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Short communication

Efficacy of novel benznidazole solutions during the experimental infection with *Trypanosoma cruzi*

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ABSTRACT

Chagas' disease is caused by the protozoan parasite *Trypanosoma cruzi*. About 8 million people throughout Latin America are infected causing approximately 10,000 deaths annually. Benznidazole, available as unique 100 mg tablets in many of the endemic countries, is currently the drug of choice for the specific treatment of this condition. Despite of the large number of pediatric patients infected, there are no commercial liquid dosage forms available to treat this trypanosomiasis. This work showed that novel benznidazole–water–polyethylene glycol 400 solutions are active against *T. cruzi* in a murine model of Chagas' disease. Present results constitute the first demonstration on the usefulness of benznidazole solutions in infected mice.

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Chagas' disease or American tripanosomiasis is caused by the protozoan parasite *Trypanosoma cruzi* (*T. cruzi*). About 8 million people throughout Latin America are infected causing approximately 10,000 deaths annually [1]. In addition, Chagas' disease has become a major concern due to globalization, because of the immigration of infected individuals to non-endemic regions, spreading the disease to countries such as Australia, Canada, France, Germany, Italy, Japan, Spain and the United States [2,3]. Chagas' disease is characterized by two clinical phases: the acute and chronic ones. The acute phase begins when the parasite enters the mammalian host. It is characterized by an absence of antibodies and manifestation of parasitemia starting 1 or 2 weeks after parasite entry. The chronic phase, which is initially asymptomatic, begins with the decline of parasitemia and can last for the patient's lifetime. The main chronic forms are cardiac (chronic chagasic cardiomyopathy) and digestive compromise [4]. The parasite *T. cruzi* is transmitted mainly by insect vectors, but congenital and transfusion-borne infections occasionally occur. Congenital transmission, affecting 1–10% of babies born to infected mothers, is becoming the most important route of infection in both endemic, due to bug control programs, and non-endemic regions [5].

Benznidazole (BZL) is one of the two active compounds with significant trypanocidal activity in the acute and early chronic phases [4]. It was developed more than 40 years ago, and it is available as 100 mg tablets. Since then, there was an extremely low priority for funding the development of suitable dosage forms to treat this protozoan infection by the governments and pharmaceutical industry [6]. To date, the available solid dosage form is usually fractionated by hand to be used in newborns and children. This situation may result in some unwanted consequences, such as improper dosages and further risks of developing side-effects [7]. However, LAFEPE with the financial and technical support from Drugs for Neglected Diseases initiative (DNDi) recently registered in Brazil a new formulation of BZL available as 12.5 mg dispersible tablets, which is easy to disintegrate [http://dndi.org/press-releases/1016-paedbenz.html]. This may constitute an important step in the treatment of Chagas' disease for the pediatric population. Within this setting, our group has been preparing and testing several solutions of BZL using cosolvent systems. Such system was able to greatly increase the drug solubility up to 10 mg/mL (BZL solubility in water is 0.4 mg/mL) and was not toxic against mammalian cells or parasites *in vitro*, when assayed at 0.2 to 0.05% [8,9]. The objective of the present study was to evaluate the therapeutic efficacy of BZL in solution. As such, three BZL liquid formulations (BZL 20, 40, 60 mg/kg body weight) were prepared at pH 2.5 in a mixture of polyethylene glycol 400 (PEG_{400})/water. The efficacy of these solutions was evaluated in mice to establish whether parasitemia in an acute model of Chagas' disease may be reduced and/or eliminated. In parallel, formulations in which BZL was dispersed were also tested; the vehicle

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Table 1
Parasitemia after treatment with different formulations of BZL.

