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ORIGINAL PAPER

Different forms of administration of biotherapy 7dH in mice experimentally infected by *Trypanosoma cruzi* produce different effects

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Objective: **To evaluate the effects of different forms of administration of the blood trypomastigotes biotherapy 7dH in mice experimentally infected with *Trypanosoma cruzi*.**
Material and methods: **Male swiss mice were inoculated with 1400 blood trypomastigotes of the Y strain of *T. cruzi* and allocated into 5 treatment groups: IC (distilled water); TCBZ (benznidazole); TBA_{7dH} (biotherapy 7dH 20 days after infection); $TBB_{7dH}7$ (biotherapy 7dH seven days before infection); $TBB_{7dH}30$ (biotherapy 7dH 30 days before infection). Parasitological parameters assessed included pre-patent and patent periods, parasitemia peak, total parasitemia, mortality and survival rates. Cure index was obtained by fresh blood examination, hemoculture and polymerase chain reaction (PCR).**
Results: **The $TBB_{7dH}7$ group showed a reduction in parasitemia peak, parasitemia area under the curve and total parasitemia. $TBB_{7dH}30$ showed a tendency to increased pre-patent and survival periods, peak parasitemia was increased without increased total parasitemia. TBA_{7dH} did not present significant alterations in the parasitological parameters analyzed.**
Conclusions: **Biotherapy 7dH given before infection (7 or 30 days) produces different effects suggesting modulation of the host's immune system. The effects range from reduced parasitemia to its effective increase. The use of biotherapy to treat *T. cruzi* infection including dose, potency and schedule deserves further investigation.** *Homeopathy* (2011) **100**, 237–243.

Keywords: *Trypanosoma cruzi*; Nosode; Chagas disease; Homeopathy; Biotherapy

Introduction

Trypanosoma cruzi (Chagas, 1909) is the causal agent of Chagas disease. The distribution of this infection in humans stretches from the south of the United States to the south of Argentina, affecting approximately eight million people.[1] Two drugs, benznidazole (BZ) and nifurtimox are available for the aetiological treatment of Chagas's disease. In Brazil, only BZ is commercially available. In addition to causing significant adverse effects, this drug is around 80% effective during the acute phase of the infection, but largely ineffective during the chronic phase.[2,3] Even though Chagas's disease is considered a neglected disease, the search for more effective drugs continues to be the main focus of many researchers.[4–9]

Homeopathy is one of the complementary/alternative medical methods most commonly employed around the world, and the WHO (World Health Organization) has been stimulating the use of traditional/complementary/alternative medicines within health systems, integrated with more classical forms of treatment. In Brazil, the Health Ministry has recently approved the 'National Policy for Integrative and Complementary Practices in the Public National Health Service',[10] aiming at motivating and

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providing support to homeopathic assistance, teaching and research projects within the several levels of the Public National Health System together with other non-conventional practices.[11]

Homeopathic medicine is based on Hahnemann's Principle of Similars. It is characterized by the use of highly dilute, dynamized doses of substances capable of stimulating, in a healthy individual, manifestations similar to those symptoms presented by a sick person, to whom this substance is recommended as treatment.[12]

One of the earliest and most notable innovations of homeopathy is isopathy or isotherapy.[13] Within the isopathic system we find those medicines known as biotherapies or nosodes, which are defined as medicines prepared from chemically undefined biological products, such as secretions, excretions, tissues and organs (pathological or not), as well as products of microbial or allergen origin.[14] Biotherapies are widely used in homeopathy, and their use in the treatment and prophylaxis of infectious or parasitic diseases has been investigated by several researchers.[6,7,15,16]

Over the last years there has been an increase in the numbers of controlled scientific experiments both *in vitro* and *in vivo*, carried out to assess the effectiveness of homeopathic medicines.[17] The use of mice as an experimental model for studies *in vivo* of *T. cruzi* infection reliably reproduces many of the physiological, biochemical, immunologic and pathological mechanisms related of Chagas's disease. The murine model enables the study of new therapeutic targets for the control or cure of infection, aiming at improving Chagas's patients' quality of life.[18]

The objective of this work was to assess *in vivo* the effect of a biotherapy prepared from blood *T cruzi* trypomastigotes in the 7dH dilution in different forms of administration on mice experimentally infected with *T. cruzi*.

