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ORIGINAL PAPER

Different treatment schemes and dynamizations of *Trypanosoma cruzi* biotherapies: what information do they transfer to the organism in infected mice?

Fabiana Nabarro Ferraz[1,*], Franciele Karina da Veiga[1], Denise Lessa Aleixo[1], Miguel Spack Júnior[2] and Silvana Marques de Araújo[1]

[1]*Department of Basic Health Science, Laboratory of Parasitology, Universidade Estadual de Maringá, Maringá, Paraná, Brazil*
[2]*Clinic of Homeopathy, Rua Luiz Gama, 485, 87014-110, Maringá, Paraná, Brazil*

Background: **The use of biotherapies in *Trypanosoma cruzi* infection can provide an understanding about effects of these highly diluted medications.**
Objectives: **To evaluate different treatment schemes and dynamizations of biotherapies prepared from blood trypomastigotes (buffy coat) in mice infected with *T. cruzi*.**
Methods: **Swiss mice infected with Y strain of *T. cruzi* were divided into two experiments. Experiment 1, all treated groups received biotherapy 7dH (10 μL/mL *ad libitum*) in different treatment schemes: TB_{7dH} – treated 3 days before infection; TBA_{7dH} – treated 3 days before and after infection; $TBAe.d._{7dH}$ – treated 3 days before infection and every day after infection and IC – infection control. Experiment 2, all treated groups received medication in different dynamizations 3 days before and after infection (10 μL/mL *ad libitum*): TBA_{15dH} – treated with biotherapy 15dH; TBA_{16dH} – treated with biotherapy 16dH; TBA_{17dH} – treated with biotherapy 17dH; $TBA_{p.chords}$ – treated with biotherapy 'potency chords' and IC – infection control. We evaluated parasitological and clinical parameters.**
Results: **Experiment 1 showed that different treatment schemes with biotherapy 7dH produced different effects on infection evolution. TBA_{7dH} group had the best outcome, with lower parasitemia, higher survival, and better clinical evolution compared to IC. Experiment 2 showed that biotherapy 'potency chords' had effects different from the individual dynamizations that it contained (15dH, 16dH, and 17dH). Animals that had patent parasitemia had delayed emergence of parasites in blood and subsequent increase in parasitemia, but had better clinical evolution compared to IC.**
Conclusions: **The effects of *T. cruzi* biotherapies depend on frequency at which they are administered, dynamization, and host–parasite relationship/individual susceptibility of treated organism. Biotherapy appeared to transfer to infected organism 'antigenic information' related to parasite and 'disease information' related to molecules produced by host's immune response and contained in the buffy coat used to prepare the medication.**
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*Correspondence: Fabiana Nabarro Ferraz, Universidade Estadual de Maringá, Departamento de Ciências Básicas da Saúde/Setor de Parasitologia, Avenida Colombo, 5790, Jd. Universitário, bloco I90, sala 11, 87020-900, Maringá, Paraná, Brazil.
E-mail: fabiana_nabarro@hotmail.com, karinafranciele@yahoo.com.br, deniseparasito@gmail.com, clinicah@pop.com.br, smaraujo@uem.br
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Introduction

Chagas disease is caused by the flagellate protozoan *Trypanosoma cruzi*.[1] It is currently a neglected disease that affects 6−7 million people worldwide, mostly in Latin America where Chagas disease is endemic.[2]

In Brazil, only benznidazole is available for the treatment of Chagas disease. Benznidazole treatment is considered safe,[3] this drug has effectiveness of approximately 80% in the acute phase of the disease, but very low efficiency in the chronic phase, besides adverse drug reactions which may induce some patients to discontinue treatment.[3−5] Thus, the search for more effective medications with less adverse reactions is one of the main focuses of many studies.[6−12]

Homeopathy has been used for the treatment of many pathologies. Several *in vivo* experimental models have been used to evaluate the effects of highly diluted medications.[7−14] Biotherapies are highly diluted medications prepared according to homeopathic techniques, which are defined as medications produced from chemically undefined biological products, such as secretions, excretions, tissues, organs, and products of microbial or allergen origin.[15]

The use of *T. cruzi* biotherapies as an intervention in mice infected with *T. cruzi* is a possible means to understand the effects of these highly diluted medications and discover new candidates to treat Chagas disease.[10] Treatment with *T. cruzi* biotherapy seeks to stimulate the host's immune response. Several studies have reported that highly diluted antigens can modulate the immune system, culminating in balance or disorder of this system. The prophylactic and therapeutic actions of *T. cruzi* biotherapies in experimental *T. cruzi* infection have been investigated. Most studies have reported beneficial parasitological, clinical, histopathological, hematological, and immunological effects.[7−12]

Currently, no studies have investigated the effects of different dynamizations and 'potency chords' of *T. cruzi* biotherapies in experimental infection. 'Potency chords' are homeopathic pharmaceutical preparations that contain, in equal proportions, different dynamizations of highly diluted medications that are prepared based on the same original substance.[16] Studies suggest that such formulations may be more effective than a single dynamization, and the effects of 'potency chords' can be clearly differentiated from the individual effects of the dynamizations that it contains.[17,18] However, little information about 'potency chords' is available in the literature,[18] especially as a treatment for *T. cruzi* infection, since no totally effective biotherapy has been described.

In this context, the present study evaluated the effects of different treatment schemes of *T. cruzi* biotherapy (dynamization 7dH), different dynamizations of *T. cruzi* biotherapy (15dH, 16dH, and 17dH), and 'potency chords' (that contain, in equal proportions, dynamizations 15dH, 16dH, and 17dH) in acute infection with *T. cruzi*, in order to understand what information these highly diluted medications prepared from blood trypomastigotes (buffy coat + parasite) transfer to the infected organism.

Material and methods

Ethics

The study was approved by the Ethics Committee for Experiments in Animals (protocol no. 030/2008), Universidade Estadual de Maringá (UEM). All of the recommendations of National Law no. 11,794 (October 8, 2008)[19] for the scientific management of animals were respected.

