J Antimicrob Chemother
doi:10.1093/jac/dkaa101

Journal of Antimicrobial Chemotherapy

Efficacy of continuous versus intermittent administration of nanoformulated benznidazole during the chronic phase of *Trypanosoma cruzi* Nicaragua infection in mice

M. S. Rial[1], E. C. Arrúa[2], M. A. Natale [1,3], J. Bua[1,3], M. I. Esteva[1], N. G. Prado[1], S. A. Laucella[1,3], C. J. Salomon[2,3] and L. E. Fichera[1,3]*

[1]*Instituto Nacional de Parasitología "Dr. Mario Fatala Chaben", ANLIS "Dr. Carlos G. Malbrán", Ministerio de Salud de la Nación, Buenos Aires, Argentina;* [2]*Area Técnica Farmacéutica, Departamento de Farmacia, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Argentina;* [3]*Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina*

*Corresponding author. E-mail: lfichera@yahoo.com

Received 25 October 2019; returned 24 December 2019; revised 10 February 2020; accepted 20 February 2020

Background: Benznidazole and nifurtimox are effective drugs used to treat Chagas' disease; however, their administration in patients in the chronic phase of the disease is still limited, mainly due to their limited efficacy in the later chronic stage of the disease and to the adverse effects related to these drugs.

Objectives: To evaluate the effect of low doses of nanoformulated benznidazole using a chronic model of *Trypanosoma cruzi* Nicaragua infection in C57BL/6J mice.

Methods: Nanoformulations were administered in two different schemes: one daily dose for 30 days or one dose every 7 days, 13 times.

Results: Both treatment schemes showed promising outcomes, such as the elimination of parasitaemia, a reduction in the levels of *T. cruzi*-specific antibodies and a reduction in *T. cruzi*-specific IFN-γ-producing cells, as well as an improvement in electrocardiographic alterations and a reduction in inflammation and fibrosis in the heart compared with untreated *T. cruzi*-infected animals. These results were also compared with those from our previous work on benznidazole administration, which was shown to be effective in the same chronic model.

Conclusions: In this experimental model, intermittently administered benznidazole nanoformulations were as effective as those administered continuously; however, the total dose administered in the intermittent scheme was lower, indicating a promising therapeutic approach to Chagas' disease.

Introduction

Chagas' disease, a neglected parasitic infectious disease, is caused by *Trypanosoma cruzi*. It is a major public health problem in Latin America and is becoming increasingly prevalent in other regions as the result of migration.[1,2] In addition to the 6–7 million people infected worldwide, 100 000 people a year are at risk of contracting *T. cruzi* infection.[3] Nearly 30% of those infected develop a chronic cardiomyopathy and/or digestive megasyndromes. Benznidazole, a nitrobenzimidazole derivative, is one of the effective and available drugs used to treat this neglected infection[4] and it is prescribed for both the acute and chronic phases in children and adult patients.[5] The Benznidazole Evaluation For Interrupting Trypanosomiasis (BENEFIT) randomized trial involving patients with severe cardiomyopathy showed that benznidazole had a trypanocidal effect, as indicated by decreased parasitic loads, but this effect was not associated with improved clinical outcomes.[6] In another randomized trial with benznidazole, Treatment in Adult Patients (TRAENA), similar trypanocidal effects and decreased levels of *T. cruzi*-specific IgG were observed, without modifications of clinical events.[7] One of the main disadvantages of benznidazole pharmacotherapy is the high doses commonly administered, which usually lead to more pronounced undesirable side effects[8] and in many cases the treatment is discontinued.[9] Additionally, in experimental murine models, the administration of lower doses of benznidazole alone or combined with other drugs,[10,11] as well as the intermittent administration of benznidazole, which was first reported by Bustamante *et al.*[12] and then by our laboratory,[11] supports the hypothesis that treatment with lower benznidazole doses might be as effective as the conventional doses of benznidazole used.[13]

The bioavailability of benznidazole is limited due to its low aqueous solubility (0.4 mg/mL) and dissolution rate. In this regard,

several approaches have been carried out to manufacture novel formulations of benznidazole with more appropriate physicochemical properties, including solid dispersions,[14–16] cyclodextrin complexation,[17,18] microencapsulation[19] and cosolvency.[20] In the last decade, the reduction in particle size to the nanometric scale has become an attractive alternative for the production of medicines with better pharmacotherapeutic performance.[21] Recently, Eudragrit®-based benznidazole nanoparticles (200–300 nm) were obtained by means of the nanoprecipitation process and showed an increased drug dissolution rate compared with the raw drug.[22] These benznidazole nanoparticles also displayed parasiticidal effects *in vitro* and in murine acute infections.[23,24]

The aim of this study was to evaluate the effectiveness of benznidazole nanoparticles (benznidazole-NP) administered continuously or intermittently to mice with chronic infections with the *T. cruzi* Nicaragua (*Tc*N) isolate and to compare benznidazole-NP with the lowest doses of benznidazole that were effective in our previous studies.[11]

Materials and methods

Ethics

All procedures involving animals followed the rules of the ethical legislation and regulatory entities established in Argentina and were approved by the Bioethics Committee of the National Institute of Parasitology "Dr. Mario Fatala Chaben" (Register RENIS No. 000028). The international recommendations for the use of laboratory animals (World Medical Association in the Declaration of Helsinki) were also followed.

Materials

Benznidazole (lot 9978 A; Laboratorios Elea, Buenos Aires, Argentina) was provided by the Instituto Nacional de Parasitología "Dr. Mario Fatala Chaben", Administración Nacional de Laboratorios e Institutos de Salud "Dr. Carlos G. Malbrán", Ministerio de Salud de la Nación (INP-ANLIS, Buenos Aires, Argentina) and Lutrol® F-68 (P188) was donated by BASF SE (Ludwigshafen, Germany). FBS was purchased from Natocor (Córdoba, Argentina) and horse serum was obtained from Internegocios SA (Córdoba, Argentina). Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma Chemical Co. (St Louis, MO, USA). African green monkey kidney epithelial cells (Vero cells) were obtained from Asociación Banco Argentino de Células (ABAC, Pergamino, Argentina). Cyclophosphamide was from Microsules Argentina (Buenos Aires, Argentina). All other reagents and chemicals used for analytical purposes were of chromatography grade.

Preparation of benznidazole-NP

The preparation of benznidazole-NP, using P188 as a stabilizer, was carried out according to our previous work.[23] Following nanoprecipitation methodology, small particles in a size range of 61–65 nm with a zeta potential of −18.30 ±1.0 mV and a size distribution (polydispersity index) of 3.35 ±0.1 were obtained.

Parasites

Trypomastigotes of the *Tc*N parasite isolate previously characterized in our laboratory were obtained from cell cultures using Vero cells.[25]

Animal model

Five groups ($n = 8$–10 per group) of 1-month-old female C57BL/6J mice of similar weight (mean ∓ SEM 20.8±1.5 g), were intraperitoneally inoculated with 3000 culture-derived trypomastigotes of the *Tc*N isolate. At 90 days post-infection (p.i.), the chronic phase was reached, characterized by a clear serological response and active chronic myocarditis with sclerotic sequelae and by other intramyocardial perivascular lesions[11] and, at that point, *T. cruzi*-infected mice were randomly divided into the following groups: (1) untreated (olive oil only); (2) treated with 30 daily doses of benznidazole-NP at 50 mg/kg/day (benznidazole-NPc 50); (3) treated with 30 daily doses of benznidazole-NP at 25 mg/kg/day (benznidazole-NPc 25); (4) treated with 13 intermittent doses of benznidazole-NP at 75 mg/kg of (benznidazole-NPit 75); and (5) treated with 13 intermittent doses of benznidazole-NP at 50 mg/kg (benznidazole-NPit 50). The intermittent treatment (groups 4 and 5) consisted of one dose every 7 days according to our previous study.[11] The schedules of all treatments are shown in Figure 1 and the total doses administered in each schedule are shown in Table 1. Benznidazole-NP doses were dispersed in olive oil and administered to mice by oral gavage using a top-cut tip, whereas the control group received a mock treatment with olive oil alone. *T. cruzi*-infected mice that were treated in the chronic phase at 90 days p.i. and untreated mice were euthanized after 7 months of follow-up (Figure 1). The findings of these treatment schemes with benznidazole-NP in this experimental model were compared with our previous results using the same infection model with benznidazole treatment.[11]

