Promising Efficacy of Benznidazole Nanoparticles in acute *Trypanosoma cruzi* Murine Model: In-Vitro and In-Vivo Studies

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Abstract. The aim of this study was to evaluate the effectiveness of benznidazole nanoparticles (BNZ-nps) on trypomastigote forms and on intracellular infection in mammalian cells and primary cardiac myocyte cells. Its effectiveness was also evaluated on acute *Trypanosoma cruzi* Nicaragua mice infection. Trypomastigotes from culture were treated with different concentrations of BNZ-nps to determine the drug concentration that lyses 50% of trypomastigotes (LC_{50}). Infected mammalian cells were incubated with different concentrations of BNZ-nps to determine the percentage of amastigote inhibition. C_3H/HeN mice with lethal acute infection were treated with 10, 25, and 50 mg/kg/day of BNZ-nps for 30 and 15 days to control the survival rate of animals. BNZ-nps having a mean particle size of 63.3 nm, a size distribution of 3.35, and a zeta potential of −18.30 were successfully prepared using poloxamer 188 as a stabilizer. BNZ-nps 25 and 50 μg/mL showed no significant differences in the percentage of inhibition of infected mammalian cells. Infected mice treated with BNZ-nps (50, 25, and 10 mg/kg/day) for 30 days and with BNZ-nps (50 and 25 mg/kg/day) for 15 days presented a 100% survival, whereas the animals treated with 10 mg/kg/day for 15 days of BNZ-nps showed a 70% survival rate. The results obtained demonstrate, for the first time, that benznidazole nanoparticles are a useful and attractive approach to treat Chagas disease in infected mice.

INTRODUCTION

Chagas disease, a neglected parasitic infection, is caused by the protozoan *Trypanosoma cruzi* and affects more than 10 million people, mainly in Latin America. Recently, as a consequence of migratory events, it has been disseminated to North America and Europe, becoming a serious health problem in non-endemic regions.[1] Benznidazole (BNZ) is one of the two available drugs prescribed to treat the circulating forms of the parasite in the acute phase of the infection, but its efficacy to treat the chronic stage of the disease is still controversial.[2,3] The treatment of Chagas disease should start immediately after positive diagnosis (acute phase). In this case, after 60 days of treatment, up to 80% of the patients can expect its remission.[4] However, chemotherapy with BNZ presents some drawbacks including the dosage regimen, long treatments, and often appearance of several side effects such as allergic dermatitis, peripheral neuropathy, and anorexia. BNZ exhibits low water solubility, which may produce low and/or variable bioavailability after oral administration. As a consequence, the search of novel BNZ delivery systems that could be effective for Chagas disease is still a major priority to control this neglected infection.[5] Nanotechnology is a convenient tool to improve the aqueous solubility and further bioavailability of hydrophobic drugs. Even though nanotechnology is widely applied to develop nanomedicines for several pathologies, including cancer, cardiovascular and neurodegenerative disorders, little is known about nanoformulations for the treatment of

Chagas disease.[6,7] Thus, the aim of this study was to evaluate the effectiveness of BNZ nanoparticles (BNZ-nps) on trypomastigotes forms and on intracellular infection in mammalian cells and primary cardiac myocyte cells as compared with raw BNZ (R-BNZ). Its effectiveness was also evaluated on acute *T. cruzi* Nicaragua (*Tc*N) mice infection.[8,9]

MATERIALS AND METHODS

Materials. BNZ (lot 9978 A; Laboratorios Elea, Buenos Aires, Argentina) was provided by Instituto Nacional de Parasitología, ANLIS Malbrán, Ministerio de Salud de la Nación, (Buenos Aires, Argentina). Lutrol® F-68 (P188) was donated by BASF SE (Ludwigshafen, Germany). The following compounds were used in this study: MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide purchased from Sigma Aldrich (Buchs SG, Switzerland); fetal bovine serum (FBS), Hanks' balanced salt solution (HBSS), and Dulbecco's modified Eagle's medium (DMEM) purchased from Gibco (Rockville, MD); horse serum purchased from Internegocios SA (Córdoba, Argentina); kidney epithelial cells of the African green monkey, Vero cells, obtained from ABAC (Pergamino, Argentina). All the other reagents and chemicals used for analytical purpose were of chromatography grade.