Parasite and inoculum

Forty-five 28-day-old male Swiss mice from the Central Vivarium, UEM, were intraperitoneally inoculated with 1400 blood trypomastigotes of the Y strain of *T. cruzi*.[20] The animals were maintained in the Vivarium of Parasitology, UEM, under controlled temperature (22 ± 2°C), a 12 h/12 h light/dark cycle and received commercial food and water *ad libitum*.

Experimental design and treatment schemes

Blind, controlled, and randomized trials were performed twice. To blind the experiments, the treatments were performed by a researcher who did not participate in the evaluation of parasitological and clinical parameters. To evaluate these parameters, each experimental group received a code such that the researchers were unaware of the treatment that was received by each group.

Two independent experiments were performed (Experiments 1 and 2). The mice were divided into groups such that the mean weights of each group were statistically equal. The treatment that each group received was determined by a random drawing.

In Experiment 1, the effects of biotherapy 7dH were evaluated according to different treatment schemes: before infection to stimulate the immune system of the host without the presence of the parasite[7−9] and/or after infection to assess the actions of the biotherapy and immune system together against the parasite.[10,12]

The dynamization 7dH was chosen based on the results reported by Ferraz et al.[9] These authors reported effects against experimental *T. cruzi* infection when biotherapy 7dH was administered 7 days before infection with reduction in parasitemia, without significant increase in survival of infected animals and when the biotherapy 7dH was administered 30 days before infection with increase in parasitemia peak, without changing in survival of infected animals. The homeopathic physician of our research group, based on these results and his clinical experience,

suggested a short period of stimulation with treatment 3 days before infection.

The mice were divided into four groups ($n = 5$/group): IC (infection control; the animals were treated with water),[21] TB_{7dH} (the animals were treated with biotherapy 7dH 3 days before infection), TBA_{7dH} (the animals were treated with biotherapy 7dH 3 days before and 3 days after infection), and $TBAe.d._{7dH}$ (the animals were treated with biotherapy 7dH 3 days before infection and every day [e.d.] after infection until the death of the animals).

In Experiment 2, dynamizations 15dH, 16dH, 17dH and 'potency chords' (consisteing of equal proportions of 15dH, 16dH, and 17dH) were chosen based on the clinical experience of the homeopathic physician of our research group, considering the oscillatory−effect curve of highly diluted medications. The effects of the biotherapies were evaluated using the treatment scheme that had the best outcome in Experiment 1 (i.e., biotherapy administered 3 days before and 3 days after infection). The mice were divided into five groups ($n = 5$/group): IC (infection control; the animals were treated with water),[21] TBA_{15dH} (the animals were treated with biotherapy 15dH), TBA_{16dH} (the animals were treated with biotherapy 16dH), TBA_{17dH} (the animals were treated with biotherapy 17dH), $TBA_{p.chords}$ (the animals were treated with biotherapy 'potency chords').

The medications were diluted in water (10 μL/mL) and offered to the animals *ad libitum* in a sterile amber bottle according to Aleixo et al.[10] In this study, water was used as the treatment in the IC group because previous study by our group demonstrated that a 7% alcohol solution (medication vehicle) and water, successed and unsuccessed, in the present experimental animal model did not alter the parasitological or clinical course of *T. cruzi* infection.[21]

T. cruzi biotherapies

The biotherapy was produced with Y strain blood trypomastigotes of *T. cruzi* that were collected from mice on day 7 after infection. The blood was centrifuged, and the buffy coat, which has a higher concentration of trypomastigotes, was collected. The biotherapy was prepared by adding 0.9 mL of *T. cruzi* concentrate/buffy coat (4.1×10^7 trypomastigotes/mL) to 9.1 mL of distilled water in a sterile amber glass flask. Dilution and succussion were performed in accordance with the Brazilian Homeopathic Pharmacopoeia[15] on a decimal scale (dH) until the dynamizations 7dH, 15dH, 16dH, and 17dH. The 'potency chords' biotherapy was prepared by mixing equal proportions of dynamizations 15dH, 16dH, and 17dH in a sterile amber glass flask. The latter dynamizations were prepared in 7% alcohol solution as the vehicle for treatment of the animals, stored at room temperature, and protected from light.

Parasitological parameters

Parasitemia was assessed using Brener's technique.[22] Parasite counts were performed of the day 4−26 after infection. The parasitemia curve was plotted using the mean parasitemia of each group. Based on the parasitemia curve, we determined the prepatent period (i.e., the mean time, in days, between inoculation and the day when fresh blood was positive), patent period (i.e., the mean time, in days, with parasitemia was detected in fresh blood), parasitemia peak (i.e., the highest mean parasitemia observed in fresh blood), total parasitemia (i.e., the mean sum of parasitemia of each mouse over the entire experiment), mortality rate (i.e., the total number of dead animals relative to the number of animals infected, observed for up to 180 days after infection), and survival rate (i.e., the mean survival time, in days, after infection, observed for up to 180 days).

For animals that showed negative parasitemia and survived to infection, blood was collected (in duplicate) for polymerase chain reaction (PCR) analysis[23,24] on days 58 (acute phase) and 450 (chronic phase) after infection. Samples that showed a 330 bp fragment were considered positive.

Clinical parameters

The animals were clinically evaluated 3 days before infection and on days 0, 4, 8, 11, 15, 18, 21, 25, and 28 after infection using a standard schedule. Body mass (g) was measured using a semi-analytical balance (BEL Engineering, Class Mark II). Body temperature (°C) was measured on the front region of the left hindleg because of its smaller amount of fur using an Icel thermometer (model no. TD-920.0387). Food (g) and water (mL) intake was measured in each group by considering the amount consumed subtracted from initial amount offered to the animals. The values were divided by the number of animals to estimate the individual values.[25]

Statistical analysis

The parasitological and clinical data were compared using BioEstat 5.0 software at a 5% level of significance. The D'Agostino Pearson or Shapiro−Wilk test was performed to verify normality of the data. Variables with a normal distribution were compared using Student's *t*-test. Data with a nonparametric distribution were compared using the Mann−Whitney test. The log-rank test was used to compare survival curves.