Formulation	14 days p.i.	21 days p.i.
Placebo (PEG_{400}/water)	20 (15–150)	52 (32–161)$^{\zeta}$
BZL_{20}–PEG[a]	0 (0–20)*	0 (0–0)*
BZL_{40}–PEG[b]	0 (0–0)*	0 (0–0)*
BZL_{60}–PEG[c]	0 (0–1)*	0 (0–0)*
Placebo (CMC/water)	20 (10–130)	60 (35–156)$^{\zeta}$
BZL_{20}–CMC[a]	0 (0–0)*	0 (0–0)*
BZL_{40}–CMC[b]	0 (0–0)*	0 (0–0)*
BZL_{60}–CMC[c]	0 (0–0)*	0 (0–0)*

All data are represented as median (rank) values of parasites/50 fields from 7 mice/group, except for ζ (data are represented as median (rank) of 5 (2 mice death)–7 mice/group). Data were analyzed by the Kruskal–Wallis non parametric analysis of variance followed by the Mann–Whitney *U* test. * Statistically different from its respective placebo, $p<0.05$.
[a] BZL_{20} means benznidazole 20 mg/kg of body weight.
[b] BZL_{40} means benznidazole 40 mg/kg of body weight.
[c] BZL_{60} means benznidazole 60 mg/kg of body weight.

used in this case was carboxymethylcellulose (CMC). BZL (Roche, Argentina and Brazil) solutions were prepared in a mixture of water (30%) and PEG_{400} (70%) (Aldrich Chemical Co., USA). The latter vehicle is considered one of the most useful solubility enhancers with an overall acceptability in terms of side-effect profile [10]. Similar to many other nitroimidazole derivatives, the solubility of BZL increases by decreasing the pH solution due to the protonation of the nitrogen atom of the drug, as described in the literature [11]. Therefore, the pH was regulated by potassium biphthalate buffer solution (pH 2.5). Male C57BL/6 mice (8–10 weeks of age) were intraperitoneally infected with 150 viable trypomastigotes of Tulahuén strain of *T. cruzi* and then treated with a BZL–PEG_{400} aqueous solution or BZL–CMC suspensions at a dose of 60, 40 y 20 mg/kg body weight, as appropriate. The drug was administered to mice orally with an intubation syringe for animal feeding. The therapy was given once a day, starting 7 days post-infection (pi), for 14 days.

First, the anti-trypanosoma effect in mice was evaluated after administration of BZL–PEG_{400} or BZL–CMC. Untreated mice infected with Tulahuén strain presented a 71.4% survival at day 21 pi, whereas a survival rate of 100% was obtained with BZL given orally in PEG_{400}/water solution or CMC/water suspension at every dose tested. As is usually observed for this model [12], mortality at 25 days pi in all infected animals treated with placebo was 100% and 0% in the BZL–PEG_{400} and BZL–CMC groups, respectively. Concerning the drug-treated mice, the absence of parasites in blood was confirmed by control of parasitemia. Monitoring of *in vivo* acute infection was evaluated by assessing bloodstream forms of *T. cruzi* by direct microscopic observation of 5 μL of heparinized tail venous blood at different days pi and measured as parasites/50 fields. Oral treatment of mice with BZL led a significant reduction of parasitemia from day 14 pi to the end of experiment in comparison with untreated mice. It is noteworthy to mention that after 40 days pi there was no reactivation of parasitemia in any of the infected animals treated with the different formulations of BZL. As shown in Table 1, the levels of parasitemia increased through acute infection, and mice treated with these BZL solutions showed significant differences compared with the placebo group at 14 and 21 days pi ($p<0.05$); however, the efficacy is the same that observed in suspension.

Specific antibodies produced by *T. cruzi*-infected treated and not-treated mice were measured by ELISA. Microtiter plates coated with recombinant antigens of *T. cruzi* were incubated with diluted 1:3 mouse serum. Specific IgG isotype was detected by incubation with rabbit IgG anti-mouse IgG peroxidase-conjugated diluted 1:1000. The samples were read at 450 nm after incubation with H_2O_2 and TMB. Fig. 1 shows that at 21 days pi animals infected and treated with placebo (PEG_{400}/water and CMC/water, respectively) presented elevated levels of these specific anti-*T. cruzi* antibodies; however, their levels were significantly decreased after treatment with BZL ($p=0.001$), regardless of the formulation and the doses of BZL used. As expected, the results of specific antibody levels are related to the parasitemias. Taking together, soluble formulations of BZL (20 and 40 mg/kg of body weight) not only reduced the patent parasitemia to zero, but also there was no reactivation of the infection after 40 days pi. It is clear that OD values for anti-*T. cruzi* antibodies after treatment are equal to those of the control animals, suggesting a drastic reduction of the parasite load of treated animals.