Preparation of BNZ-nps. BNZ-nps were prepared by a solvent diffusion method. BNZ (200 mg) was dissolved in ethanol (10 mL). The solution was injected (1 mL/minute) into water (20 mL) containing P188 (300 mg) under stirring (1,000 rpm/60 minutes). The resulting solution was stirred (500 rpm) for 18 hours at room temperature to allow solvent evaporation. Nanoparticles were then recovered by centrifugation for 20 minutes (15,000 rpm), washed twice with distilled water, and frozen overnight at −20°C and freeze-dried (48 hours).

Particle size and Zeta potential measurement. The particle size of BNZ-nps was determined by photon correlation spectroscopy using an SZ-100 Horiba equipment (HORIBA

Instruments Inc., Irvine, CA). The samples were prepared by 10-fold dilution of 1 mL of the nanoparticles with distilled water. Zeta potential was determined by the electrophoretic mobility of nanoparticles at 25°C, using an SZ-100 Horiba equipment. These determinations were carried out in triplicate.

Cell toxicity assay. The viability of the kidney epithelial cells of the African green monkey (Vero) cells was determined by reduction of yellow tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a blue crystalline formazan product (MTT assay).[10]

Cells (1 × 105) were seeded in a 96-well plate and were incubated over night at 37°C with increasing concentrations of BNZ-nps (10, 25, and 50 μg/mL). Next, MTT was added, and, to dissolve it, a solution of dimethylformamide and sodium dodecyl sulfate was added after 2–4 hours. Color developed was measured at 540 nm in a microplate reader (model 3550; Bio-Rad, Philadelphia, PA). Each experiment was done in triplicate.

Hemolytic assay. A 4% suspension of fresh defibrinated human blood was prepared in a sterile 5% glucose solution and treated with BNZ-nps (100, 50, 25, and 10 μg/mL) for 24 hours at 37°C.[11] After centrifugation, the supernatant absorbance was determined at 540 nm to assess percent hemolysis. R-BNZ was used as the hemolytic reference drug and Triton X-100 (Sigma Aldrich, St. Louis, MO) was used as the positive control.

Antitrypanosomal activity. Trypomastigotes were obtained from Vero cell culture. The assay was performed in a sterile 96-well microplate with 50,000 trypomastigotes per well. The cultures were then incubated at 37°C for 24 hours under a 5% CO_2 atmosphere with 90 μL of fresh DMEM medium supplemented with 20% FBS and 10% of mice blood, and with 10 μL of each dilution of the R-BNZ and BNZ-nps to obtain the desire concentration (5–100 μg/mL). The drug concentration at which 50% of the parasites were lysed (LC_{50}) was calculated by counting the cells according to the Brener method.[12] In addition, to detect any potential antitrypanosomal activity of the excipient, this study was performed using P188 alone. The assay was performed in duplicate for each of the three different experiments.

Preparation of ventricular myocytes. Ventricular myocytes were isolated from 5-day-old mice.[13] Heart fragments were dissociated with HBSS with 0.02% trypsin at 37°C for four cycles of 15 minutes each. The cells were resuspended in DMEM with 10% FBS and 10% horse serum and transferred to 12-mm coverslips contained in 24-well culture plates. These cultures were kept in a 5% CO_2 incubator at 37°C to allow the cells to adhere firmly to the coverslips.

Amastigote growth inhibition assay. Mice cardiac myocytes (CMs) and Vero cells were incubated in 24-well tissue-culture plates on 12-mm coverslips as described in the above section "Preparation of ventricular myocytes." After 48 hours, cultured cells were infected with *Tc*N trypomastigotes (parasites:cell ratio 10:1) and incubated for 3 hours at 37°C in a 5% CO_2 atmosphere. After CM and Vero cells were washed to remove the non-internalized parasites, they were incubated with 10, 25, and 50 μg/mL of BNZ-nps for 24 and 48 hours, respectively. The coverslips were removed, rinsed with PBS, fixed in methanol, and stained with Giemsa. In each experiment, a total of 300 cells were evaluated for each treatment in randomly selected fields. The percentage of growth inhibi-

tion was calculated as ([experimental infected cells − control infected cells]/[control infected cells]) × 100.[14]