For the clinical parameters, the days of assessment were divided into three distinct periods: before infection (3 days before infection and on day 0), first evaluation period after infection (on days 4 and 8 after infection), and second evaluation period after infection (on days 11, 15, 18, 21, 25, and 28 after infection).

The statistical effect size was also determined for the parasitological and clinical parameters using the Effect Size Calculator (http://www.uccs.edu/~lbecker/; accessed May 6, 2015). Effect sizes ≤0.2 were considered small. Effect sizes >0.2 to ≤0.5 were considered medium. Effect sizes >0.5 were considered large.[26] The calculation of the effect size complemented the statistical significance tests, thus allowing determination of the effect of an intervention.[27]

Results

Experiment 1: effect of biotherapy 7dH using different treatment schemes

Biotherapy 7dH: treatment 3 days before infection (TB_{7dH}): No significant difference was observed in parasitological (Table 1, Figure 1A, and Figure 2A) or clinical (Figure 3) parameters between the TB_{7dH} group and IC group. The mortality rate was 100% in the IC group and 80% in the TB_{7dH} group. The surviving mouse never presented patent parasitemia and did not develop characteristic clinical signs of infection, such as general edema, bristly fur, hypothermia, cachexia, or a decrease in general activity. The animal showed a negative PCR on day 58 after infection (in the two samples collected) and positive PCR on day 450 after infection (in one of the samples collected; Figure 4).

Biotherapy 7dH: treatment 3 days before and 3 days after infection (TBA_{7dH}): The parasitemia curve ($p < 0.01$) and parasitemia peak on day 15 of infection ($p < 0.05$) were significantly lower in the TBA_{7dH} group compared with the IC group (Table 1 and Figure 1A), presenting large statistical effect sizes of 0.6 and 1.3, respectively. When evaluating individual animals, 60% of the mice in the TBA_{7dH} group had a higher prepatent period ($p < 0.05$), longer survival curve ($p < 0.05$), lower parasitemia peaks (on day 8 and 15 of infection; $p < 0.05$), lower total parasitemia ($p < 0.05$), and lower parasitemia curve ($p < 0.01$) compared with the IC group (Figures 1B and 2A). A large statistical effect size (>0.8) was obtained for all of the parasitological parameters evaluated.

The analysis of the clinical parameters is presented in Figure 3. In the second evaluation period after infection (day 11−28 after infection, which is usually a period of high morbidity), the TBA_{7dH} group had significantly higher water intake ($p < 0.05$), food intake ($p < 0.05$), body mass ($p < 0.05$), and body temperature ($p < 0.05$) compared with the IC group. These outcomes had a large statistical effect size (>1.4).

The mortality rate was 60% until day 38 after infection. The surviving mice did not exhibit patent parasitemia when the parasitological parameters were evaluated until day 26 after infection and did not develop characteristic clinical signs of infection. However, on day 38 after infection, one mouse died, and the surviving mouse had a negative PCR on day 58 after infection (in the two samples collected) and positive PCR on day 450 after infection (in one of the samples collected; Figure 4).

Biotherapy 7dH: treatment 3 days before infection and every day after infection ($TBAe.d._{7dH}$): In animals that presented patent parasitemia, the parasitemia curve ($p < 0.01$) and parasitemia peak on day 15 after infection ($p < 0.05$) were significantly higher in the $TBAe.d._{7dH}$ group than in the IC group (Table 1, Figure 1A). These outcomes represent large statistical effect sizes (0.6 and 1.9, respectively). No significant difference was observed in analysis of the survival curves (Figure 2A) and clinical parameters between the $TBAe.d._{7dH}$ group and IC group (Figure 3).

Table 1 Parasitological parameters (mean ± standard deviation) assessed in groups of mice infected with *T. cruzi* and submitted to different treatment schemes using biotherapy 7dH

Group	*Prepatent period (days)*	*Patent period (days)*	*Parasitemia peak 8 day of infection (trypomastigotes/mL)*	*Parasitemia peak 15 day of infection (trypomastigotes/mL)*	*Total parasitemia (trypomastigotes/mL)*	*Parasitemia curve (trypomastigotes/mL)*	*Survival (days)*	*Mortality (%)*
IC	5.8 ± 1.3	13.5 ± 2.4	(14.7 ± 8.4) × 10^5	(10.9 ± 8.3) × 10^5	(58.0 ± 34.3) × 10^5	(5.7 ± 6.9) × 10^5	15.8 ± 1.5	100
TB_{7dH}	6.2 ± 1.7	12.0 ± 7.6	(8.9 ± 8.7) × 10^5	(24.8 ± 35.3) × 10^5	(64.8 ± 39.5) × 10^5	(5.9 ± 12.8) × 10^5	50.6 ± 72.5	80
TBA_{7dH}	12.5 ± 9.5	8.6 ± 7.9	(7.1 ± 9.7) × 10^5	(2.9 ± 3.0) × 10^{5*}	(24.9 ± 27.3) × 10^5	(2.2 ± 4.5) × 10^{5*}	54.8 ± 70.6	80
$TBAe.d._{7dH}$	5.2 ± 1.3	14.4 ± 2.9	(21.2 ± 12.9) × 10^5	(49.2 ± 27.3) × 10^{5*}	(132.0 ± 78.5) × 10^5	(12.7 ± 19.3) × 10^{5*}	44.8 ± 66.3	80

IC – infection control; TB_{7dH} – animals were treated with biotherapy 7dH 3 days before infection; TBA_{7dH} – animals were treated with biotherapy 7dH 3 days before and 3 days after infection; $TBAe.d._{7dH}$ – animals were treated with biotherapy 7dH 3 days before infection and every day after infection.
* Statistical significance ($p < 0.05$) compared with the IC group.