Conclusions

Cosolvent systems of PEG_{400}/water at pH 2.5 were useful vehicles to highly improve the solubility of BZL. Previous physical and chemical stability studies showed that the dosage forms could be kept at room temperature for long-term storage. Parasitological evaluations, either by direct microscopic observation of bloodstream forms or by detection of anti-parasite antibodies by ELISA in acutely infected mice treated with these BZL novel formulations revealed promising results of drug efficacy in solution. Although the efficacy of these new solutions of

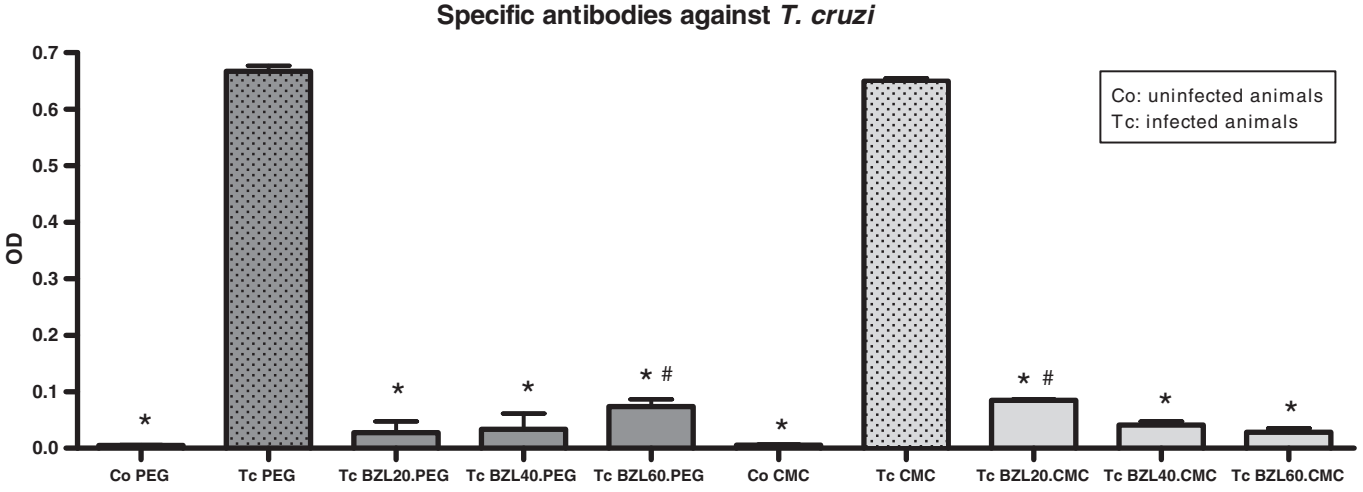

Fig. 1. Detection of specific antibodies against *Trypanosoma cruzi*. Specific IgG was determined by ELISA at 21 days pi. Microtiter plates coated with recombinant antigens of *T. cruzi* (Wiener Lab., Argentina) were incubated with diluted 1:3 mouse serum from uninfected (Co) and infected (Tc) and treated mice. Specific IgG isotype was detected by incubation with rabbit IgG anti-mouse IgG peroxidase-conjugated (Sigma, EEUU) diluted 1:1000. IgG values are presented as mean ± SD of OD (450 nm). Data were statistically analyzed by ANOVA followed by Tukey's test for multiple comparisons. * Statistically different from infected animals treated with placebo, $p=0.001$; # Statistically different from uninfected animals treated with placebo, $p=0.01$.

BZL is the same as that obtained in suspension, the advantage of BZL solution for pediatric patients is that they avoid the need to manually fragment and disperse the currently available tablets, which can always lead to dosing errors. True solutions are also better than suspensions needing to be homogenized before use. In essence, this experimental work provides a suitable reference to the development of BZL solutions, which have poorly water-soluble property, as a simple and useful alternative for the treatment for Chagas' disease.

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