Animal model. Eight groups, each consisting of 10 1-month-old female C3H/HeN mice, were inoculated intraperitoneally with 1,000 culture-derived trypomastigotes of the *Tc*N isolate. Infected mice were divided into the following groups: 1) infected mice without treatment, 2) mice treated with R-BNZ with daily doses of 50 mg/kg body weight for 15 days (2–17 days post infection [dpi]) (R-BNZ), 3) mice treated with BNZ-nps for 15 days with daily doses of 50 mg/kg/day (BNZ-nps 50-15), 4) mice treated with BNZ-nps for 30 days with daily doses of 50 mg/kg/day (BNZ-nps 50-30), 5) mice treated with BNZ-nps for 15 days with daily doses of 25 mg/kg/day (BNZ-nps 25-15), 6) mice treated with BNZ-nps for 30 days with daily doses of 25 mg/kg/day (BNZ-nps 25-30), 7) mice treated with BNZ-nps for 15 days with daily doses of 10 mg/kg/day (BNZ-nps 10-15), 8) mice treated with BNZ-nps for 30 days with daily doses of 10 mg/kg/day (BNZ-nps 10-30), and 9) mice without infection treated with BNZ nanoparticles for 30 days with daily doses of 50 mg/kg/day, as reference formulation (BNZ-nps 50 F). R-BNZ and BNZ-nps were dispersed in olive oil and administered to mice through oral gavage. Mortality was recorded every day. All procedures involving experimental protocols in animals were conducted in accordance with 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" (registered RENIS N°: 000028), and met the international recommendations for the use of laboratory animals (World Medical Association in the Declaration of Helsinki).

Statistical methods. The statistical significant data ($P < 0.05$) were analyzed by the Student's *t* test and analysis of variance test of the LC_{50} of trypomastigotes and percentage of amastigotes inhibition. To assess differences between survival curves, a log-rank test of Kaplan–Meier was performed. All tests were performed using GraphPad software (La Jolla, CA) (Prism 5.0).

RESULTS AND DISCUSSION

BNZ-nps were prepared by using the nanoprecipitation technique with P188 as a stabilizer. This technique was chosen because of its simplicity, as compared with the emulsification process. Thus, complex purification of oily residues was avoided during the BNZ-np production. BNZ is a hydrophobic molecule highly soluble in ethanol, a solvent miscible with water. Once the drug is dissolved in such solvent and poured dropwise into the aqueous solution containing P188, it becomes nontransparent showing the generation of BNZ-nps.[15] Its characterization indicated a mean particle size of 63.3 ± 2.82 nm, a zeta potential of −18.30 ± 1.0, and a size distribution (polydispersity index) of 3.35 ± 0.1. It is known that the development of novel nanocarriers is a promising approach to improve the efficacy of chemotherapeutic agents. Therefore, taking into account the clinical importance of BNZ, both biocompatibility and toxicity issues need to be evaluated. Cell viability studies were performed in relation to the BNZ-nps toxicity issues by means of the MTT assay (Table 1). No significant differences were observed between the optical densities of cells treated with BNZ-nps (10, 25, and 50 μg/mL) and R-BNZ (50 μg/mL) and the

TABLE 1
Cells toxicity assay

	OD mean	Standard deviation
Vero cells	0.765	0.104
R-BNZ (50 μg/mL)	0.782	0.098
BNZ-nps (50 μg/mL)	0.672	0.026
BNZ-nps (25 μg/mL)	0.695	0.009
BNZ-nps (10 μg/mL)	0.717	0.015

BNZ-nps = benznidazole nanoparticles; OD = optical density; R-BNZ = raw benznidazole. The OD was determined spectrophotometrically at a wavelength of 578 nm. The OD of Vero cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assay showed no significant differences after the addition of BNZ-nps or R-BNZ. Data were collected from three independent experiments in duplicate.

FIGURE 1. Effects of raw benznidazole (R-BNZ) and BNZ nanoparticles (BNZ-nps) on Vero-culture-derived trypomastigotes. After a 24-hour treatment, antitrypanosomal activity was evaluated as the drug concentration at which 50% of the parasites were lysed (LC_{50}). Curves represent log of drug concentration (**A**) R-BNZ and (**B**) BNZ-nps in relation to trypomastigotes percentage by Brenner method. Controls without treatment represent 100% of trypomastigotes.

optical densities of untreated cells. In addition, no morphological change or destabilization of the cell membrane was visible by optical microscopy, indicating that such BNZ-nps would present desirable properties in terms of cell viability.[16]