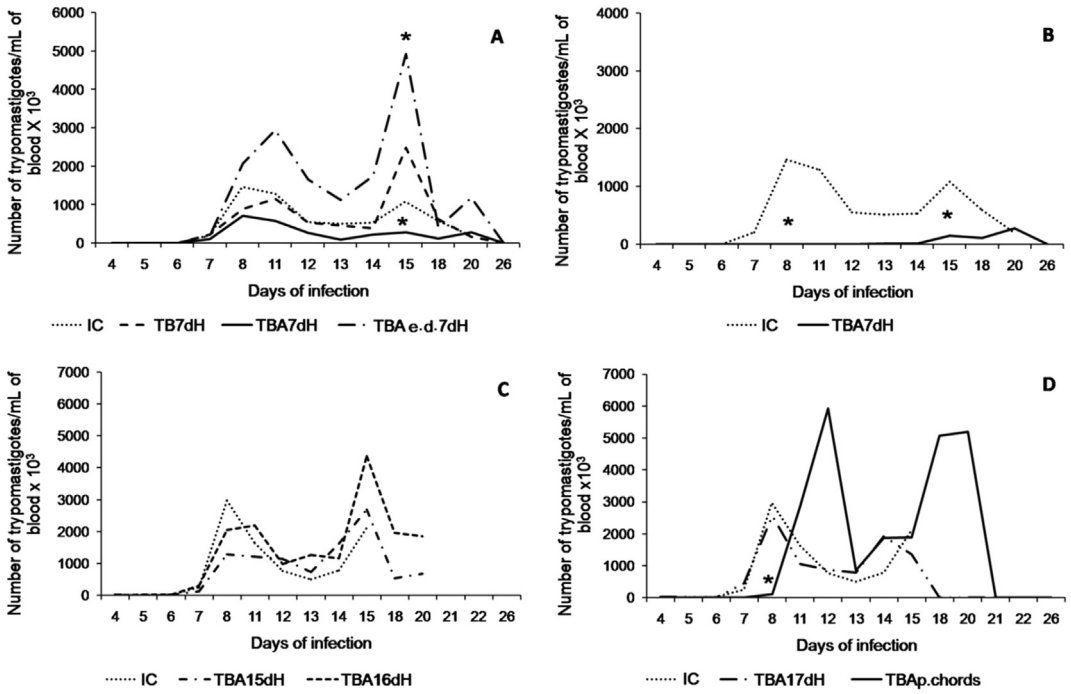

Figure 1 Experiment 1: A-Parasitemia curve of mice infected with *T. cruzi* and submitted to different treatment schemes using biotherapy 7dH. Groups: IC (infection control); TB_{7dH} (animals were treated with biotherapy 7dH 3 days before infection); TBA_{7dH} (animals were treated with biotherapy 7dH 3 days before and 3 days after infection) and $TBAe.d._{7dH}$ (animals were treated with biotherapy 7dH 3 days before infection and every day after infection). B-Parasitemia curve of mice infected with *T. cruzi* belonging to TBA_{7dH} that progressed with lower parasitemia compared to IC group. Experiment 2: C, D-Parasitemia curve of mice infected with *T. cruzi* and submitted to the treatment with different dynamizations of biotherapies. Groups: TBA_{15dH} (animals were treated with biotherapy 15dH); TBA_{16dH} (animals were treated with biotherapy 16dH); TBA_{17dH} (animals were treated with biotherapy 17dH); $TBA_{p.chords}$ (animals were treated with biotherapy "potency chords"). Biotherapies were administered to animals 3 days before and 3 days after infection. *Statistical significance ($p < 0.05$) compared with the IC group.

The mortality rate was 80% in the $TBAe.d._{7dH}$ group. The surviving mouse never exhibited patent parasitemia and did not develop characteristic clinical signs of infection. The mouse showed a positive PCR on day 58 after infection (in the two samples collected) and day 450 after infection (in the two samples collected; Figure 4).

Experiment 2: effect of biotherapies 15dH, 16dH, and 17dH and 'potency chords' administered 3 days before and 3 days after infection

Biotherapies 15dH, 16dH, and 17dH administered 3 days before infection and 3 days after infection (TBA_{15dH}, TBA_{16dH}, and TBA_{17dH}): No significant difference was observed in the parasitological parameters (Table 2,

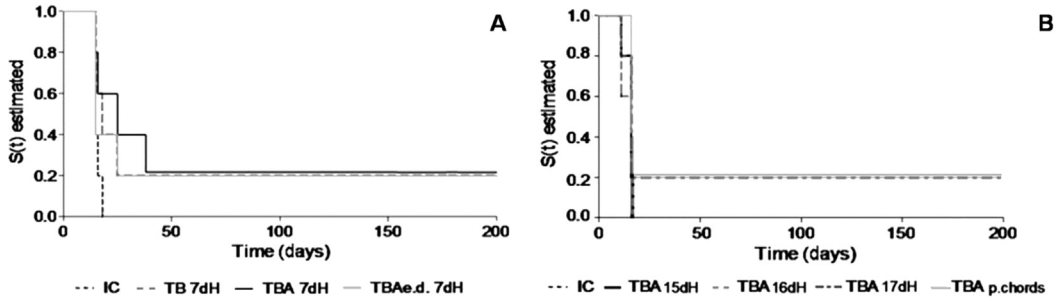

Figure 2 **A**-Survival curve of mice infected with *T. cruzi* and submitted to different treatment schemes using biotherapy 7dH. Groups: IC – infection control; TB_{7dH} – animals were treated with biotherapy 7dH 3 days before infection; TBA_{7dH} – animals were treated with biotherapy 7dH 3 days before and 3 days after infection; $TBAe.d._{7dH}$ – animals were treated with biotherapy 7dH 3 days before infection and every day after infection. **B**-Survival curve of mice infected with *T. cruzi* and submitted to the treatment with different dynamizations of biotherapies. Groups: IC – infection control; TBA_{15dH} – animals were treated with biotherapy 15dH; TBA_{16dH} – animals were treated with biotherapy 16dH; TBA_{17dH} – animals were treated with biotherapy 17dH; TBAp.chords – animals were treated with biotherapy "potency chords". Biotherapies were administered to animals 3 days before and 3 days after infection.

