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Original Article

Randomized study using biotherapeutic "*T. cruzi* 3dH" impairs experimental infection by *Trypanosoma cruzi*

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ABSTRACT

Objective: The aim of this study was to evaluate the effect of a biotherapeutic in very low dynamization (3dH) in murine infection by *Trypanosoma cruzi*. **Materials and Methods:** Swiss male mice (*Mus musculus*) infected by *T. cruzi* Y strain were divided into three groups according to their treatment: Control infection - 7% ethanol-water solution; 3dH each day (ED) - daily biotherapeutic *T. cruzi* 3dH, from the day of infection until the end of experiment; 3dH single day (SD) - biotherapeutic *T. cruzi* 3dH, on the day of infection for 12 h. Parasitological (total parasitemia, peak of parasites, day of the peaks, prepatent period, patent period, and survival) and clinical (body temperature and body weight) parameters were evaluated. **Results:** No significant differences were observed among groups for parasitological evaluation. 3dH ED group presented an earlier mortality compared to control. Clinical parameters showed that animals treated with biotherapeutic had worse outcome when compared with control animals. **Conclusion:** The low dynamization of *T. cruzi* biotherapeutic worsened its murine infection by means of clinical evaluation. Considering other studies using biotherapeutic of *T. cruzi*, suggests the possibility for an efficacy of different dynamizations regarding oscillatory potency-effect-curves.

KEY WORDS: Biotherapeutic, oscillatory potency-effect-curve, *Trypanosoma cruzi*

INTRODUCTION

Biotherapeutic medicines are prepared according to homeopathic pharmacotechnics from organic products [1]. Several studies approaching the use of biotherapeutics in murine infection by *Trypanosoma cruzi* were carried out with encouraging results [2-5].

The infection by *T. cruzi* was studied in experimental murine models using Y strain [6]. In those models, the strain shows well-defined characteristics such as high parasitemia, peak of parasites on 8th day of infection, death of all untreated infected animals; thus, being an excellent experimental model, which may be useful for testing medications that may be later used in humans [7].

The homeopathic medication is used in several dynamizations and schedules of treatment since the time of Hahnemann, the "father" of Homeopathy [8,9]. Hahnemann reports that the most appropriate medication depends not only on the correct homeopathic selection but also the dose and frequency [8]. He observed that homeopathic medicines administered improperly can cause harm greater than the disease itself and mentions the necessity for regulation of the homeopathic medicinal doses as much as possible, to make them suitable for each patient [9].

Some studies point out that lower dynamizations are often used in acute cases to provide the organism with the added stimulation required [10], however, Deroukakis [11] designated that there are many theories about potency selection. Indeed, potency selection is a complicated issue, considering that homeopathic treatments generally have a sinusoidal behavior, in which the idea of an oscillatory potency-effect-curve could explain the non-linearity of homeopathic medicine effects, and

the fact that some potencies are effective, and some potencies are no more effective in certain cases [12].

Considering murine *T. cruzi* infection as an acute case, the use of lower dynamizations could improve the infection evolution [7,13]. In this context, the aim of this study was to evaluate the effect of biotherapeutic of *T. cruzi* in very low dynamization (3dH) in murine infection by the protozoan, by measuring of parasitological and clinical parameters using two different schedules of treatment.

MATERIALS AND METHODS

The experiment was approved by Ethics Committee for Animal Experimentation (CEEA/UEM) under protocol number 030/2008 following Brazilian Guidelines for Care and Animals Use (DBCA) [14] elaborated by the National Council of Animal Experimentation Control (CONCEA).

Experimental Animals

The study involved 42, 56-day-old male Swiss mice, *Mus musculus*, from the Central Animal Facility of Universidade Estadual de Maringa. The animals were infected intraperitonealy with 1,400 blood trypomastigotes of *T. cruzi* Y Strain [7]. They were kept in controlled conditions: Temperature 22.7 ± 1.2°C, light/dark cycles of 12 h, filtered water and food *ad libitum*.