In addition, because of its potential importance in clinical research, in vitro hemolytic assay was performed to evaluate whether BNZ-nps might damage red blood cells.[17] The optical density values of treated cells with BNZ-nps and R-BNZ were similar to the optical density values of the control untreated blood cells (Table 2), while the positive control, Triton X-100 (10%), produced a complete lysis of erythrocytes. It is worth mentioning that nanoparticles could be considered safe drug delivery systems to be used as trypanocides, since no detectable destabilization of the membranes of red blood cells was observed, as occurs with other nanoparticles evaluated as carriers for cancer therapy.[18]

Next, an assay involving nonproliferative trypomastigotes of TcN was carried out. As observed in Figure 1A, R-BNZ decreased the survival rate of the infective and nonproliferative trypomastigote stage with a LC_{50} of 49 μg/mL. In contrast, as seen in Figure 1B, a LC_{50} of 36 μg/mL was obtained in the case of the BNZ-nps, indicating the suitability of BNZ-nps to treat both the acute and chronic phase infections. It is worth mentioning that P188 did not produce any lytic effect on the nonproliferative trypomastigotes of TcN (data not shown). Controls without treatment represent 100% of trypomastigotes. The lower concentration of R-BNZ reduced the number of parasites to 80% with respect to untreated controls while BNZ-nps reduced the parasites to 60% with the same drug concentration. It is known that the range of parasite susceptibility to BNZ depends on the strain prevailing in different geographical areas, and that T. cruzi discrete typing unit (DTU) I, frequent in endemic areas of northern South America, is generally resistant to BNZ while isolates of T. cruzi DTU II were

TABLE 2
Hemolytic assay

	OD mean	Standard deviation
Control blood	0.466	0.092
R-BNZ (50 μg/mL)	0.659	0.35
BNZ-nps (100 μg/mL)	0.592	0.328
BNZ-nps (50 μg/mL)	0.384	0.028
BNZ-nps (25 μg/mL)	0.413	0.013
BNZ-nps (10 μg/mL)	0.454	0.03
Triton X-100 (10%)	2.617	0.039

BNZ-nps = benznidazole nanoparticles; OD = optical density; R-BNZ = raw benznidazole. The OD was determined spectrophotometrically at a measuring wavelength of 540 nm. The OD of blood showed no significant differences before and after addition of BNZ-nps. R-BNZ was used as the hemolytic reference drug and Triton X-100 (10%) was used as the positive control. Data were collected from three independent experiments in duplicate.
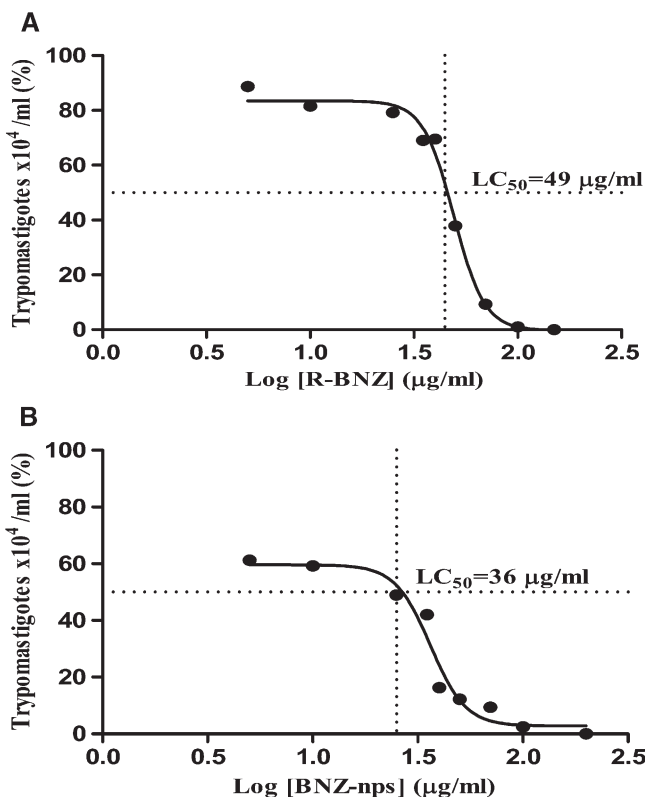
partially resistant to BNZ.[19] Previously, it was observed that TcN epimastigotes DTU I were more resistant to BNZ than CL Brener epimastigotes DTU VI.[8] In this regard, although there are limits to determine the efficacy of BNZ, its affinity for cardiac tissue and resistance to BNZ makes it interesting to study these BNZ-nps in the isolated TcN.