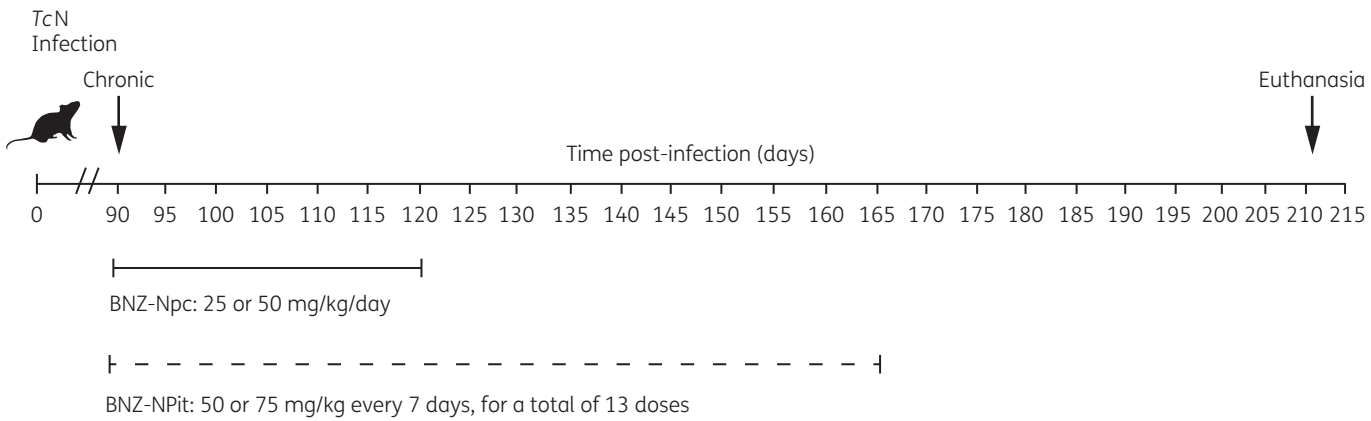

Figure 1. Treatment schedules for benznidazole-NP (BNZ-NP) in the experimental murine model of chronic *T. cruzi* infection with *Tc*N. Continuous treatment (BNZ-NPc) with 25 or 50 mg/kg/day for 30 days (solid line) and intermittent treatment (BNZ-NPit) with 13 doses of 50 or 75 mg/kg at 7 day intervals for a total of 91 days (dotted line).

Table 1. Single and total doses of benznidazole administered in each treatment scheme

Treatment schedule	Single dose[a]	Total dose
Continuous[b]	25 mg/kg/day	750 mg/kg
	50 mg/kg/day	1500 mg/kg
Intermittent[c]	50 mg/kg/day	650 mg/kg
	75 mg/kg/day	975 mg/kg

[a]The total amount of benznidazole administered within the nanoformulation was calculated as previously reported.[23]
[b]Continuous administration consisted of 30 daily doses of benznidazole-NP.
[c]Intermittent administration consisted of one dose every 7 days for a total of 13 doses.

The animals were obtained from the bioterium at the National Institute of Parasitology "Dr. Mario Fatala Chaben", ANLIS "Dr. Carlos G. Malbrán", Buenos Aires, Argentina, under specific pathogen-free conditions.

Mice were housed and assayed in a controlled room at a temperature of 22°C under a circadian light cycle of 12 h light/12 h dark, with water and food *ad libitum*, and were infected and randomly assigned to the treatment groups. All the animals were monitored for physical distress twice daily. Physical adverse events that were monitored included inability to move, reduced appetite, extreme pallor, ruffled hair and body weight loss (16%). Animals were euthanized if they exhibited any sign of a clinical adverse event that appeared to be highly stressful.

Induction of immunosuppression

A group of mice ($n = 6$) treated with benznidazole-NPit 75 were immunosuppressed with cyclophosphamide 30 days post-treatment. The immunosuppression protocol consisted of three cycles of 50 mg of cyclophosphamide/kg of body weight, for 4 consecutive days, with an interval of 3 days between each cycle.[26] After the last cycle of cyclophosphamide treatment, parasitaemia was evaluated in fresh blood collected from the tail of each mouse for 10 days and the number of parasites was estimated as described by Brener[27] and by quantitative PCR (qPCR).

Parasitaemia detected by DNA amplification

A volume of blood, collected from euthanized *T. cruzi*-infected and uninfected mice between 6 and 7 months p.i. ($n = 5$–9 samples per treatment), after the completion of drug treatment, was mixed with an equal volume of 6 M guanidine hydrochloride and 0.1 M EDTA at pH 8 and then kept at room temperature for 1 week, followed by storage at 4°C until use. DNA was isolated from 0.3 mL of the guanidine/EDTA buffer mixture using a commercial kit (High Pure PCR Template Preparation Kit, Roche) and then eluted in 0.1 mL according to the manufacturer's protocol. Thereafter, DNA amplification was performed; the samples were run in duplicate using a commercial kit (SYBR® GreenER® qPCR SuperMix Universal; Invitrogen, Life Technologies, USA) as previously described.[28] Epimastigotes of the *Tc*N isolate were used as a standard in artificially spiked mouse blood. The calibration curve, negative samples and non-template DNA control were included in every run. The cut-off value was determined to be equal to 0.14 parasite equivalents (Eq)/mL. Samples below the cut-off value were considered negative.

ECGs

ECGs were performed with an electrocardiograph Cardimax FX-2111 to assess cardiac electrical alterations in mice from all the experimental groups while under anaesthesia (Avertin, Sigma).[29] The heart rate (HR), atrioventricular node conduction time (PR interval), QRS and QT intervals in the ECGs were determined using ImageJ software.

Measurement of antibody response

Blood from benznidazole-treated and untreated *T. cruzi*-infected mice ($n = 5$–9 samples per treatment) was collected from the orbital venous sinus (500 μL). Serum samples were analysed for IgG antibody levels by ELISA. A lysate from *T. cruzi* Tulahuen strain epimastigotes (20 μg/mL) was used as the source of antigen. Briefly, flat-bottomed 96-well plates were coated overnight at 4°C with 50 μL/well of antigen diluted in carbonate buffer at pH 9.6. Plates were blocked for 1 h at room temperature with 100 μL/well of 5% skimmed milk in PBS. After washing three times with PBS/0.05% Tween 20 (PBS-T), serum samples were added to the plates (1:50–1:400 dilutions, 50 μL/well) for 30 min at 37°C. After washing with PBS-T, 50 μL/well of horseradish peroxidase-labelled goat anti-mouse IgG (Jackson) was added for 30 min at room temperature. The reaction was developed with 50 μL/well of *o*-phenylenediamine dihydrochloride (OPD) and stopped with 1 M sulphuric acid. The OD was read at 490 nm with an ELISA microplate reader (MINDRAY ME-96A). A cut-off value for antibody levels was set as the mean + 2 SD of the OD obtained from the sera of uninfected control mice, which was 0.08.[24]

Histopathological studies

Chronically infected mice were euthanized after 6–7 months of follow-up ($n = 5$–9 per treatment group), following the completion of treatment. Hearts were removed from treated and untreated *T. cruzi*-infected animals, fixed in a 10% formaldehyde solution and embedded in paraffin. Tissue sections (5 μm) were stained with haematoxylin–eosin (H&E) and Masson's trichrome collagen stain and evaluated by light microscopy, recording the number of parasite nests, the extent of mononuclear infiltrates and fibrosis. The degree of the infection was evaluated as previously described.[30,31] Briefly, eight different areas of the heart were evaluated (left and right atria, upper and lower halves of each ventricular wall and septum). The presence of inflammatory cells was scored as follows: 0, absent/none; 1, focal or mild myocarditis with only one focus; 2, moderate with multiple inflammatory foci; 3, extensive with inflammatory foci or disseminated inflammation with minimal 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 observed by microscopy: 0, absent/mild; 1, small and less than four isolated foci of fibrosis; 2, moderate and diffuse connective tissue that partially compromised the wall; 3, severe and diffuse connective tissue that compromised the whole wall. A numeric sum for each heart section represented the inflammation or fibrosis index.