Animal Groups

The animals were divided into three groups according to the treatment: (1) control infection (CI) group (n = 12) - infected animals treated with 7% ethanol-water solution, in water (10 μl/ml) offered *ad libitum* in an amber bottle; (2) 3dH each day (ED) group (n = 15) - infected animals treated with the biotherapeutic *T. cruzi* 3dH, in water (10 μl/ml) offered *ad libitum* in an amber bottle from the day of infection until the end of the experiment; (3) 3dH single day (SD) group (n = 15) – infected animals treated with the biotherapic *T. cruzi* 3dH, in water (10 μl/ml) offered *ad libitum* in an amber bottle on the day of infection for 12 h.

The animals were divided into cages so that the mean initial weights of mice in each group were statistically equal. The sample size considered statistical analysis [15] and the 3R's emphasizing reduction, refinement, and replacement of animal use [16].

Biotherapeutic *T. cruzi* 3dH

The medication was produced from the buffy coat of blood collected from the orbital plexus of three mice, containing blood trypomastigotes on the 7th day of infection with *T. cruzi* Y strain. The medication was prepared by adding 0.9 ml of *T. cruzi* concentrate (4.1×10^7 trypomastigotes/ml) to 9.1 ml of sterile bidistilled water in a 30 ml glass bottle (1 dH). The dynamization 2dH was made in a 70% ethanol-water solution. The dynamization used, 3dH, was prepared with 7% ethanol-water solution [1] and stored in comercial glass. The water used was bidistilled and sterilized. The microbiological test and biological risk were negative, according to the regulations of the Brazilian Ministry of Health (RDC n 67) [17].

Parameters Evaluated

Parasitological and clinical parameters were evaluated. Parasitemia was evaluated by daily counts of blood trypomastigotes from the day of infection, using the technique of Brener [18]. Using the parasitemia curve, the following data were obtained: Total parasitemia (P_{total}), calculated as the sum of the mean daily parasitemia levels for each mouse in each group; maximum peak of parasites (P_{max}), the mean of the highest level of parasitemia observed in each animal throughout the experiment; the day on which these peaks occurred (DP_{max}); prepatent period (PPP), which is the time from infection to detection of the parasite in blood; patent period (PP), the period when the parasitemia can be detected; and survival computed during the course of the experiment. The animals were clinically evaluated for body temperature and body weight to measure the welfare of each mouse.

Experimental Design

The experiment was performed twice, as a blind randomized controlled trial to minimize effects of subjective bias. The report was based according to ARRIVE - Animals in Research: Reporting *in vivo* Experiments) [19]; and REHBaR - Reporting Experiments in Homeopathic Basic Research [20] guidelines, used to improve reporting of research using animals and homeopathy.

Statistical Analysis

The results were compared statistically using Mann–Whitney U test by the software BioStat 5.0. The survival analysis and the curve of survival were obtained using the log-rank test in the program Prisma 6.0. The acceptable level of significance was 5% for all analyses.

RESULTS

To our knowledge, this is the first study to evaluate the effect of a biotherapeutic in very low dynamization (3dH) in a murine model of *T. cruzi* infection, comparing two different schedules of treatment.

Parasitological Parameters

Parasitological parameters are shown in Figure 1 and Table 1. The parasitemia curve [Figure 1] shows a slight reduction of daily parasitemia with no statistical difference compared with control group, in groups treated with biotherapeutic *T. cruzi* 3dH (P = 0.339 for 3dH ED, and P = 0.308 for 3dH SD). There was also no difference among treated groups for this parameter (P = 0.979).