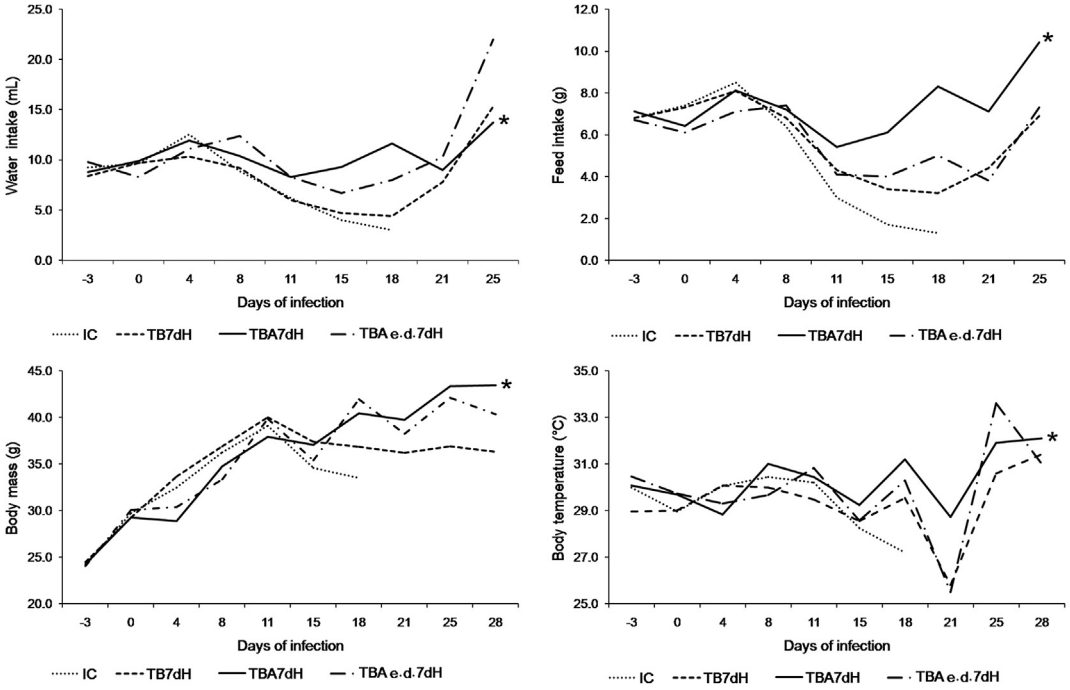

Figure 3 Clinical parameters assessed in groups of mice infected with *T. cruzi* and submitted to different treatment schemes using biotherapy 7dH. Groups: IC – infection control; TB_{7dH} – animals were treated with biotherapy 7dH 3 days before infection; TBA_{7dH} – animals were treated with biotherapy 7dH 3 days before and 3 days after infection; $TBAe.d._{7dH}$ – animals were treated with biotherapy 7dH 3 days before infection and every day after infection. *Statistical significance ($p < 0.05$), on days 11–28 after infection compared with the IC group.

Figure 1C, D and Figure 2B) or clinical parameters (Figure 5) between the TBA_{15dH}, TBA_{16dH}, and TBA_{17dH} groups and IC group. The mortality rates in the TBA_{15dH}, TBA_{16dH}, and IC groups were 100%. The mortality rate in the TBA_{17dH} group was 80%. The surviving mouse never presented patent parasitemia and did not develop characteristic clinical signs of infection. The mouse showed a negative PCR on day 58 after infection (in the two samples collected) and a positive PCR on day 450 after infection (in the two samples collected; Figure 4).

Biotherapy 'potency chords' administered 3 days before infection and 3 days after infection ($TBA_{p.chords}$): The $TBA_{p.chords}$ group presented a parasitemia curve that was different from the IC group and single dynamization groups ($p < 0.05$), with higher total parasitemia ($p < 0.05$) in animals that presented patent parasitemia. The prepatent period was significantly longer in the $TBA_{p.chords}$ group compared with the IC group ($p < 0.05$). The increase in the prepatent period resulted in a decrease in the patent period ($p < 0.05$; Table 2) and a delay of the parasitemia peaks on days 12 and 20 after infection (Figure 1D). These parasitological outcomes represented a large statistical effect size (>2.0). No significant difference was observed in analysis of the survival curves (Figure 2B) between the $TBA_{p.chords}$ group and IC group.

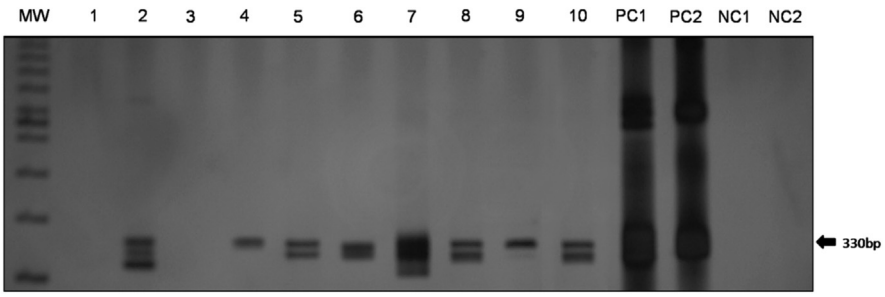

Figure 4 Polyacrylamide gel shows the 330 bp *T. cruzi* kDNA specific fragments in blood samples of surviving mice. Samples were collected in duplicate on day 450 after infection. (1) and (2) TB_{7dH} – animals were treated with biotherapy 7dH 3 days before infection; (3) and (4) TBA_{7dH} – animals were treated with biotherapy 7dH 3 days before and 3 days after infection; (5) and (6) $TBAe.d._{7dH}$ – animals were treated with biotherapy 7dH 3 days before infection and every day after infection; (7) and (8) TBA_{17dH} – animals were treated with biotherapy 17dH 3 days before and 3 days after infection; (9) and (10) $TBA_{p.chords}$ – animal were treated with biotherapy "potency chords" 3 days before and 3 days after infection. MW – 100 bp, molecular weight DNA ladder; PC1 – positive control for PCR; PC2 – positive control for extraction; NC1 – no DNA in the reaction mixture for PCR amplification; NC2 – negative control for extraction; bp – base pairs.