Material and methods

Ethics

The study design was approved by the State University of Maringá (UEM) Ethics Committee for Experiments in Animals – Registration n° 030/2008. The surviving animals were sacrificed by deepening anesthesia with ketamine chlorhydrate (50 mg/kg) and xylazine (10 mg/kg) intraperitoneally. All recommendations of the National Law (N° 6.638, November, 5th 1979) for scientific management of animals were respected. The animals had free access to food and water.

Animals

Male Swiss mice with four weeks of age, supplied by the UEM Central Bioterium were used in the study and allocated into 5 treatment groups:

IC – Infection Control, animals were treated with distilled water.
TCBZ – Treatment Control, animals were treated with BZ.
TBA_{7dH} – Animals were treated with biotherapy 7dH during 20 days after infection.
$TBB_{7dH}7$ – Animals were treated with biotherapy 7dH seven days before infection.
$TBB_{7dH}30$ – Animals were treated with biotherapy 7dH 30 days before infection.

There was no statistically significant difference in the average weight of between groups. After weighing, the animals were placed in unlabelled individual cages. The mice were randomly assigned to treatments. After infection, the treatment, which each group received, was determined by drawing of lots. 7–13 animals in each group were used in each experimental group.

Parasite and inoculum

Animals were intraperitoneally inoculated with 1400 blood trypomastigotes of the Y strain of *T. cruzi*. This strain was isolated from a patient during acute phase of Chagas disease. It belongs to the *T. cruzi* II genetic group,[19] which is a reticulotropic, highly virulent strain, with parasitemia peak in the eighth day of infection and produces 100% mortality in Swiss mice.[20]

Biotherapy 7dH

The biotherapies used in this work were produced in a specialized homeopathic pharmacy from blood containing blood trypomastigotes collected from the orbital plexus of three mice on the seventh day of infection by the Y strain of *T. cruzi*. Blood was centrifuged at 840 rpm for 10 min. The leukocyte layer (with higher concentration of trypomastigotes) was carefully collected for use. The biotherapy preparation was carried out by adding 0.9 mL of a *T. cruzi* concentrate (4.1×10^7 trypomastigotes/mL) to 9.1 mL of distilled water in a 15 mL amber glass flask, and succussed 100 times. Dynamisations were performed in an 85°GL alcoholic solution up to 6dH, and 7% alcohol solution in dynamisation 7dH for the treatment of animals.[14]

Treatment plan

BZ (Rochagan – ROCHE), a standard treatment control drug, was orally administered at one dose of 100 mg/kg/day/animal for 20 consecutive days to the animals in TCBZ groups. Biotherapy 7dH was orally administered at a volume of 0.2 mL/day/animal for 20 consecutive days to the mice in TBA_{7dH}. IC groups received orally for 20 consecutive days 0.2 mL/day/animal of distilled water. Distilled water was used as control after additional separate experiments showed no statistical difference in outcome in the control group treated with distilled water or the alcoholic solution used for biotherapy preparation. For all these four groups the treatment was only initiated after infection by *T. cruzi* had been evidenced (4th day after inoculation). For the animals in the two groups treated with biotherapy 7dH before infection ($TBB_{7dH}7$ and $TBB_{7dH}30$), 0.2 mL/day/animal of the medicament was given orally for 7 and 30 consecutive days before the animals were infected with *T. cruzi*, respectively. The treatments were given by one person who did not take part in assessment of the parasitological parameters. In addition to visual control of intake of each

dose, animals were not allowed water 1 h before treatment was used to ensure the entire dose was ingested.

Assessment parameters

Parasitological parameters: Parasitemia was assessed using Brener's technique: 5 μL of blood collected from the caudal vein and examined on microscope slides.[21] Parasites count was performed daily from the 4th day after infection until the animal's death. The parasitemia curve was drawn by using the mean parasitemia values of the animals inoculated in each group. From this parasitemia curve the following information was obtained: ***pre-patent period*** (mean time, in days, between inoculation day and the day when the fresh blood examination (FBE) was found positive); ***patent period*** (mean time, in days, with parasitemia detected in the FBE); ***parasitemia peak*** (highest mean parasitemia observed); ***total parasitemia*** (mean of the sum of parasitemia of each mice over the experiment); ***mortality rate*** (total number of dead animals in relation to the number of animals infected, observed up to 120 days after infection); and ***survival rate*** (mean life time of the surviving animals, in days, after infection, observed up to 120 days). The parasitemia of the mice of all groups was obtained blind, those who assessed the parasitemia were not aware of the treatment the animals had received.