Table 1: Parasitological parameters of animals subjected to different treatment schedules using biotherapeutic *T. cruzi* 3dH

Group	P_{total} ($\times 10^5$)	P_{max} ($\times 10^5$)	DP_{max} ($\times 10^5$)	PPP (days)	PP (days)	Survival (days)
CI	13.75±8.09	7.09±4.57	9.09±1.97	4.45±1.21	10.36±1.28	15.8±1.68
3dH ED	9.94±4.65	5.17±2.81	8.2±0.77	4.86±1.18	9.06±1.66*	13.8±1.74
3dH SD	11.21±5.02	6.46±4.02	8.2±0.56	4.73±1.09	9.80±1.52	14.57±1.45

*$P<0.05$, *T. cruzi: Trypanosoma cruzi*, SD: Single day, ED: Each day

Similarly, no significance was observed in treatment groups to the control group for P_{total}, P_{max}, and PPP ($P > 0.05$) [Table 1]. For PP, significant difference in the comparison was observed between control and 3dH ED group ($P = 0.039$), however, this data is directly related to the early death of some animals observed in this treated group, which presented lower surviving compared with control ($P = 0.004$) compatible with clinical worsening of infection.

Survival

The survival curve is shown in Figure 2. Infected animals treated with biotherapic *T. cruzi* 3dH showed early death compared to the control group. The group 3dH ED presented a significant early death ($P = 0.004$) compared to control. Although presented early death, the difference in 3dH SD group was not significant ($P = 0.089$). Furthermore, there was no difference for survival both treated groups ($P = 0.118$).

Clinical Parameters

The results for clinical parameters (temperature and weight) are shown in Figure 3. We observed that animals treated with *T. cruzi* 3dH presented worse clinical outcome compared to the control group. The lower weight observed in the treated groups compared to control, mainly in 3dH SD group ($P < 0.001$), reflected this worse outcome.

DISCUSSION

In the murine infection by *T. cruzi*, considering more specifically mice infection, some authors consider that the parasitemia is directly related to morbidity [6,21]. Series of trials reported the effect of biotherapeutic *T. cruzi* 3dH in the experimental infection by *T. cruzi*, showing reduction of parasitemia and tissue parasitism, which can be understood as a benefit of treatment [22-26]. However, no detailed information about the methodology used in this study was displayed. Aleixo *et al.* [2], using the biotherapeutic 17dH, showed that in the frequently treated group a better outcome of the infection was present by means of lower parasitemia levels compared with the control group, with a better evolution of the infection, agreeing with the concept that parasitemia and morbidity is directly proportional in mice infection by *T. cruzi*. In another study, Ferraz *et al.* [27] showed only minor effect to 15dH dynamization in the same model of infection, with similar data of parasitological parameters. The same team by Ferraz *et al.* [5] reported an intermediary effect in parasitological evaluation to 7dH; the 7dH biotherapic reduced total parasitemia and parasite peak in schedule of treatment in which the substance was administered 7 days before infection, and the schedule

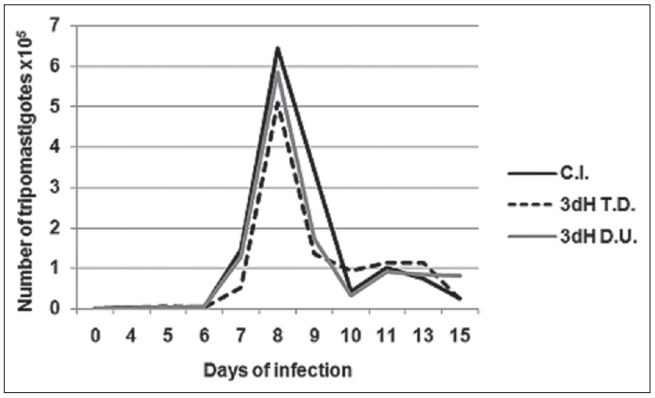

Figure 1: Curve of parasitemia of animals infected by ***Trypanosoma cruzi*** and subjected to different treatment schedules using biotherapeutic *T. cruzi* 3dH and control group