The evaluation of clinical parameters in the $TBA_{p.chords}$ group in the second period of evaluation after infection (day 11−28 after infection) revealed significantly higher food intake ($p < 0.05$), body mass ($p < 0.05$), and body temperature ($p < 0.05$) compared with the IC group (Figure 5). These clinical parameters had a large statistical effect size (>1.0).

The mortality rate was 80% in the $TBA_{p.chords}$ group. The surviving mouse never exhibited patent parasitemia and did not develop characteristic clinical signs of infection. The mouse showed a negative PCR on day 58 after infection (in the two samples collected) and a positive PCR on day 450 after infection (in the two samples collected; Figure 4).

Discussion

In Experiment 1, biotherapy 7dH was administered 3 days before infection (TB_{7dH}) and did not significantly affect any of the parasitological or clinical parameters. In contrast, when administered 3 days before infection and 3 days after infection (TBA_{7dH}), the animals had lower parasitemia and a longer survival curve compared with the control group, reflected by a better clinical evolution (i.e., stable body temperature, higher body weight gain, and higher food and water intake compared with the control group) on day 11−28 after infection, a period of high morbidity when animals usually start to present a reaction to the infection.[25]

In this experimental model (Swiss mice are highly susceptible to infection), the evolution of the disease is irreversible, culminating in the death of untreated animals (IC group).[28] Thus, an increase in the survival period, a decrease in parasitemia, and better clinical evolution in the host are indicators of a good treatment scheme, independent of the presence of the parasite.[10] The surviving animals in the TB_{7dH} and TBA_{7dH} groups showed a positive PCR on day 450 after infection in only one sample that was collected in each group. Furthermore, these mice never presented patent parasitemia and did not develop characteristic clinical signs of infection (e.g., general edema, bristly fur, hypothermia, cachexia, or a decrease in general activity). These outcomes are consistent with low levels of parasitemia.

Biotherapy 7dH treatment for 3 days before infection and every day after infection ($TBAe.d._{7dH}$) in animals that had patent parasitemia caused higher parasitemia without significantly altering any of the clinical parameters compared with the control group. With this treatment scheme, one mouse survived and never presented patent parasitemia and did not develop characteristic clinical signs of infection, confirming the importance of the host−parasite relationship associated with individual reactions of the organism to highly diluted medications.[10] This animal showed a positive PCR on days 58 and 450 after infection in the two samples that were collected for each day of assessment.

In fact, contradictory and non-linear effects may be observed in complex systems that involve the fine modulation of biological functions, mainly after the use of highly diluted medications.[9,11] According to some authors, the use of highly diluted medications in the acute phase of infection requires a higher stimulus frequency.[10,29] However, overstimulation with biotherapy 7dH that was given 3 days before infection and every day after infection may be directly related to an increase in parasitemia in most of the animals. This was similar to Ferraz et al.,[9] in which the animals were treated with *T. cruzi* biotherapy 7dH 30 days before infection. Probably, repeated stimuli may cause a less effective immunomodulation for controlling parasitemia. Thus, further studies evaluating the production of antibodies, proinflammatory and antiinflammatory cytokines in mice treated with *T. cruzi* biotherapy using this treatment regimen and dynamization may be interesting to deepen this research.

The evaluation of the parasitological and clinical parameters showed that the different treatment schemes with biotherapy 7dH produced different effects on the evolution of *T. cruzi* infection. The present results are consistent with Ferraz et al.[9] These authors used the same experimental model and found that *T. cruzi* biotherapy 7dH that was administered 7 days before infection (0.2 mL/day by gavage) decreased parasitemia, which was different from administration 30 days before infection and 20 days after infection (0.2 mL/day by gavage).

In Experiment 2, biotherapies 15dH, 16dH, and 17dH administered 3 days before infection and 3 days after infection (TBA_{15dH}, TBA_{16dH}, and TBA_{17dH}) did not significantly affect the parameters assessed. Highly diluted medicines generally have an oscillatory potency−effect curve, which could explain the non-linear effects and fact that some dynamizations are effective and others are ineffective.[30] Sandri et al.[12] used the same experimental model (28-day-old mice) and *T. cruzi* biotherapy 17dH that was administered after infection (0.2 mL/day by gavage) and also found no difference in the parasitological parameters that were assessed. However, Aleixo et al.[10] also used the *T. cruzi* biotherapy 17dH and the animals that were treated after infection with this biotherapy *ad libitum* (10 μL/mL, diluted in water) presented a better evolution of the parasitological and clinical parameters than animals that were treated after infection with biotherapy 17dH by gavage (0.2 mL/day), which had a worse evolution of infection compared with the control group. These findings indicate that the treatment scheme is an important variable that is associated with the specific dynamization and can directly influence the effects of highly diluted medications.