Cure index: The mouse was considered to be cured when it presented negative results for all the three methods described below. Cure index, for each method, was obtained by the ratio between the number of animals cured, in a given treatment, and the total number of treated animals × 100.

a) *FBE*: 5 μL of blood was collected from the caudal vein of mice on day 30 after the end of the treatment.[21]

b) *Hemoculture (HE)*: Thirty days after the end of treatment, 500 μL of blood collected from the orbital plexus of each mouse was distributed into two test tubes containing 3 mL of Liver Infusion Tryptose (LIT) medium and incubated at 28°C. The material was examined on day 30, 60 and 90 after HE was performed.[22]

c) *Polymerase Chain Reaction (PCR)*: Thirty days after the end of the treatment, 100 μL of venous blood of each animal were added to the 200 μL Guanidine/EDTA (Ethylenediamine Tetraacetic Acid) 6.0 M and kept in room temperature for at least 7 days.[23] One week later, the lysate was boiled for 7 min and kept at 4°C. DNA extraction and precipitation were carried out according to Wincker *et al.*,[24] modified by Gomes *et al.*[25] DNA was amplified, using specific primers 121 (5'AATAATGTACGGG(T/G)GAGATGCATGA3') and 122 (5'GGGTTCGATTGGGGTTGGTGT3'), according to Gomes *et al.*[26] Briefly: 2 μL of DNA solution from each sample was added to a solution containing 10 mM Tris–HCl (pH 9.0), 75 mM KCl, 3.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mM dATP (deoxyadenosine triphosphate), dCTP (deoxycytidine triphosphate), dGTP (deoxyguanosine triphosphate) and dTTP (deoxythymidine triphosphate) (Sigma Company Ltd., USA), 10 pmol of each primer, 1 U of Taq DNA polymerase (Invitrogen, USA) and milli-Q water to complete 10 μL. To this mixture were added 30 μL of mineral oil, the reaction was performed in an automatic thermocycler (MJ Research – PTC 150). The tubes were subjected to 35 amplification cycles: DNA denaturation at 95°C for 1 min (with longer initial step for 5 min), annealing of primers at 65°C for 1 min and extension at 72°C for 1 min (the final step of 10 min.). Amplification products were submitted to polyacrylamide gel electrophoresis at 4.5% and visualized through the impregnation with silver salts.[27]

Study design

This work was carried out in a series of three experiments. Each experiment consisted of an IC group, a treatment control group (TCBZ), and one of the following three experimental groups: Experiment 1: IC + TCBZ + TBA_{7dH}; Experiment 2: IC + TCBZ + $TBB_{7dH}7$; Experiment 3: IC + TCBZ + $TBB_{7dH}30$. All experiments were carried out in duplicate.

Statistical analysis

Data were compared using Statistica 6.0 Software, at a significance level of 5%. For each variable analyzed, normality Kolmogorov–Smirnov (K–S) test was performed. For those with a normal distribution, Analysis of Variance (ANOVA) test and Least Significant Difference (LSD) test were carried out. For variables that were not normally distributed, Median Test was performed. Chi-square test was used for comparing percentages.

Results

Biotherapy 7dH – treatment 7 days before infection (Experiment 1)

Table 1 shows the parasitemia parameters for each experimental group. The area under the parasitemia curve ($p = 0.029$), parasitemia peak (7 days after infection – $p < 0.001$) and total parasitemia ($p < 0.001$) were significantly lower for $TBB_{7dH}7$ in relation to the IC (Figure 1A).

Considering survival time, group $TBB_{7dH}7$ presented survival 6.2% higher than that of IC. Mortality rate for $TBB_{7dH}7$ and IC was 100%. There were no deaths in the TCBZ group during the 120 days of observation. Cure index could only be calculated for TCBZ, being 71% (FBE), 43% (HE), and 29% (PCR).