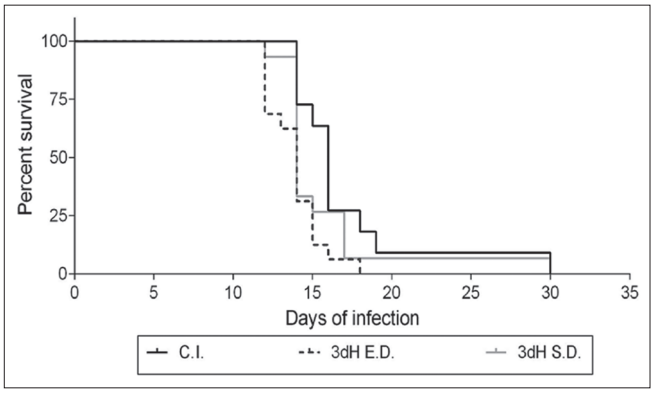

Figure 2: Survival curve of animals infected by ***Trypanosoma cruzi*** and subjected to different treatment schedules using biotherapeutic *T. cruzi* 3dH and control group

of 30 days before infection showed a higher PPP. These data reinforce the literature in which authors report that the schedule and dynamization are important to determine the response to medication in each organism [2,5,9,11]. In the present study, no statistical difference was observed among treated groups and control group for parasitological parameters; so, we can suppose that very low dynamization (3dH) can not encourage the organism to defend itself against the parasite.

Several studies performed by our group showed a tendency for the higher survival of animals treated with diluted and dynamized drug in this experimental model [2,5,27]. Ferraz *et al.* [27] showed an earlier mortality to 16dH biotherapeutic; Aleixo *et al.* [2] showed a lower mortality in 17dH; and again Ferraz *et al.*, in another study [5], using 7dH in a schedule of 30 days before infection showed a higher survival too, with 7.7% of cure index.

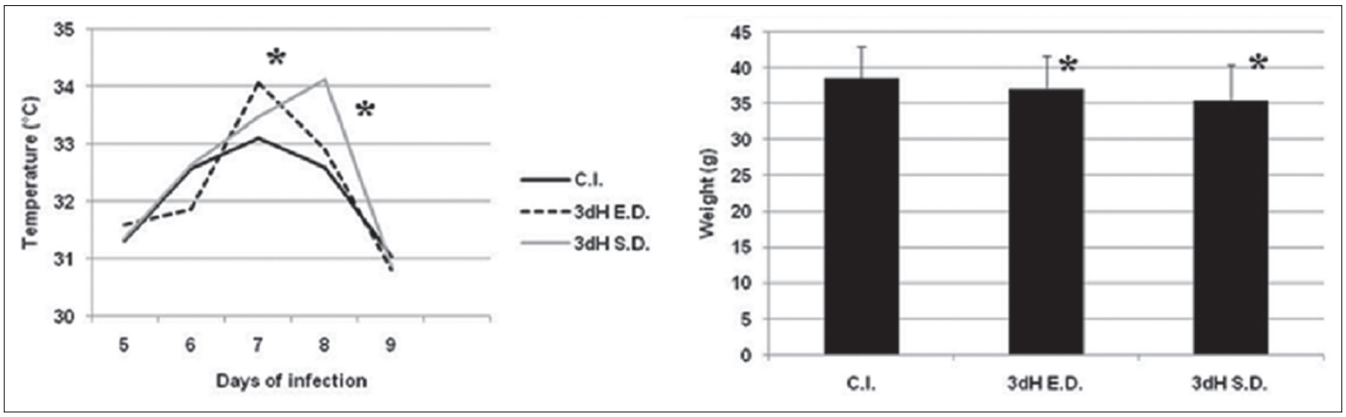

Figure 3: Clinical parameters (temperature and weight) of animals subjected to different treatment schedules using biotherapeutic *Trypanosoma cruzi* 3dH.