Furthermore, considering that TcN also invades cardiac cells, the effects of BNZ-nps in infected primary cardiac myocyte cultures were also studied and compared with infected Vero cells.[8] As shown in Figure 2, treatments with BNZ-nps 25, 50 μg/mL and R-BNZ 50 μg/mL exhibited no significant differences in the percentage of amastigote growth inhibition on Vero and cardiac myocyte cells. It is worth mentioning that with half the dose of BNZ-nps, the same inhibitory effect on the growth of amastigotes is achieved. This result may suggest that BNZ-nps improve the absorption of BNZ and, therefore, its inhibitory activity against these infected cell lines.[20,21] In contrast, a lower inhibitory activity against the infected cells in both cell cultures was observed when the assay was performed using 10 μg/mL of BNZ-nps. However, taking into account the reported adverse effects of BNZ on infected patients, these results are remarkably important in terms of a potential reduction of the BNZ dose up to 25 μg/mL without losing its efficacy. Moreover, the inhibitory effects of the formulation observed on the infected cells may confirm the ability of such nanoparticles to cross
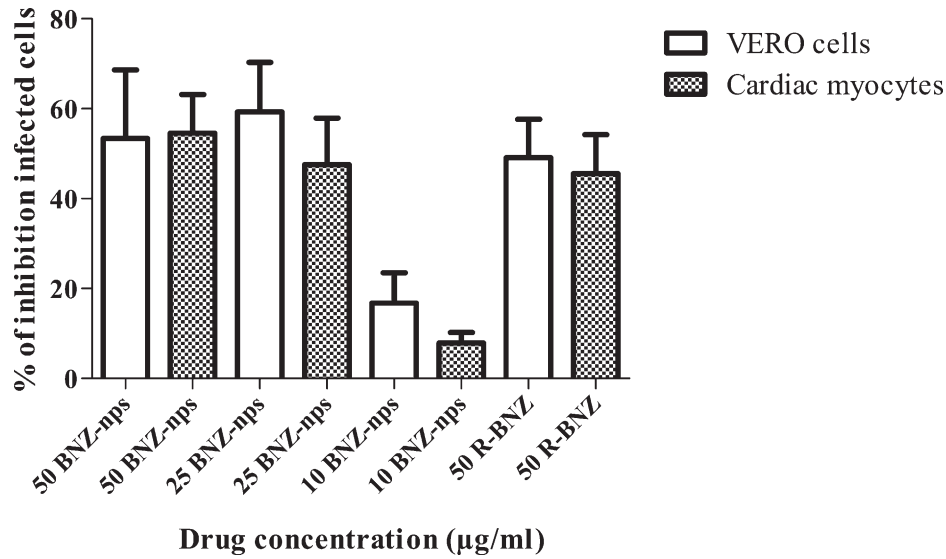
FIGURE 2. Percentage of inhibition of *Trypanosoma cruzi* Nicaragua infected Vero cells and cardiac myocytes primary culture, exposed to benznidazole nanoparticles (BNZ-nps; 10, 25, and 50 μg/mL) and raw BNZ (R-BNZ; 50 μg/mL). In analysis of variance statistical test, no significant differences were observed between BNZ-nps (25 μg/mL), BNZ-nps (50 μg/mL), and R-BNZ (50 μg/mL) treatments. In Student's *t* test, no significant differences were observed between the responses of both cell lines.
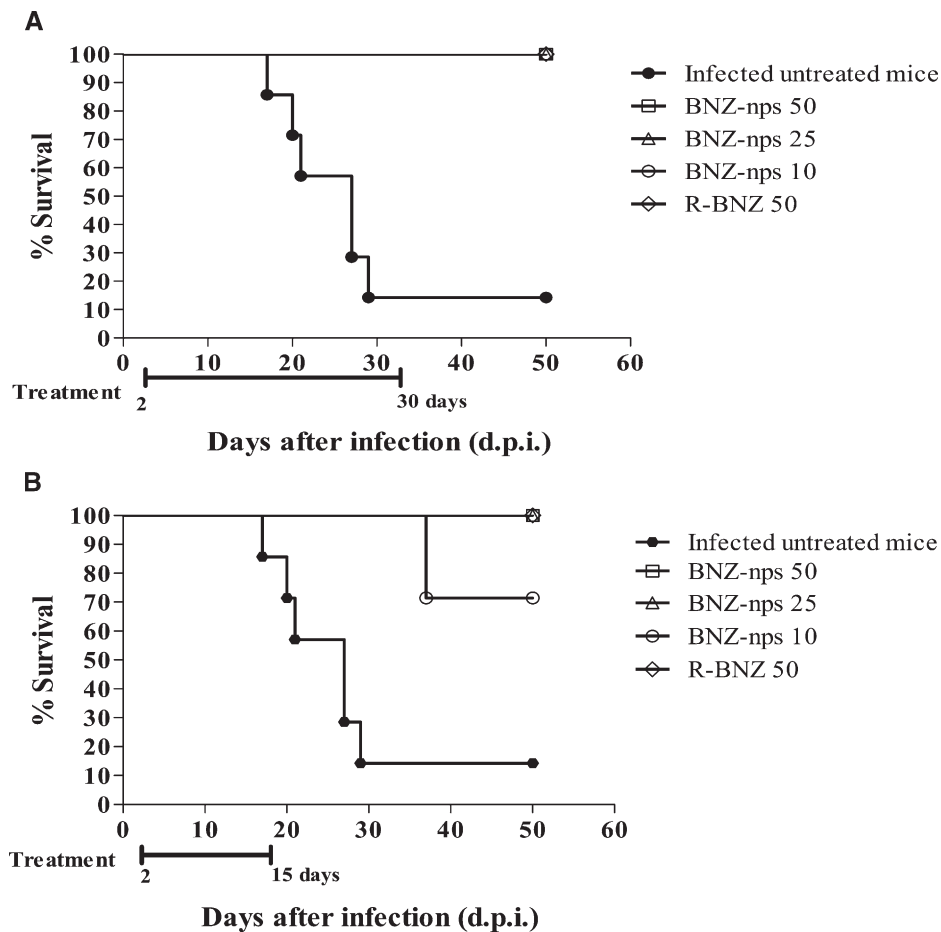