Biotherapy 7dH – treatment 30 days before infection (Experiment 2)

Table 2 shows the parasitological parameters results obtained for biotherapy 7dH used as treatment 30 days before infection. Group $TBB_{7dH}30$ presented a pre-patent period 19% longer than the other groups ($p = 0.086$). $TBB_{7dH}30$ showed shorter patent period than IC. Parasitemia peak (8 days after infection) was different among groups ($p < 0.001$, Figure 1B), being higher in $TBB_{7dH}30$ than IC ($p = 0.044$). Total parasitemia ($p = 0.683$) and area under the parasitemia curve ($p = 0.285$) were not different between

Table 1 Parasitological parameters assessed in groups of male Swiss mice experimentally infected with *T. cruzi*, submitted to a treatment with blood trypomastigotes biotherapy 7dH seven days before infection

Group	*Pre-patent period (days)**	*Patent period (days)**	*Parasitemia peak (trypomastigotes/mL)**	*Total parasitemia (trypomastigotes/mL)**	*Post-infection survival (days)**	*Mortality (%)*	*Mortality (N/T)*
IC	4.0 ± 0.0	9.16 ± 1.16^{a}	$7.54 \times 10^{5} \pm 3.18 \times 10^{5a}$	$2.39 \times 10^{6} \pm 8.66 \times 10^{5a}$	9.17 ± 1.17^{a}	100^{a}	7/7
$TBB_{7dH}7$	4.0 ± 0.0	9.78 ± 0.07^{a}	$1.88 \times 10^{5} \pm 9.99 \times 10^{4b}$	$1.11 \times 10^{6} \pm 5.29 \times 10^{5b}$	9.78 ± 0.07^{a}	100^{a}	9/9
TCBZ	4.0 ± 0.0	1.71 ± 0.76^{b}	0.0 ± 0.0^{c}	$1.78 \times 10^{4} \pm 1.13 \times 10^{4c}$	180 ± 0.0^{b}	0^{b}	0/7

IC – Infected Control; $TBB_{7dH}7$ – Treated with biotherapy 7dH seven days before infection; TCBZ – Treatment Control with BZ. N/T – number of dead/total mice in the group. Different letters in a column means significant difference ($p \leq 0.05$).
* Mean ± standard deviation.

IC and $TBB_{7dH}30$. Mortality rate was statistically different ($p < 0.0001$) among groups, being 92.3% for $TBB_{7dH}30$ and 100% for IC. Survival rate was also different among groups ($p = 0.004$), with $TBB_{7dH}30$ presenting the second longest survival period. Cure index for TCBZ, was 88% (FBE), 75% (HE and PCR). For $TBB_{7dH}30$, the surviving mouse presented negative FBE, positive HE (only for one tube) after 60 days of culture, and PCR was negative on day 30, and positive (one sample) on day 90 after treatment, giving a cure index 7.7% for the FBE and 0% for HE and PCR.

Biotherapy 7dH – treatment after infection (Experiments 3)

No statistical difference was observed in any parameters (pre-patent period, patent period, parasitemia peak, total parasitemia, mortality and survival) for TBA_{7dH} in relation to IC. Only mice which received TCBZ survived, their cure index was 100% (FBE and HE) and 83.3% (PCR). The cure index obtained for TBA_{7dH} group was 0%.

Reproducibility

All experiments were repeated at least twice. Apart from the fact that the experiments reproduced essentially the same results when repeated, when considering all experiments no statistical difference between parasitemia curves was found between IC groups ($p = 0.129$). Cure indices found for TCBZ groups in each experiment for each technique did not show any statistical difference either (FBE, $p = 0.918$; HE, $p = 0.634$; PCR, $p = 0.510$).

Discussion

This work assessed *in vivo* the effect of different treatment plans with blood trypomastigotes biotherapy 7dH given to mice experimentally infected with *T. cruzi*.

Biotherapy 7dH, when used as treatment 7 days before infection, decreased the area under the parasitemia curve, parasitemia peak and total parasitemia of the infected mice without, however, decreasing mortality rate or increasing survival time. In experimental infection by *T. cruzi*, parasitemia means morbity,[28] and its decrease indicates that the treatment used was beneficial. Almeida *et al.*,[6] using a pre-infection treatment with a *T. cruzi* biotherapy 12dH and a different therapeutic plan and lineage of mice, observed a decreased pre-patent period and decreased parasitemia in some assessment days, without a significant decrease in parasitemia peak and lower rate of mortality. Queiroz *et al.*,[7] using a biotherapy prepared from culture trypomastigotes, in 30dH, and a different pre-infection treatment plan, observed a 50% reduction in mortality, an increase in survival time without decreased parasitemia. Projects involving experimental treatment of mice infected by *T. cruzi* with biotherapy were planned and executed very differently.[6,7] If some of them show more promising results, others added important new information, which leads to the conclusion that the complexity of the parasite–host relationship is an important factor in producing the effect of treatment with ultradiluted substance, and in all cases some effect was observed.