IFN-γ ELISA, *T. cruzi* amastigote lysate and splenocytes

Splenocytes isolated from the spleens of the different groups of mice (untreated and benznidazole-treated *Tc*N-infected mice) were examined for the number of *T. cruzi*-specific IFN-γ-producing T cells by ELISpot assays for IFN-γ using a commercial kit (BD Biosciences) according to the instructions of the manufacturer. Splenocytes were seeded into wells in triplicate at a concentration of 4×10^5 cells/well in RPMI medium with 10% FBS and stimulated with 10 μg/mL of a *T. cruzi* lysate preparation, 20 ng/mL of PMA (Sigma) plus 500 ng/mL ionomycin (Sigma) (positive control) or medium alone (negative control). The number of specific IFN-γ-secreting cells was calculated by subtracting the value of the wells containing media alone from the value of the lysate-stimulated wells.[32] For the *T. cruzi* amastigote-enriched lysate preparation, *Tc*N trypomastigotes were cultured overnight in DMEM (Mediatech; pH 5.0) to transform trypomastigotes into amastigotes. Amastigotes were then washed in cold PBS and frozen at −20°C until several parasite pellets had been obtained and pooled into a small volume

of PBS. Thereafter, the sample was subjected to four freeze/thaw cycles at −70°C, followed by sonication. The supernatant following centrifugation at 12 000 **g** was collected and filter-sterilized and the protein concentration was determined. Splenocytes were isolated from spleens and cryopreserved using 80% FBS plus 20% DMSO in liquid nitrogen until use.

Statistical analysis

The normality of the variable distribution was assessed by using the Shapiro–Wilk normality test. Differences among groups were evaluated by analysis of variance (ANOVA) or Kruskal–Wallis test as appropriate, followed by Bonferroni or Dunn's multiple comparison tests. Statistical significance was considered at $P < 0.05$ (two-tailed). All experiments were performed blind. Data analyses and graphs were performed and generated, respectively, with GraphPad Prism 7.0 software.

Results

Course of infection and quantified parasite load in drug-treated TcN-infected mice

All *T. cruzi*-infected mice, untreated and treated with benznidazole-NP, survived until the end of the experiment and were euthanized (210 days p.i.) (Figure S1, available as Supplementary data at *JAC* Online) and no changes were observed in their general condition or behaviour. The treatment efficacy of both benznidazole-NP schemes against *Tc*N infection was assessed by qPCR. Untreated C57BL/6J mice infected with *Tc*N had a median parasite load of 8.9 Eq/mL at 210 days p.i. Of the group of mice treated with benznidazole-NPit 50, 80% (4/5 animals) exhibited negative qPCR results, whereas no parasite load could be detected in any of the animals for the other benznidazole-NP treatment schedules (Figure 2). With respect to the benznidazole treatment schemes, the benznidazole-c 75 (BNZc 75) group presented 80% (8/10 animals) without parasitaemia and the benznidazole-it 100 (BNZit 100) group presented 75% (6/8 animals) (Figure 2).

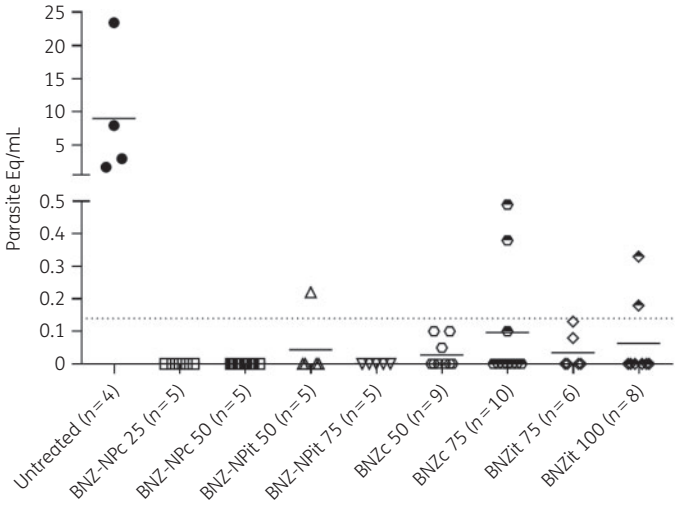

Figure 2. Parasite load in *T. cruzi*-infected mice treated with benznidazole-NP. Animals were treated with a daily dose of 25 or 50 mg/kg/day of benznidazole-NP (BNZ-NPc) for a total of 30 doses or with 13 doses of 50 or 75 mg/kg of benznidazole-NP (BNZ-NPit) administered at 7 day intervals for a total of 91 days. Data for animals treated with a daily dose of 50 or 75 mg/kg/day of benznidazole (BNZc) for a total of 30 doses or with 13 doses of 75 or 100 mg/kg of benznidazole (BNZit) administered at 7 day intervals for a total of 91 days are from Rial et al., 2019.[11] Benznidazole-c 50 (BNZc 50), benznidazole-c 75 (BNZc 75), benznidazole-it 75 (BNZit 75) and benznidazole-it 100 (BNZit 100).

T. cruzi-specific humoral immune responses following chemotherapy

T. cruzi-specific antibody levels were decreased in all the treatment schedules compared with the untreated animals (Figure 3). Benznidazole-NPit was more effective at reducing parasite-specific antibody levels compared with benznidazole-NPc, with comparable efficacy to that previously reported for benznidazole-c 75 and benznidazole-it 100 (Figure 3).[11] On an individual basis, all the animals in the benznidazole-NPit 50-treated group and 8/9 (89%) animals in the benznidazole-NPit 75-treated group had *T. cruzi*-specific antibody levels equal to or below the cut-off value.

ECGs

ECG parameters, such as the HR, PR, QRS and QT intervals, in uninfected and *T. cruzi*-infected untreated and treated mice were evaluated at 6–7 months p.i. Untreated *T. cruzi*-infected animals presented a significant decrease in HR, along with an increase in the atrioventricular nodule conduction time (PR) compared with the uninfected mice (Figures 4a and b and 5c and d) as previously reported in other mouse models.[31,33] These conduction alterations produced by *T. cruzi* and evidenced by HR and PR were reversed after both continuous and intermittent benznidazole-NP administration (Figures 4a and b and 5e–h), resembling the ECGs of healthy mice (Figure 5a and b). For HR, the most effective treatment was benznidazole-NPc 50 (Figure 4a); for PR, benznidazole-NPc 25 and benznidazole-NPit 50 were the most effective with respect to the untreated group (Figure 4b). Benznidazole-NP was as effective as benznidazole at all of the doses assessed.

Histopathological studies

A significant reduction in mononuclear cell infiltrates and connective tissue patches of fibrosis was observed in *T. cruzi*-infected animals after administration of benznidazole-NPc 50 compared with untreated *T. cruzi*-infected animals (Figures 6a and 7a). Although not statistically significant, a reduction in inflammation was also observed with benznidazole-NPit 50 and benznidazole-NPit 75 (Figures 6a and 7c–e). Benznidazole-NPc 50 was as effective as benznidazole-c 75 and benznidazole-it 100.

Intermittent administration of benznidazole-NP, either with the 50 or 75 mg/kg dose, showed the highest reduction in fibrosis compared with the untreated animals (Figures 6b and 8d and e). Benznidazole-NPit 75 was more effective than benznidazole-NPc 50, but comparable to benznidazole-it 100 (Figures 6b and 8c and e).