FIGURE 3. Effects of benznidazole nanoparticles (BNZ-nps) on treatment of infected mice. Survival curve during the acute phase in C3H/HeN mice infected with 1,000 trypomastigotes of *Trypanosoma cruzi* Nicaragua and treated with (**A**) 30 oral doses and (**B**) 15 oral doses of BNZ-nps. To assess differences between survival curves, a log-rank test of Kaplan–Meier was performed, which showed significant differences between treated and untreated infected mice ($P < 0.0001$).

the coronary vasculature to inhibit the intracellular growth of *T. cruzi* amastigotes.

To ascertain whether BNZ-nps could be used as a novel treatment of Chagas disease, an assay in the acute phase of *Tc*N-infected mice was performed. It is also known that parasitemia in non-treated C3H/HeN mice inoculated with 1,000 trypomastigotes of *Tc*N, peaks at 30–35 dpi and decreases by 45–50 dpi, resulting only in a 15% survival rate.[9] To determine whether differences in infection outcome would be correlated with different doses of BNZ-nps, a treatment over 30 consecutive days in infected mice was carried out. As shown in Figure 3A, the infected mice treated with 50, 25, and 10 mg/kg/day BNZ-nps for 30 days, survived, at least, 50 days. In addition, the antiparasitic effect of BNZ-nps was also evaluated over 15 consecutive days of treatment in infected mice. As observed in Figure 3B, the infected mice treated with BNZ-nps (50 and 25 mg/kg/day) survived, at least, 50 days, while the infected mice group treated with BNZ-nps (10 mg/kg/day) exhibited a survival rate of 70% after 38 days and till the end of the experiment, confirming that BNZ formulated as nanoparticles has an in vivo antiparasitic effect in dose-dependent manner. In addition, it was found that both uninfected untreated and uninfected mice treated with BNZ-nps 50 mg/kg/day had the same weight, appearance, and behavior confirming the suitability of such BNZ-nps in suppressing parasitemia and preventing death of infected *Tc*N mice.

CONCLUSIONS

In summary, for the first time it was shown that nanoparticles of BNZ exhibited a remarkable effect on *T. cruzi* amastigote growth inhibition in primary cardiac myocytes. It was also confirmed that all mice survived acute infection with 15 doses of 25 or 50 mg/kg/day BNZ-nps during the assay. These findings led to the conclusion that the BNZ-nps treatment is a very convenient approach, in different doses and schedules, to successfully treat Chagas disease in an experimental model of acute *T. cruzi* infection.

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