Our results, as well as those obtained by other authors[6,7,29] suggest that a biotherapy given before infection is capable of

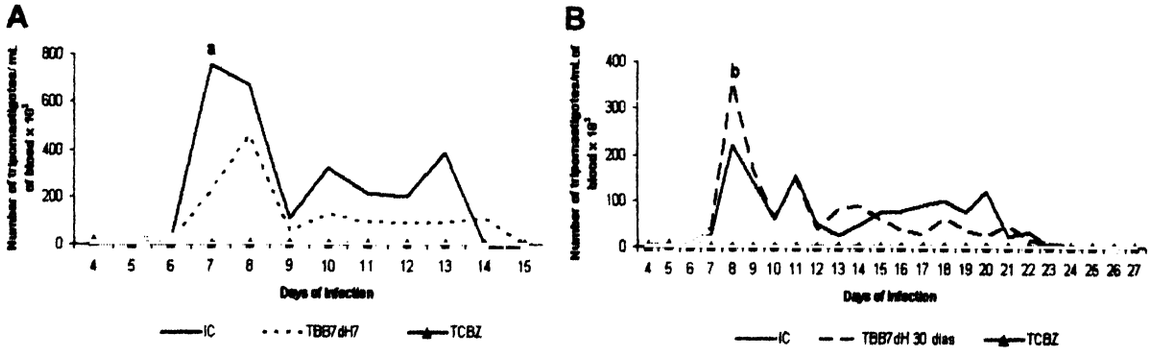

Figure 1 A – Parasitemia curve of male Swiss mice infected with 1400 blood trypomastigotes, Y strain of *T. cruzi* for groups $TBB_{7dH}7$, IC and TCBZ ($^{a}p = 0.000$). B – Parasitemia curve of male Swiss mice infected with 1400 blood trypomastigotes, Y strain of *T. cruzi* for groups $TBB_{7dH}30$, IC and TCBZ ($^{b}p = 0.000$).

Table 2 Parasitological parameters assessed in groups of male Swiss mice experimentally infected with *T. cruzi*, submitted to a treatment with blood trypomastigotes biotherapy 7dH thirty days before infection

Group	*Pre-patent period (days)**	*Patent period (days)**	*Parasitemia peak (trypomastigotes/mL)**	*Total parasitemia (trypomastigotes/mL)**	*Post-infection survival (days)**	*Mortality (%)*	*Mortality (N/T)*
IC	4.00 ± 0.00^{a}	13.63 ± 2.45^{a}	$2.21 \times 10^{5} \pm 1.29 \times 10^{5a}$	$9.58 \times 10^{5} \pm 2.08 \times 10^{5a}$	13.75 ± 2.49^{a}	100^{a}	8/8
$TBB_{7dH}30$	4.92 ± 1.55^{a}	11.92 ± 2.84^{a}	$3.53 \times 10^{5} \pm 2.07 \times 10^{5b}$	$1.07 \times 10^{6} \pm 2.99 \times 10^{5b}$	25.46 ± 46.48^{a}	92.3^{a}	12/13
TCBZ	4.00 ± 0.00^{a}	2.11 ± 0.76^{b}	0.0 ± 0.0^{c}	$8.09 \times 10^{3} \pm 7.97 \times 10^{3c}$	180 ± 0.00^{b}	0^{b}	0/9

IC – Infected Control; $TBB_{7dH}30$ – Treated with biotherapy 7dH 30 days before infection; TCBZ – Treatment Control with BZ. N/T – number of dead/total mice in the group. Different letters in a column means significant difference ($p < 0.05$).
* Mean ± standard deviation.

stimulating the host's immune system, protecting it when the organism is exposed to the respective antigen, in a similar way as observed for vaccines produced with antigens of *T. cruzi*.[30,31] This hypothesis is supported by the present study due to the low dynamisation of the biotherapy, which contains molecules of the parasite.[32]

In contrast, biotherapy 7dH, when used as treatment 30 days before infection, showed a tendency to increase the pre-patent period and survival time of the infected animals and decrease mortality, despite increased peak parasitemia without increasing total parasitemia and parasitemia areas under the curve. These data suggest a homeopathic effect, with parasitemia peak exacerbation, without an increase in total parasitemia, together with a tendency to decreased mortality and increased survival time. These results suggest that timing is important, these effects were not observed when biotherapy 7dH was administered 7 days before infection.