Decrease in IFN-γ-producing splenocytes in response to T. cruzi in mice treated with benznidazole-NP

Splenocytes of mice chronically infected with *Tc*N showed IFN-γ production in response to *T. cruzi* antigens (Figure 9). *T. cruzi*-specific T cell responses decreased significantly after all treatments, although this was more evident after intermittent

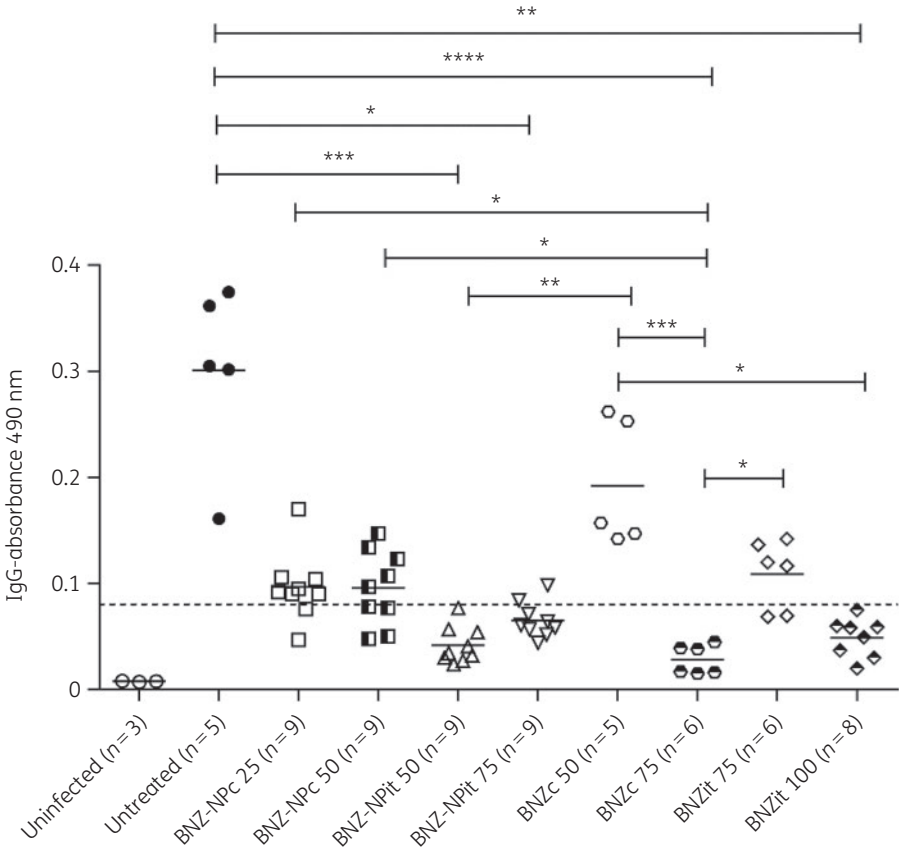

Figure 3. *T. cruzi*-specific antibody levels in serum samples from *Tc*N-infected treated and untreated mice after 6–7 months of follow-up. The cut-off value for antibody levels, as described in the Materials and methods section, is represented by the horizontal dashed line. Each symbol represents an individual mouse. **P* < 0.05, ***P* < 0.001, ****P* < 0.001, *****P*<0.0001 as determined by the Kruskal–Wallis test. Data for BNZc 50, BNZc 75, BNZit 75 and BNZit 100 are from Rial *et al*., 2019.[11]

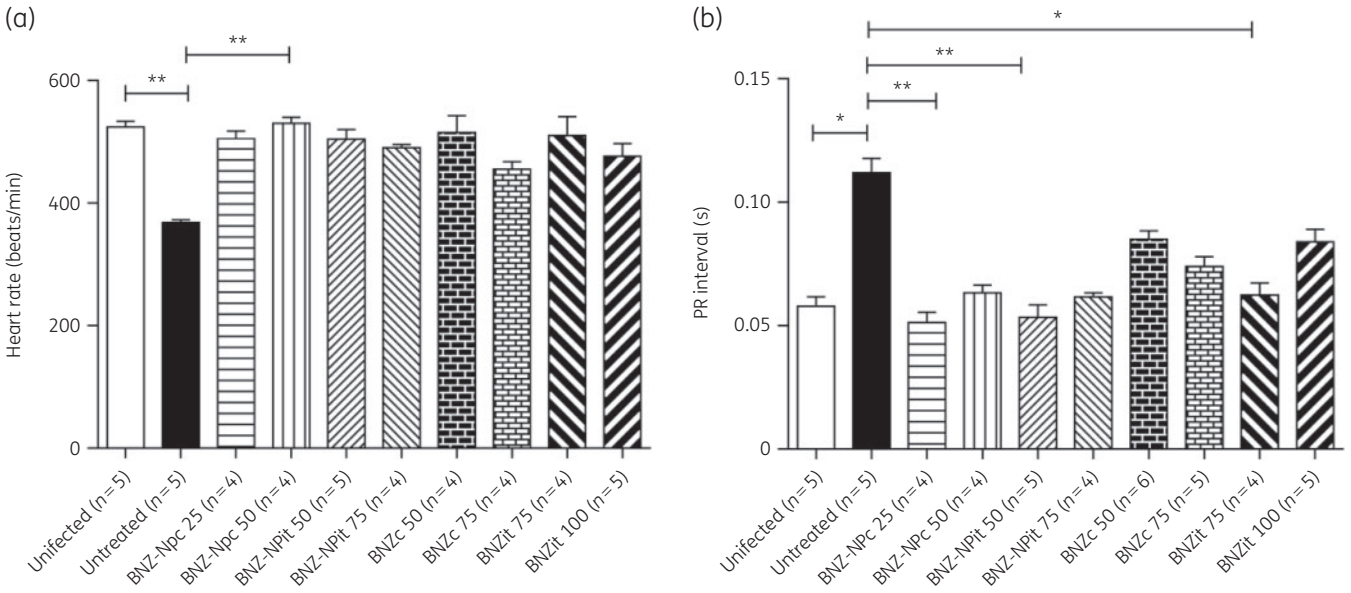

Figure 4. Electrocardiographic evaluation of *Tc*N-infected mice treated with benznidazole-NP. ECGs were conducted in benznidazole-NP-treated and untreated animals, as described in the Materials and methods section, after 6–7 months of follow-up. HR (a) and PR interval (b). **P* < 0.05, ***P* < 0.001, analysed by the Kruskal–Wallis test. Data for BNZc 50, BNZc 75, BNZit 75 and BNZit 100 are from Rial *et al*., 2019.[11]

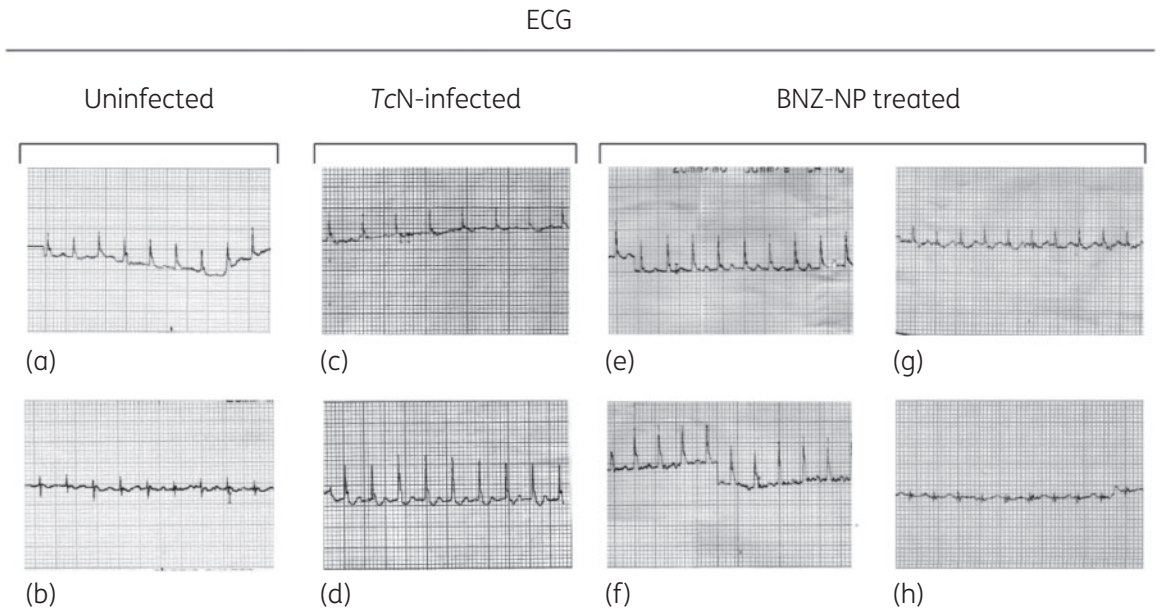

Figure 5. Representative ECGs of C57BL/6J *T. cruzi*-infected mice following treatment with benznidazole-NP. Panels (a) and (b), normal ECGs of healthy mice; panel (c), ECG of untreated infected mice with decreased HR; panel (d), ECG untreated infected mice with increased PR interval; panels (e) and (f), ECGs of mice treated with benznidazole-NPc 50 or with benznidazole-NPc 25 mg/kg/day, respectively; and panels (g) and (h), ECGs of mice treated with benznidazole-NPit 50 mg/kg. Panels e–h show that the abnormalities were reversed following drug treatment.