The biotherapy 'potency chords' administered 3 days before infection and 3 days after infection ($TBA_{p.chords}$) caused a different parasitemia curve compared with the control group and single dynamizations. The biophysical principles that result from the 'potency chords' would probably ensure a different effect.[31] In the animals that presented patent parasitemia, we observed the delayed emergence of parasites in blood (i.e., a decrease in the patent period and delay of the parasitemia peaks), with a subsequent increase in parasitemia compared with the control

Table 2 Parasitological parameters (mean ± standard deviation) assessed in groups of mice infected with *T. cruzi* and submitted to the treatment with different dynamizations of biotherapies

Group	*Prepatent period (days)*	*Patent period (days)*	*Parasitemia peak 8 day of infection (trypomastigotes/mL)*	*Parasitemia peak 15 day of infection (trypomastigotes/mL)*	*Total parasitemia (trypomastigotes/mL)*	*Parasitemia curve (trypomastigotes/mL)*	*Survival (days)*	*Mortality (%)*
IC	5.3 ± 1.2	14.7 ± 1.2	(29.8 ± 18.8) × 10^5	(21.3 ± 18.5) × 10^5	(90.7 ± 11.9) × 10^5	(9.1 ± 12.3) × 10^5	16.0 ± 0.0	100
TBA_{15dH}	5.6 ± 3.0	13.2 ± 2.9	(12.9 ± 8.4) × 10^5	(26.7 ± 28.0) × 10^5	(85.7 ± 30.0) × 10^5	(8.4 ± 11.5) × 10^5	15.2 ± 2.4	100
TBA_{16dH}	5.0 ± 1.7	12.2 ± 1.9	(20.4 ± 15.0) × 10^5	(43.7 ± 58.9) × 10^5	(113.6 ± 59.2) × 10^5	(11.4 ± 17.9) × 10^5	14.2 ± 2.9	100
TBA_{17dH}	4.0 ± 0.0	13.5 ± 1.7	(25.7 ± 13.4) × 10^5	(13.6 ± 9.5) × 10^5	(87.4 ± 34.6) × 10^5	(8.9 ± 10.7) × 10^5	47.8 ± 73.9	80
$TBA_{p.chords}$	8.7 ± 2.1*	11.7 ± 1.5*	(1.1 ± 1.5) × 10^5*	(18.8 ± 11.8) × 10^5	(169.0 ± 52.2) × 10^5*	(15.8 ± 27.9) × 10^5	49.0 ± 73.2	80

IC – infection control; TBA_{15dH} – animals were treated with biotherapy 15dH; TBA_{16dH} – animals were treated with biotherapy 16dH; TBA_{17dH} – animals were treated with biotherapy 17dH; $TBA_{p.chords}$ – animals were treated with biotherapy "potency chords". Biotherapies were administered to animals 3 days before and 3 days after infection.
* Statistical significance ($p < 0.05$) compared with the IC group.

group. However, these animals maintained a better evolution of the clinical parameters (i.e., stable body temperature, higher weight gain, and higher food intake compared with the control group) on day 11–28 after infection. The use of a more resistant experimental model (rats or resistant mouse strains)[28] could possibly maintain a host-parasite balance early during infection, thus allowing evident beneficial effects.

For biotherapy 17dH and the 'potency chords', one mouse survived and never presented patent parasitemia and did not develop characteristic clinical signs of infection. These animals showed only positive PCR on day 450 after infection, further confirming the importance of the host–parasite relationship that is associated with individual reactions of the organism to highly diluted medications.[10]

The outcomes showed that the 'potency chords' biotherapy exerted effects on the evolution of *T. cruzi* infection that differentiated it from treatments with the individual dynamizations. A previous study by Gomez and Jorge[32] investigated the protective effects of different dynamizations (10dH, 30dH, 200dH, and 1000dH) of *Phosphorus* and 'potency chords' on hepatic metabolism in rats in an induced toxic hepatitis model. These authors showed that the 'potency chords' also exerted effects that clearly differentiated it from the individual dynamizations.

This and other studies[7–12] demonstrated that biotherapies that are prepared from *T. cruzi* alter the course of experimental infection with *T. cruzi*, suggesting that these biotherapies are capable of stimulating the host immune response. To evaluate the immunomodulatory action of the *T. cruzi* biotherapies, some authors assessed the humoral immune response in mice that were infected with *T. cruzi* and treated before infection. Queiroz et al.[7] and Almeida et al.[8] reported higher immunoglobulin G (IgG) production in mice that were pretreated with *T. cruzi* biotherapies. Sandri et al.[12] reported a decrease in serum transforming growth factor β (TGF-β) concentrations, an increase in the number of apoptotic cells, and a decrease in hepatic inflammation in animals that were treated with biotherapy 17dH after infection. In addition to immunomodulatory action, Ferraz et al.[11] reported that pretreatment with *T. cruzi* biotherapy 7dH altered the dynamics of blood cells in mice that were experimentally infected with *T. cruzi*.

Despite the evidence that biotherapies modulate the course of *T. cruzi* infection,[7–12] these experiments raise the following intriguing question: What information is transferred by *T. cruzi* biotherapies to organisms (Swiss mice) that are infected and treated with different treatment schemes and dynamizations? The aim of the treatment (pre- and/or postinfection) with *T. cruzi* biotherapies in the present study was to modulate the immune system of the host against the infection. These medications prepared from blood (buffy coat and parasites) of Swiss mice infected with *T. cruzi*, which has been used in this and other studies,[9–12] have in its constitution the 'antigenic information' that is related to the parasite and 'disease information' that is related to