Although other hypotheses should be explored to explain the higher parasitemia peak observed one possibility is the involvement of apoptosis and/or a special modulation of the immune system by changing the balance of pro and anti-inflammatory cytokines involved in Chagas disease. The higher parasitemia peak can be understood from the explanation that homeopathic formulations pass information to the organism treated, and this information is disseminated throughout the organism.[12] Homeopathic preparations may increase communication between cells.[33,34] The function of apoptosis, or programmed cell death is to remove altered cells.[35] In an attempt to eliminate infection by *T. cruzi*, infected cells undergo apoptosis and, consequently, release the parasites into the blood stream, increasing the parasitemia peak. The literature suggests homeopathic medicaments can apoptosis.[36] Apoptosis is a target to treat *T. cruzi* infection, and that BZ can suppress apoptosis.[37] Sandri demonstrated that apoptosis is augmented in mice *T. cruzi* infection treated with biotherapy.[38]

According to Carillo,[39] living organisms are complex, organized and systemic and the disease is not bad in itself, but just a movement by the organism to restore equilibrium. To treat homeopathically, therefore, is to assist this process, making the establishment of a complete re-balance quicker and more effective.

With biotherapy 7dH used as treatment 30 days before infection, one mouse survived. Analysis showed FBE negative, HE late positive in just one culture tube, and PCR positive just in one sample, 90 days after treatment. Both HE and PCR have high sensitivity to experimental murine infections,[21,40] and the results observed in this study demonstrated, for this animal, an extremely low parasitemia. It is important to point out that, for the lineage of mice, strain and inoculum of the parasite used in the present study, all animals infected, without exception, die if untreated.

Biotherapies in general act by stimulating the body's immune system.[6,7,15,16] However, mechanisms involved in modulation of the immune system must be investigated, including the balance of pro and anti-inflammatory, apoptosis and other mechanisms involved in resistance to infection with *T. cruzi*.

Biotherapy 7dH when used as treatment after infection, did not show any significant effect in terms of parasitological parameters assessed. Other authors have found similar results, using biotherapies prepared with the same strain of *T. cruzi*, but with other preparation techniques, different dynamisations, and different therapeutic plans,[6,7] though always after infection. For other types of microorganisms, there are data in the literature that show the positive effect of biotherapies as treatment after infection, which have been experimentally used in the clinical practice of human and veterinary medicine.[15,16] These contradictory results emphasise the complexity of host-parasite relationship in experimental infection with *T. cruzi*.

We also evaluated the effect of a blood trypomastigotes biotherapy 12cH prepared by trituration (data not shown). It was administered to mice for 20 days after infection. The results were similar to that of biotherapy 7dH administered after infection, in other words, did not alter the course of experimental infection with *T. cruzi*. According to Hahnemann (paragraph 270 of the Organon of the art of healing – sixth edition) the medicament prepared from triturating blood trypomastigotes in lactose should be capable of activating the latent medicinal strength present in substances.[40]

In this study, BZ, was used as a control for treating Chagas disease.[28] In all experiments the cure index for the surviving animals (treated with BZ) did not present statistically significant differences between experiments. These results confirm the reproducibility of our data obtained, in contrast to some other experiments with homeopathic medicaments.[12,41] Moreover, the data presented here confirm the importance of using several diagnosis techniques (parasitological, serological and molecular) for experimental chemotherapy studies.[22,28,42]

The parasitemia peak reduction seen in $TBB_{7dH}7$ and its increase in $TBB_{7dH}30$, showed the non-linearity of the

effects and the global reaction characteristic of homeopathy.[43] They give clues on the characterization of the phenomenon, and that it is different from traditionally known mechanisms. Therefore, there is a need to conceive new lines theories and models that can objectively interpret the diversity of results obtained in homeopathy.[43]

Conclusion

This study shows that biotherapy 7dH used as treatment in mice 7 days before experimental infection by *T. cruzi* decreased, parasitemia area under the curve, parasitemia peak and total parasitemia, but not increased survival time. When given 30 days before infection, biotherapy 7dH increased parasitemia peak without increasing total parasitemia and parasitemia area under the curve, showing a tendency to decrease mortality and increase survival time. On the other hand, as a treatment after infection, biotherapy 7dH did not significantly alter the course of infection by *T. cruzi*. As medication administered prior to infection by *T. cruzi* as well as at post-infection treatment, dose of biotherapies, the potency and the treatment schedule deserve further investigation.

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