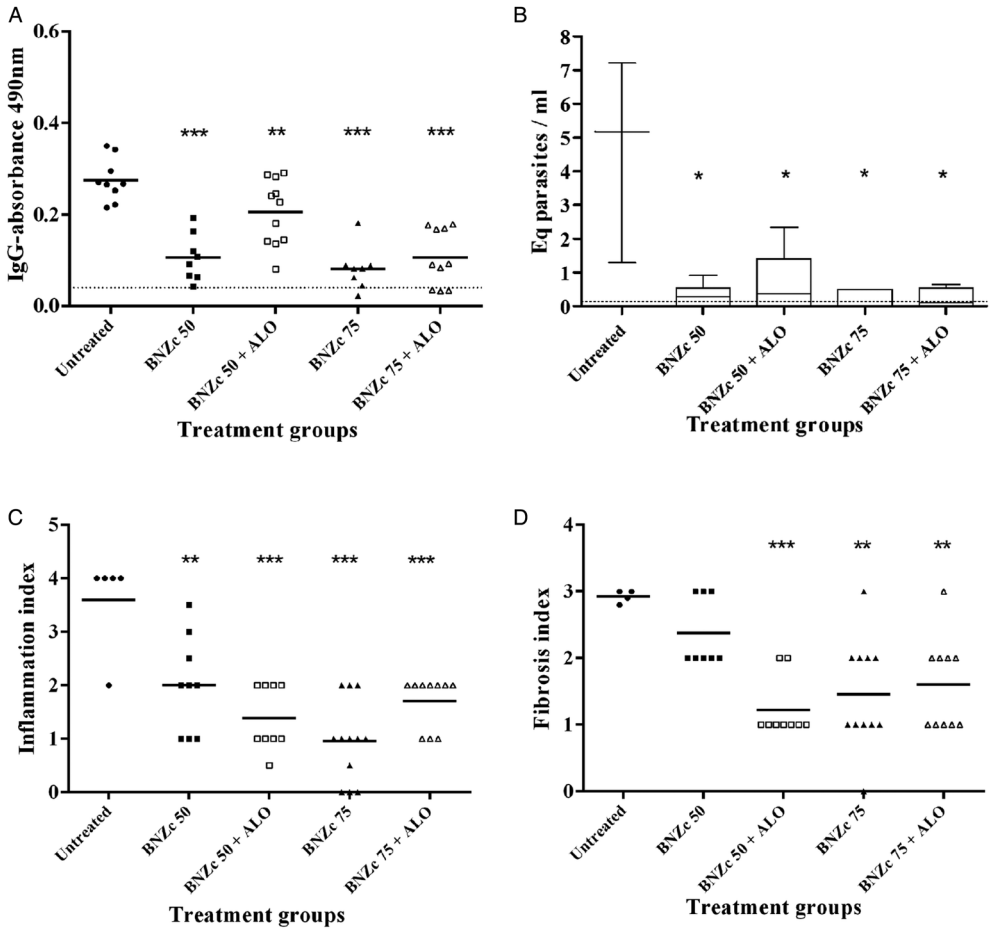

Fig. 6. *Trypanosoma cruzi*-specific antibody levels in serum samples from uninfected C3H/HeN mice and TcSylvio-X10/4-infected untreated and treated mice (A). The cut-off value to discriminate positive and negative titres (OD = 0.08), as described in the Materials and methods section, is represented by the horizontal dotted line. Parasitaemia by quantitative PCR amplification of a *T. cruzi* satellite DNA (B). Inflammation index represented of morphometric quantification of inflammatory cells in heart tissues stained with haematoxylin and eosin (C). Morphometric quantification of fibrosis stained with collagen Masson's trichrome (D). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

alone the most efficient one in the reduction of inflammatory lesions (Fig. 6C and D).

Discussion

This study on experimental trypanocidal treatment was carried out in different animal models, in line with the suggestions arising from preclinical study workshops conducted in Brazil in 2008 (Romanha *et al.*, 2010) and Argentina in 2015 (Ministry of Health of Brazil and Argentina, National Institute of Parasitology, Dr M. Fatala Chaben, ANLIS Malbrán, and Drugs for Neglected Diseases Initiative). Mice models are essential in preclinical studies to contribute in the identification of new or known trypanocidal drugs before clinical trials. However, the lack of standardization shows wide data variability that hinders the comparison of the treatments tested for efficacy (Chatelain

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and Konar, 2015). In our laboratory, the isolate *Tc*N was characterized as DTU I (Grosso *et al.*, 2010), prevalent in many endemic areas of Central and South America (Zingales, 2017). *Tc*N produced an acute infection in C3H/HeN mice with high parasitaemia and mortality levels (85%), while survival was achieved when they were treated with low doses of the conventional BNZ or with the new nano-formulated BNZ (Grosso *et al.*, 2013; Scalise *et al.*, 2016; Rial *et al.*, 2017*a*, 2017*b*). In this paper, systematic sequential combinations in the chronic model have been studied as a progression of previous results for low doses of BNZ with ALO in acute infection by *Tc*N, with courses of ALO in some arms of chronic mice treated with BNZ in the acute phase (Grosso *et al.*, 2013). The sequential therapies were based on the efficacy obtained with this regime in the acute phase, while concomitant administration impaired acute *T. cruzi* infection in mice (data not shown). Sequential and concomitant therapies are equally successful regimens in different pathologies, as in the cases of experimental Chagas disease (Diniz *et al.*, 2013; Bustamante *et al.*, 2014). Intermittent administration of BNZ plus ALO was only performed in C57BL/6J mice, since this model allows the use of more tools for better defined pathologies and electrocardiographic alterations that enabled us to evaluate the efficacy of treatments.