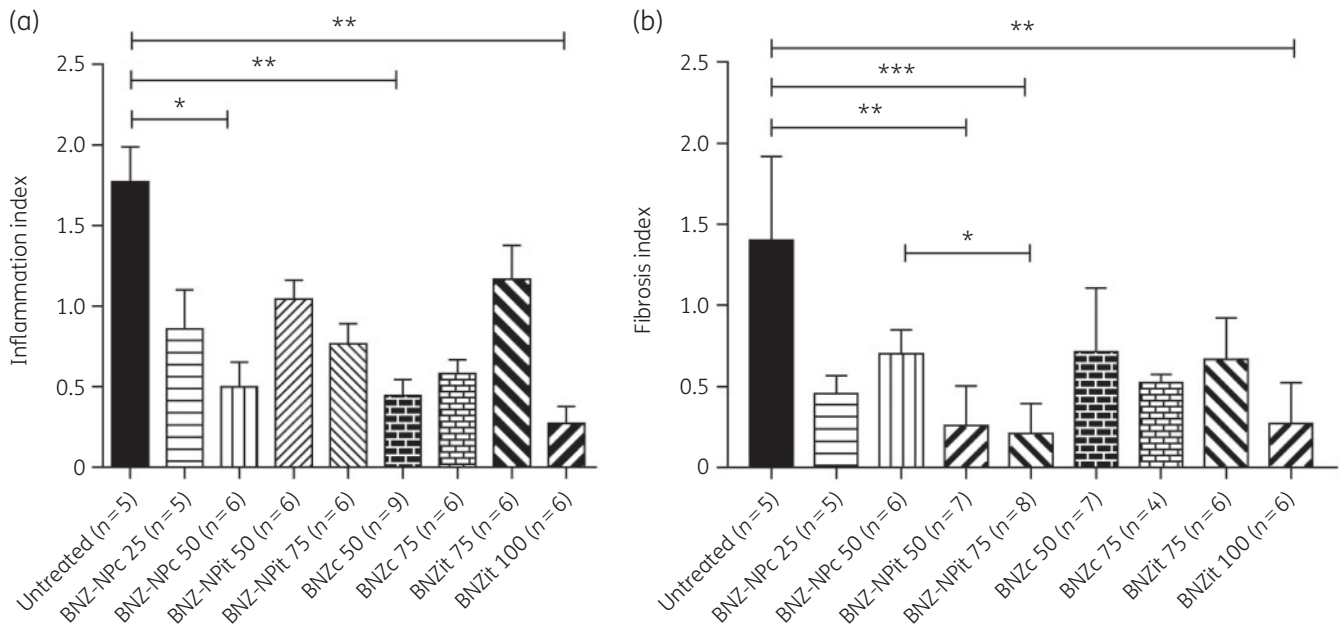

Figure 6. Evaluation of inflammation and fibrosis in chronic *T. cruzi*-infected mice treated with benznidazole and benznidazole-NP. Data represent morphometric quantification of inflammatory cells in heart tissues by staining with H&E (a) and morphometric quantification of fibrosis stained with Masson's trichrome collagen stain (b). Animals were treated every day with benznidazole-NPc at 25 or 50 mg/kg/day for a period of 30 days or treated intermittently with benznidazole-NPit at 50 or 75 mg/kg every 7 days for a total of 13 doses. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, analysed by the Kruskal–Wallis test. Data for BNZc 50, BNZc 75, BNZit 75 and BNZit 100 are from Rial *et al*., 2019.[11]

administration of both benznidazole-NP and benznidazole drugs. Benznidazole-NPit 75 was more effective at reducing the frequency of IFN-γ-producing cells compared with benznidazole-NPc 25.

Quantified parasite load in immunosuppressed mice

We next evaluated the reactivation of parasites by qPCR after immunosuppression in the mice treated with benznidazole-NPit 75, which was shown to be potentially the most effective treatment.

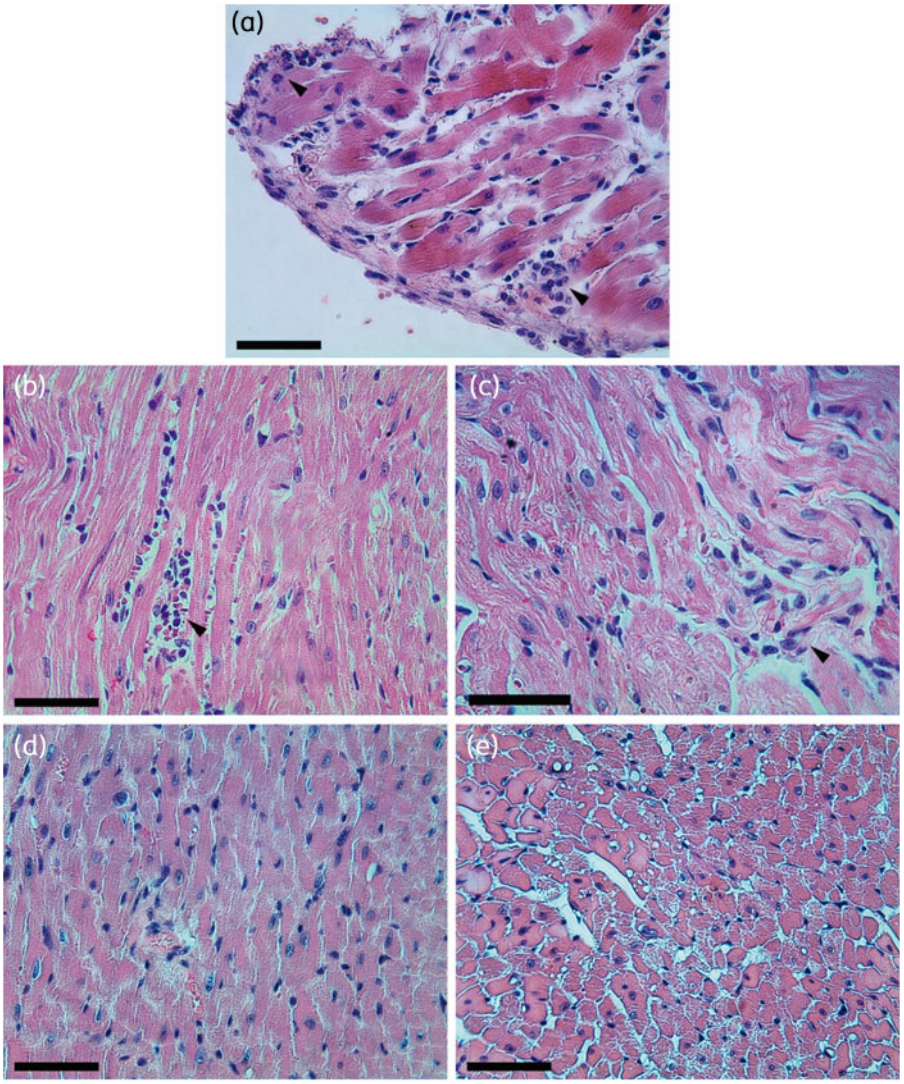

Figure 7. H&E staining of heart tissues from mice chronically infected with *T. cruzi* and treated with benznidazole-NP. Ventricles of: untreated infected mice (a); infected mice treated with benznidazole-NPc at 25 mg/kg/day (b); infected mice treated with benznidazole-NPc at 50 mg/kg/day (c); infected mice treated with benznidazole-NPit at 50 mg/kg (d); and infected mice treated with benznidazole-NPit at 75 mg/kg (e). Arrowheads indicate the inflammatory cell foci (a and b) and isolated mononuclear cells (c). Magnification: 40×; scale bars: 25 μm. This figure appears in colour in the online version of *JAC* and in black and white in the printed version of *JAC*.