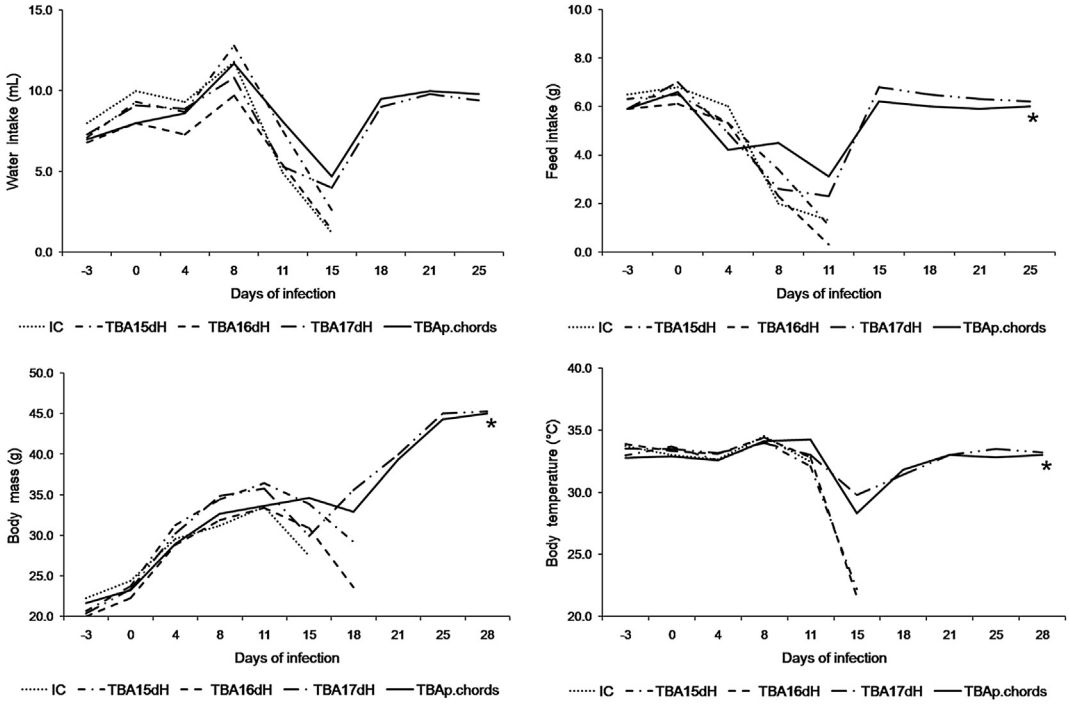

Figure 5 Clinical parameters assessed in groups of mice infected with *T. cruzi* and submitted to the treatment with different dynamizations of biotherapies. Groups: IC – infection control; TBA_{15dH} – animals were treated with biotherapy 15dH; TBA_{16dH} – animals were treated with biotherapy 16dH; TBA_{17dH} – animals were treated with biotherapy 17dH; $TBA_{p.chords}$ – animals were treated with biotherapy "potency chords". Biotherapies were administered to animals 3 days before and 3 days after infection. *Statistical significance ($p < 0.05$), on days 11–28 after infection compared with the IC group.

immune response pattern of the animal infected, with the influence of cells and molecules produced by the host's immune system (Swiss mice). Interestingly, experiments performed by our group using biotherapy prepared from mouse serum chronically infected with *T. cruzi* (contain significant concentrations of molecules that are produced by the immune response of Swiss mice – 'disease information', since the antigen concentration is possible but very low) in the experimental infection, demonstrated immune system modulation, increased parasitemia, and decreased survival of the treated animals.[33] Therefore, the 'disease information' that is transferred to a sick and susceptible organism can exacerbate blood parasitemia in an attempt to promote a reaction in the host organism. Moreover, the susceptibility (higher morbidity and mortality) of the experimental model used (Swiss mice *versus T. cruzi*–Y strain infection)[28] does not always afford beneficial modulation, with survival of infected animals. This is likely attributable to the treatment scheme (e.g., frequency of stimuli) and dynamization inadequate of the highly diluted medication associated with the host's susceptibility to infection.[10,34]

The outcomes suggested that the effects of the biotherapies that were prepared from the blood (buffy coat + parasites) of Swiss mice that were infected with *T. cruzi* are attributable to the modulatory action of 'antigenic information' that is related by the parasite and 'disease information' that is related to the molecules and cells that are produced by the infected host's immune response and contained in the buffy coat that was used to prepare the medications. These effects, beneficial or detrimental, vary according to the modulatory actions that are produced by the frequency of stimuli that are generated by different treatment schemes and dynamizations of *T. cruzi* biotherapies. The effects also depend on the host–parasite relationship and individual susceptibility of the organism to infection. According to Aleixo et al.,[10] the use of *T. cruzi* biotherapy 17dH in experimental infection with the Y strain in Swiss mice, with an appropriate treatment scheme, appears to favor mice with a tendency to develop lower levels of parasitemia (i.e., there are individual variations in the susceptibility to infection, even in susceptible species[28]), producing decrease in parasitemia, clinical improvement, and a longer survival time in treated animals compared with the control group.

Although this was a basic research, the outcomes of these and other *in vivo* and *in vitro* experiments with highly diluted medications (constitutional and biotherapies medications) demonstrate that the treatment scheme (frequency of stimuli),[9–10] dynamization,[35,36] and specific reactions of individual organisms to the medication (e.g., susceptibility of the host to infection)[10] influence the effects. As observed and described by Hahnemann,[34] such therapeutic strategies deserve attention by homeopathic physicians in clinical situations. Moreover, suggest that researches which evaluate the effect of *T. cruzi* biotherapy should be stimulated and further deepened to a clinical study in human patients,[37,38] because this highly diluted medication appears to have potential for use as preventive and/or therapeutic treatment[7–10] in populations of Chagas disease-endemic areas.

Conclusions

In summary, the evaluation of parasitological and clinical parameters showed that different treatment schemes using *T. cruzi* biotherapy 7dH produced differential effects on the evolution of *T. cruzi* infection. Biotherapy 7dH, when administered 3 days before and 3 days after infection (TBA_{7dH}), had the best outcome, including lower parasitemia, higher survival, and better clinical evolution in infected animals. The *T. cruzi* 'potency chords' biotherapy that was administered 3 days before and 3 days after infection ($TBA_{p.chords}$) presented effects that differentiated it from the individual dynamizations that it contained (15dH, 16dH, and 17dH). In animals that had patent parasitemia, delayed emergence of the parasite was observed in blood, with a subsequent increase in parasitemia. However, these animals maintained a better evolution of clinical parameters. These outcomes demonstrate that the effects of these biotherapies that were prepared from the blood (buffy coat + parasites) of Swiss mice infected with *T. cruzi* varied according to the frequency of stimuli that were generated by the different treatment schemes and dynamizations and depend on the host−parasite relationship and individual susceptibility of the organism to infection. Moreover, the results suggest that the effects are attributable to the modulatory action of 'antigenic information' that is related to the parasite and 'disease information' that is related to the molecules and cells that are produced by the infected host's immune response and contained in the buffy coat that was used to prepare the medications.

Conflict of interests

The authors declare that they have no conflict of interests.

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