The use of *in vivo* chronic models, the design of different treatment schemes and data reporting followed the ARRIVE guidelines (Kilkenny *et al.*, 2010). Beyond the different infection inocula used in each mouse strain to achieve *T. cruzi* chronic infection, all treatments reduced the serology, the parasite load and myocardial lesions at 6 months pi in *Tc*N-C57BL/6J-resistant mice, *Tc*N-C3H/HeN-sensitive mice and *Tc*Sylvio-X10/4-C3H/HeN.

BNZc 75 induced a rapid negative serological response in C57BL/6J infected with *Tc*N, while the lower doses only achieved a 40% negative serological response at 12 months pi. In the C3H/HeN mouse model infected with *Tc*N, BNZ 75 induced non-reactive serology in 100% of the mice, while in the *Tc*Sylvio-X10/4-C3H/HeN model, only 20–30% of treated mice were serologically negative with that dose.

The progression of basal myocarditis and fibrosis was more evident in the *Tc*N-C57BL/6J and *Tc*Sylvio-X10/4-C3H/HeN than in the *Tc*N-C3H/HeN model. In *Tc*N-C57BL/6J, all treatments were effective to reduce inflammation, but BNZc 75 and BNZit 100 were necessary to inhibit the development of fibrosis. BNZ 75 was more effective in *Tc*N-C3H/HeN to reduce overall pathology than in *Tc*Sylvio-X10/4-C3H/HeN, where it only showed partial efficacy.

ALO addition to the lowest dose of BNZ had a positive interaction on serology and pathology in *Tc*N-C57BL/6J, and on pathology in *Tc*Sylvio-X10/4-C3H/HeN.

These effects showed more variability and were not apparent with high doses because the effectiveness achieved concealed or inhibited the potential additive effect of ALO. The positive interaction between drugs associated with BNZ has been demonstrated in acute mice models (Diniz *et al.*, 2013; Grosso *et al.*, 2013; Strauss *et al.*, 2013; Bustamante *et al.*, 2014), in chronic models (Bustamante *et al.*, 2014), and in the pharmacokinetic interaction between BNZ and itraconazole in a murine model (Moreira da Silva *et al.*, 2012).

In the *Tc*N-C57BL/6J model, the progression of severe ECG abnormalities was inhibited by the treatment. In this sense, there was a clear association between ECG alterations in the heart and the isolate *Tc*N. These alterations were more severe than those observed in C3H/HeN with the same isolate *Tc*N. This result is in line with the observation that ECG abnormalities depend not only on the type of parasite involved, but also on the specific strain used in the mouse model, in this case, C57BL/6J mice (Postan *et al.*, 1987). Finally, this model enabled the comparison between two treatment schedules, namely BNZc and BNZit, which showed similar results. This point is worth highlighting, considering that BNZit 100 was as effective as BNZc 75. The data suggest that the efficacy of BNZ in this chronic model would be supported by a total variable dose between 1300 mg kg^{-1} 13 $days^{-1}$ (BNZit 100) and 2250 mg kg^{-1} 30 $days^{-1}$ (BNZc 75) per mouse. Pharmacokinetic studies could provide a clearer vision of these events. In this sense, it has been reported that the BNZ is widely distributed in the biological system in mice (Perin *et al.*, 2017). Human pharmacokinetic studies seem to indicate that Chagas disease patients are treated with an overdose of BNZ, and suggest that doses and treatment schedules should be reassessed by means of clinical trials (Altcheh *et al.*, 2014; Soy *et al.*, 2015).

The original idea of the intermittent scheme developed by Bustamante *et al.* (2014) was revisited in our research, extending the time of weekly administration, long enough to produce significant reductions in antibody titres and in parasitic loads, as well as to inhibit the progress of cardiac abnormalities, parameters that have historically been linked to the cure in Chagas disease. Besides, a pilot study conducted in humans with intermittent treatment showed a promising low rate of treatment exclusion and low percentages of detectable parasite loads (Álvarez *et al.*, 2016).

To sum up, low doses of BNZ induced a clear trypanocidal effect and had an impact on the reduction of pathology in chronic models. The C57BL/6J chronic model associated with isolate *Tc*N made it possible to clearly define a chronic phase, and through a series of reproducible efficacy indicators, it can be considered a good preclinical model. Further studies are needed to verify whether low doses and/or more time of treatment are useful on the pathology in human chronic Chagas disease.

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Conflict of interest. None.

Ethical standards. All procedures involving experimental protocols in animals were conducted in accordance with ethical legislation and standards issued by regulatory entities established in Argentina, and were approved by the Bioethics Committee of the National Institute of Parasitology Dr Mario Fatala Chaben (registered RENIS No.: 000 028); they also met the international recommendations for the use of laboratory animals (World Medical Association in the Declaration of Helsinki).

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