The parasite load in blood from the abovementioned group of mice was undetectable after immunosuppression.

Discussion

In previous studies, we have shown the antiparasitic activity of polymeric nanoparticles of benznidazole in acute infection with the *Tc*N isolate in mice.[23,24]

Here we evaluated the effect of treatment with benznidazole-NP administered in daily doses or every 7 days in comparison with the effect of standard benznidazole in a mouse model of chronic *T. cruzi* infection.[11] The use of this chronic model, the design of the different treatment schemes, the number of animals used and the data reporting followed the ARRIVE guidelines.[34] Both treatment schemes with benznidazole-NP, either continuously or intermittently administered, at 75 mg/kg/day or 100 mg/kg every 7 days, respectively, were as effective as the standard benznidazole treatment,[11] with all mice surviving until euthanasia at the end of the experiment. Remarkably, intermittent administration of 75 mg/kg of benznidazole-NP was equally as effective as the intermittent administration of benznidazole at 100 mg/kg, which was the lowest dose of benznidazole effective in our previous studies.[11] This was reflected by the parasite load, the levels of *T. cruzi*-specific antibodies, the degree of fibrosis and the frequency of IFN-γ-producing cells. However, a 25% reduction in dose was achieved compared with that used in our former study and this was an approximately 70% reduction of total dose compared with the standard dose of 100 mg/kg/day for 30 days generally used in mouse models of *T. cruzi* infection.[35,36] This is likely due to the smaller size of these nanoparticles compared with the standard

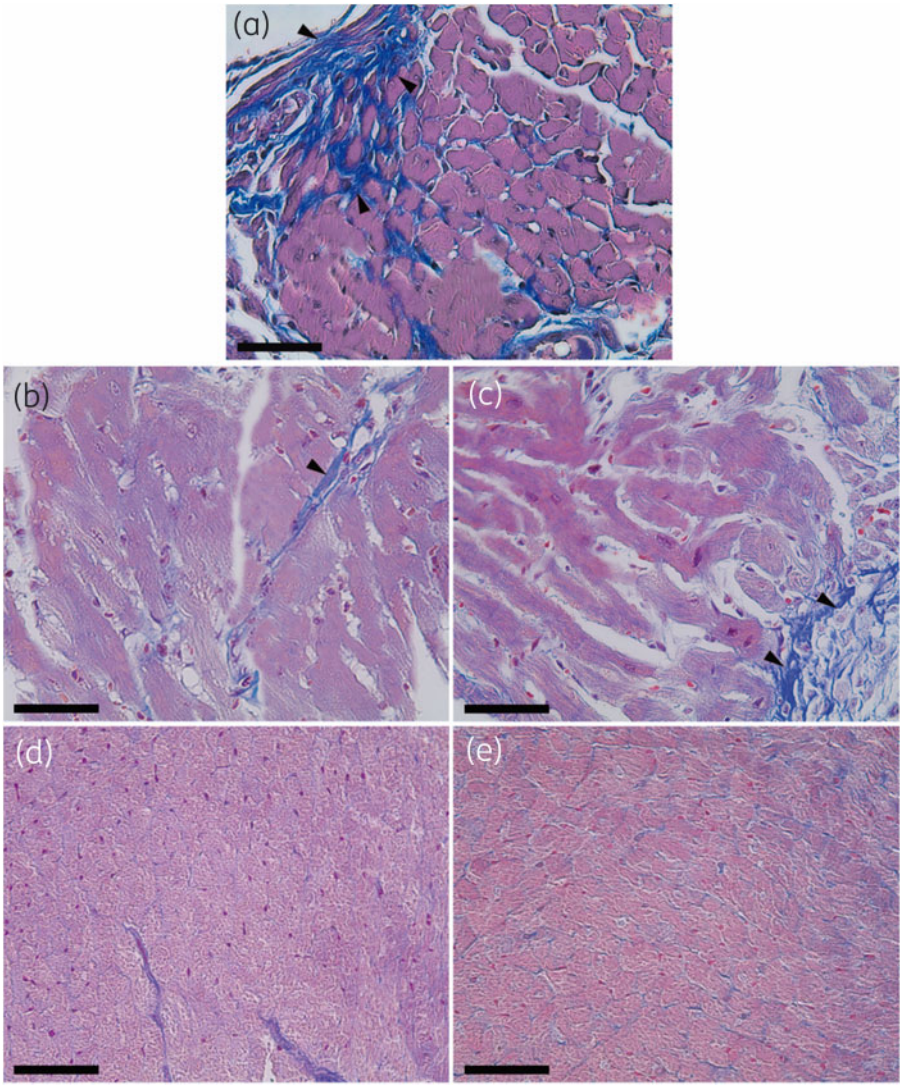

Figure 8. Masson's trichome collagen staining of heart tissues from mice chronically infected with *T. cruzi* and treated with benznidazole-NP. Ventricles of: untreated infected mice (a); infected mice treated with benznidazole-NPc at 25 mg/kg/day (b); infected mice treated with benznidazole-NPc at 50 mg/kg/day (c); infected mice treated with benznidazole-NPit at 50 mg/kg (d); and infected mice treated with benznidazole-NPit at 75 mg/kg (e). Arrowheads indicate patches of fibrosis (a–c). Magnification: 40×; scale bars: 25 μm. This figure appears in colour in the online version of *JAC* and in black and white in the printed version of *JAC*.

benznidazole, which increases their solubility from 0.4 mg/mL to 3.99 mg/mL.[22] However, benznidazole-NP and standard benznidazole were equally effective at improving the electrocardiographic alterations induced by the infection.

The benznidazole-NPit 50 and benznidazole-NPit 75 treatments induced a 42% and 57% reduction in inflammation, respectively, contrasting with the more significant reduction obtained with benznidazole-it 100 compared with the untreated mice.[11] However, this decrease in inflammation with the nanoparticle treatments seems to be a sufficient condition to inhibit fibrosis.

Several studies have shown the effectiveness of drug nanoformulations in other experimental models. In an experimental infection with toxoplasma, nanoformulations of lopinavir/ritonavir revealed the same level of effectiveness, but with a decreased effective dose of lopinavir/ritonavir.[37] Pandey and Khuller[38] showed that oral doses of streptomycin as nanoparticles administered to mice infected with *Mycobacterium tuberculosis* had an efficient therapeutic action equivalent to that produced with a triple administration of the drug in injectable form. Even though there are no available data on nanoformulated drugs in clinical trials for human infections yet, they appear to be promising alternatives, with potential benefits including the reduction in the duration of treatment and the appearance of adverse events. In this study, we observed that some doses worked better, based on the approaches and methods used in this experimental model to evaluate the effectiveness of the drug. With respect to the parasite load, parasite DNA was undetectable following treatment with benznidazole-NP in all experimental groups, with the exception of one mouse in the benznidazole-NPit 50 group that had detectable, but low parasitaemia. Nonetheless, the amount of total

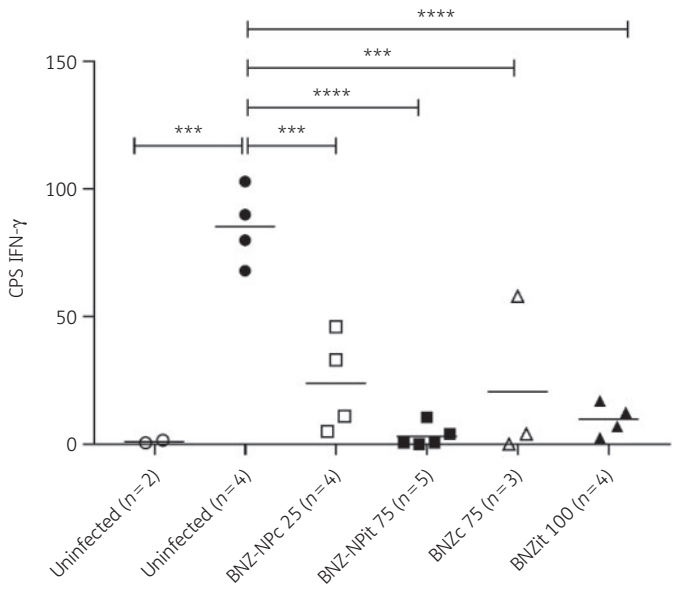

Figure 9. IFN-γ-producing T lymphocytes specific for *T. cruzi*. The number of IFN-γ-producing splenocytes was determined by ELISpot after stimulation with a *T. cruzi* parasite lysate. Each symbol represents the number of IFN-γ cells producing spots (CPS IFN-γ) in response to *T. cruzi* antigens for each mouse. ***$P < 0.001$, ****$P < 0.0001$. Data were analysed by ANOVA followed by Dunn's multiple comparison tests.