In an experimental model of infection with pathogenesis involving irreversible damage that evolves with the death of infected control animals, earliness of death observed in the treated groups indicates a worse prognosis. In *T. cruzi* infection, reduced weight values reflect cachexia, which is observed during infection [7,18,21]. Enhancing this cachexia involves a worse prognosis showing an inefficiency of this intervention.

The effect of biotherapeutic in treated groups displayed a higher temperature with abrupt drop compared to control animals. Maintaining a stable body temperature is the main aspect of homeostasis [28]. Hippocrates said that fever is favorable [29] and the actual concept that fever is beneficial during infections is based on clinical observations and several observational studies [30]. Although heat/fever is a part of the acute phase response and represent the most manifest sign of infectious illness [31], studies show that survival improves when temperature increases [30]; and in some cases of sepsis, hypothermia occurs before death [32-34], as we observed in this experiment. In our study, the increase of temperature shows an amendment of the body that can be regarded as a response to the medication in order to achieve homeostasis. Nonetheless, the abrupt drop of temperature before death, can be regarded as ineffectiveness of medication in this potency, despite the attempt to achieve balance with the initial increase of temperature.

Despite the literature data, in our study, the lowest dynamization of biotherapic *T. cruzi* 3dH shows no benefit in the murine model of *T. cruzi* infection by parasitological and clinical evaluation. Instead, the low potency promoted the untimely death of the animals, indicating a worse prognosis. In homeopathic principles, a worse prognosis can be understood like a aggravation which can be described like a temporary appearance of new symptoms, or a temporary intensification of existing symptoms, following a dose of a homeopathic medication [9] with subsequent reestablishment welfare. This is pointed as a stimulation of diluted and succeed medications which may lead the living system to restore a state of equilibrium [35], as already been reported by Hahnemann [9], who considered the homeopathic aggravation as a signal of an incipient cure.

In this experiment, the acute model of infection did not present a health reestablishment of animals, which died untimely. Even though aware that this is a model with irreversible imbalance of the system, we can conclude that 3dH dynamization, although low, provided no positive effect in controlling acute infection. This can be understood, considering the fact homeopathic treatments have senoidal behavior (dynamization waveforms) [36-40].

According this theory, the response pattern in curves is responsible for the tendency toward favorable responses in host-pathogen systems in low, medium, and high dynamizations, acting as enhancers or suppresssors [41]. Thus, some dynamizations had a positive effect, but others negative. The response of homeopathic medications follows a sinusoidal curve, where some points with no effect were observed [12]. A study to evaluate the hydrating effect of the sodium chloride in various dilutions showed negative effect on some evaluated potencies/dynamizations (3cH, 17cH), and positive effect in other ones (between 4-17cH and 17cH-19cH) [42]. Studies published by our group showed diverse outcomes for animals infected by *T. cruzi* and treated with biotherapeutics [2,5,27,38]. Animals treated with 7dH showed no statistical difference compared with infected control group [5]. Dynamizations 15dH, 16 dH, and 17dH showed positive effects against this parasite, with a crescent result and better outcome in the treatment using 17dH [5,27,43] which also presented an increase of survival. These data show the oscillatory potency-effect-curve where it presents effective and ineffective action of biotherapic in this model of infection. In the present work, 3dH biotherapeutic showed not only a lack of effect, but also detriment to the animals subjected to this treatment.

CONCLUSION

In conclusion, The low dynamization 3dH of *Trypanosoma cruzi* biotherapeutic worsened murine infection by this parasite in clinical and parasitological evaluation. This dynamization showed a nearly mortality, mainly in the group treated every day. Although lower potencies are recommended for acute cases, the 3dH dynamization acted triggering a worse prognosis. The senoidal behavior observed in homeopathic treatment effects may be the explanation for this result. The knowledge of oscillatory potency-effect-curve of *T. cruzi* biotherapeutic

can optimize the choice of different dynamizations for clinical use in humans and also taken into account for each organism.

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