benznidazole drug used in the benznidazole-NPit 50 treatment was lower than the benznidazole-c 50 and benznidazole-NPit 75 treatment schemes. The effectiveness of intermittently administered treatment with benznidazole-NP was also demonstrated by the rate of anti-*T. cruzi* specific antibody titres below the cut-off and the reduction in *T. cruzi*-specific IFN-γ-producing cells, which might be the result of a lower parasite load, as observed in patients with chronic Chagas' disease treated with benznidazole[39,40] and also by the absence of circulating parasites after immunosuppression of mice.

We might speculate that the intermittent administration of benznidazole-NP might eventually allow for treatments over longer periods with fewer side effects, which may be effective against the dormant form of this parasite. This is particularly important since it was demonstrated that the intracellular form of *T. cruzi* has the ability to establish dormancy,[41] which might be a factor for drug resistance to benznidazole.

The fact that treatment with low doses of benznidazole was reported to be highly effective in decreasing the parasite burden in *T. cruzi*-infected mice and that different studies in humans have demonstrated that patients are being treated with an overdose of benznidazole supports the claim that benznidazole doses and treatment schemes should be re-evaluated through new clinical trials.[11,42,43] In this regard, alternative treatment schemes for benznidazole administration have been assessed in mice.[12,36,44] A pilot study in patients with chronic Chagas' disease treated with intermittent doses of benznidazole showed low percentages of detectable parasite loads, along with a reduction in the rate of treatment suspension due to adverse events.[45]

It is known that the half-life of benznidazole is approximately 12 h, hence the reason for giving benznidazole twice a day in humans; however, in mice it is 1–2 h.[12] The initial studies presumed that the effectiveness of benznidazole depended on maintaining circulating levels above the MIC. However, the effectiveness observed with intermittent treatment suggests that it is more likely it acts through a mechanism of maximum concentration.[12] Medications that work through this mechanism have prolonged post-antibiotic effects, which affect the growth of the pathogen after complete elimination of the compound.[46] The side effects of benznidazole treatment could be eliminated with a reduction in the cumulative dose of benznidazole.[47]

In conclusion, both daily and intermittent treatment schemes with benznidazole-NP represent a promising therapeutic approach for Chagas' disease. In particular, intermittent administration of benznidazole-NP could well be an attractive alternative scheme for clinical trials in Chagas' disease patients, with the possible reduction of adverse effects.

Acknowledgements

We thank the animal facility staff of the Instituto Nacional de Parasitología "Dr. Mario Fatala Chaben" (INP-ANLIS): Gabriela Barja, Laura Potenza and Leticia Orellana. We are also very grateful to Claudia Nose for her excellent technical assistance. We greatly appreciate Dr Karina Gómez from the Instituto de Investigaciones en Ingeniería Genética y Biología Molecular, INGEBI, CONICET, for allowing the use of the Cardimax FX-2111 electrocardiograph equipment to perform the ECGs. We are very grateful to INP-ANLIS (Argentina), CONICET (Argentina), Agencia Santafesina de Ciencia, Tecnología e Innovación (ASACTEI, Santa Fe, Argentina) and the Universidad Nacional de Rosario (Santa Fe, Argentina) for their financial support. E.C.A. and M.A.N. acknowledge CONICET for fellowships. Part of the results shown in this manuscript were presented as posters at the XXX Annual Meeting of the Argentine Society of Protozoology held in Resistencia, Chaco, Argentina, 1–4 November 2018 (DyT 20: 'Efectos de nanoformulaciones de benznidazol sobre *Trypanosoma cruzi* y sobre la progresión de la patología cardíaca en la infección crónica murina') and at the Drug Discovery for Neglected Diseases International Congress, held in Buenos Aires, Argentina, 4–6 December 2018 (36: 'Experimental therapy using nanoformulations of benznidazole in mouse model of *Trypanosoma cruzi* Nicaragua chronic infection').

Funding

This work was partially supported by the Ministerio de Salud de la Nación, Argentina, by the Instituto Nacional de Parasitología (INP-ANLIS)[Grant Fondos Concursables ANLIS (FOCANLIS 2017) 1704 to M.S.R.] and by the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), [Grant Proyectos de Investigación Plurianuales (PIP) 0037 to S.A.L.].

Transparency declarations

None to declare.

Author contributions

L.E.F. and C.J.S. conceived and designed the study. M.S.R., E.C.A., M.I.E., M.A.N. and J.B. performed the experiments. L.E.F., M.S.R., J.B., M.A.N and N.G.P. analysed and interpreted the results. C.J.S., S.A.L., M.S.R. and L.E.F. wrote the first draft of the manuscript and supplied all test compounds. All authors read and approved the final manuscript.

Supplementary data

Figure S1 is available as Supplementary data at *JAC* Online.

References

1 Moncayo A, Silveira AC. Current epidemiological trends for Chagas disease in Latin America and future challenges in epidemiology, surveillance and health policy. *Mem Inst Oswaldo Cruz* 2009; **104**: 17–30.

2 Rassi A Jr, Rassi A, Marin-Neto JA. Chagas disease. *Lancet* 2010; **375**: 1388–402.

3 WHO. Chagas Disease (American Trypanosomiasis). 2017. Fact Sheet: 340. http://www.who.int/mediacentre/factsheets/fs340/en/.

4 Marin-Neto JA, Rassi A Jr, Morillo CA *et al*. Rationale and design of a randomized placebo-controlled trial assessing the effects of etiologic treatment in Chagas' cardiomyopathy: the Benznidazole Evaluation for Interrupting Trypanosomiasis (BENEFIT). *Am Heart J* 2008; **156**: 37–43.

5 Sosa Estani S, Segura EL, Ruiz AM *et al*. Efficacy of chemotherapy with benznidazole in children in the indeterminate phase of Chagas' disease. *Am J Trop Med Hyg* 1998; **59**: 526–9.

6 Morillo CA, Marin-Neto JA, Avezum A *et al*. Randomized trial of benznidazole for chronic Chagas' cardiomyopathy. *N Engl J Med* 2015; **373**: 1295–306.

7 Riarte A, Prado NG, De Rissio AM *et al*. TRAENA: benznidazole treatment in adult patients with low risk chronic Chagas disease—a phase 3 randomized clinical trial. *Plataform DNDi Newsl* 2016; **6**: 15–7.

8 Ferraz LRM, Alves AÉG, Nascimento D *et al*. Technological innovation strategies for the specific treatment of Chagas disease based on Benznidazole. *Acta Trop* 2018; **185**: 127–32.

9 Vinuesa T, Herráez R, Oliver L *et al*. Benznidazole nanoformulates: a chance to improve therapeutics for Chagas disease. *Am J Trop Med Hyg* 2017; **97**: 1469–76.

10 Grosso NL, Alarcon ML, Bua J *et al*. Combined treatment with benznidazole and allopurinol in mice infected with a virulent *Trypanosoma cruzi* isolate from Nicaragua. *Parasitology* 2013; **140**: 1225–33.

11 Rial MS, Scalise ML, López Alarcón M *et al*. Experimental combination therapy using low doses of benznidazole and allopurinol in mouse models of *Trypanosoma cruzi* chronic infection. *Parasitology* 2019; **146**: 305–13.

12 Bustamante JM, Craft JM, Crowe BD *et al*. New combined and reduced dosing treatment protocols cure *Trypanosoma cruzi* infection in mice. *J Infect Dis* 2014; **209**: 150–62.

13 Fernández ML, Marson ME, Ramirez JC *et al*. Pharmacokinetic and pharmacodynamic responses in adult patients with Chagas disease treated with a new formulation of benznidazole. *Mem Inst Oswaldo Cruz* 2016; **111**: 218–21.

14 Lima AA, Soares-Sobrinho JL, Silva JL *et al*. The use of solid dispersion systems in hydrophilic carriers to increase benzonidazole solubility. *J Pharm Sci* 2011; **100**: 2443–51.

15 Leonardi D, Salomon CJ. Unexpected performance of physical mixtures over solid dispersions on the dissolution behavior of benznidazole from tablets. *J Pharm Sci* 2013; **102**: 1016–23.

16 Fonseca-Berzal C, Palmeiro-Roldán R, Escario JA *et al*. Novel solid dispersions of benznidazole: preparation, dissolution profile and biological evaluation as alternative antichagasic drug delivery system. *Exp Parasitol* 2015; **149**: 84–91.

17 Leonardi D, Bombardiere ME, Salomon CJ. Effects of benznidazole: cyclodextrin complexes on the drug bioavailability upon oral administration to rats. *Int J Biol Macromol* 2013; **62**: 543–8.

18 Soares-Sobrinho JL, Santos FL, Lyra MA *et al*. Benznidazole drug delivery by binary and multicomponent inclusion complexes using cyclodextrins and polymers. *Carbohydr Polym* 2012; **89**: 323–30.

19 García MC, Martinelli M, Ponce NE *et al*. Multi-kinetic release of benznidazole-loaded multiparticulate drug delivery systems based on polymethacrylate interpolyelectrolyte complexes. *Eur J Pharm Sci* 2018; **120**: 107–22.

20 Santos Souza HF, Real D, Leonardi D *et al*. Development and *in vitro*/*in vivo* evaluation of a novel benznidazole liquid dosage form using a quality-by-design approach. *Trop Med Int Health* 2017; **22**: 1514–22.

21 Khalil NM, de Mattos AC, Carraro T *et al*. Nanotechnological strategies for the treatment of neglected diseases. *Curr Pharm Des* 2013; **19**: 7316–29.

22 Seremeta KP, Arrúa EC, Okulik NB *et al*. Development and characterization of benznidazole nano- and microparticles: a new tool for pediatric treatment of Chagas disease? *Colloids Surf B Biointerfaces* 2019; **177**: 169–77.

23 Scalise ML, Arrúa EC, Rial MS *et al*. Promising efficacy of benznidazole nanoparticles in acute *Trypanosoma cruzi* murine model: in-vitro and in-vivo studies. *Am J Trop Med Hyg* 2016; **95**: 388–93.

24 Rial MS, Scalise ML, Arrúa EC *et al*. Elucidating the impact of low doses of nano-formulated benznidazole in acute experimental Chagas disease. *PLoS Negl Trop Dis* 2017; **11**: e0006119.

25 Grosso N, Bua J, Perrone A *et al*. *Trypanosoma cruzi*: biological characterization of a isolate from an endemic area and its susceptibility to conventional drugs. *Exp Parasitol* 2010; **126**: 239–44.

26 Dos Santos FM, Caldas S, de Assis Cáu SB *et al*. *T. cruzi*: induction of benznidazole resistance *in vivo* and its modulation by *in vitro* culturing and mice infection. *Exp Parasitol* 2008; **120**: 385–90.

27 Brener Z. Therapeutic activity and criterion of cure on mice experimentally infected with *T. cruzi*. *Rev Inst Med Trop Sao Paulo* 1962; **4**: 389–96.

28 Bua J, Volta BJ, Perrone AE *et al*. How to improve the early diagnosis of *Trypanosoma cruzi* infection: relationship between validated conventional diagnosis and quantitative DNA amplification in congenitally infected children. *PLoS Negl Trop Dis* 2013; **7**: e2476.

29 Myung JK, Ji EL, Bermseok O. Validation of non-invasive method for electrocardiogram recording in mouse using lead II. *Biomed Sci Lett* 2015; **21**: 135–43.

30 Gupta S, Garg NJ. Prophylactic efficacy of TcVac2 against *Trypanosoma cruzi* in mice. *PLoS Negl Trop Dis* 2010; **4**: e797.

31 Postan M, Bailey JJ, Dvorak JA *et al*. Studies of *Trypanosoma cruzi* clones in inbred mice. III. Histopathological and electrocardiographical responses to chronic infection. *Am J Trop Med Hyg* 1987; **7**: 541–9.

32 Laucella SA, Postan M, Martin D *et al*. Frequency of interferon-γ-producing T cells specific for *Trypanosoma cruzi* inversely correlates with disease severity in chronic human Chagas disease. *J Infect Dis* 2004; **189**: 909–18.

33 Garcia S, Ramos CO, Senra JF *et al*. Treatment with benznidazole during the chronic phase of experimental Chagas' disease decreases cardiac alterations. *Antimicrob Agents Chemother* 2005; **49**: 1521–8.

34 Kilkenny C, Browne WJ, Cuthill IC *et al*. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *PLoS Biol* 2010; **8**: e1000412.

35 Diniz L de F, Urbina JA, de Andrade IM *et al*. Benznidazole and posaconazole in experimental Chagas disease: positive interaction in concomitant and sequential treatments. *PLoS Negl Trop Dis* 2013; **7**: e2367.

36 Mazzeti AL, Diniz L de F, Gonçalves KR *et al*. Synergic effect of allopurinol in combination with nitro-heterocyclic compounds against *Trypanosoma cruzi*. *Antimicrob Agents Chemother* 2019; **63**: e02264–18.

37 Abou-El-Naga IF, El Kerdany ED, Mady RF *et al*. The effect of lopinavir/ritonavir and lopinavir/ritonavir loaded PLGA nanoparticles on experimental toxoplasmosis. *Parasitol Int* 2017; **66**: 735–47.

38 Pandey R, Khuller GK. Nanoparticle-based oral drug delivery system for an injectable antibiotic—streptomycin. *Chemotherapy* 2007; **53**: 437–41.

39 Laucella SA, Mazliah DP, Bertocchi G *et al*. Changes in *Trypanosoma cruzi*-specific immune responses after treatment: surrogate markers of treatment efficacy. *Clin Infect Dis* 2009; **49**: 1675–84.

40 Albareda MC, Natale MA, De Rissio AM *et al*. Distinct treatment outcomes of antiparasitic therapy in *Trypanosoma cruzi*-infected children is associated with early changes in cytokines, chemokines, and T-cell phenotypes. *Front Immunol* 2018; **9**: 1–15.

41 Barrett MP, Kyle DE, Sibley LD *et al*. Protozoan persister-like cells and drug treatment failure. *Nat Rev Microbiol* 2019; **17**: 607–20.

42 Altcheh J, Moscatelli G, Mastrantonio G *et al*. Population pharmacokinetic study of benznidazole in pediatric Chagas disease suggests efficacy despite lower plasma concentrations than in adults. *PLoS Negl Trop Dis* 2014; **8**: e2907.

43 Soy D, Aldasoro E, Guerrero L *et al*. Population pharmacokinetics of benznidazole in adult patients with Chagas disease. *Antimicrob Agents Chemother* 2015; **59**: 3342–9.

44 Strauss M, Lo Presti MS, Bazán PC *et al*. Clomipramine and benznidazole association for the treatment of acute experimental *Trypanosoma cruzi* infection. *Parasitol Int* 2013; **62**: 293–9.

45 Álvarez MG, Hernández Y, Bertocchi G *et al*. New scheme of intermittent benznidazole administration in patients chronically infected with *Trypanosoma cruzi*: a pilot short-term follow-up study with adult patients. *Antimicrob Agents Chemother* 2016; **60**: 833–7.

46 Craig WA. Pharmacokinetic/pharmacodynamic parameters: rationale for antibacterial dosing of mice and men. *Clin Infect Dis* 1998; **26**: 1–12.

47 Viotti R, Vigliano C, Lococo B *et al*. Side effects of benznidazole as treatment in chronic Chagas disease: fears and realities. *Expert Rev Anti Infect Ther* 2009; **7**: 157–63.