Biotherapic 200dH is harmful to acute murine infection with *Trypanosoma cruzi*.

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Abstract

Biotherapics employed to treat mice infected by *Trypanosoma cruzi* were carried out with encouraging results. The aim of this study was evaluated the effect of biotherapic of *Trypanosoma cruzi* 200dH, using two different schedules of treatment. Swiss male mice, aged 56 days-old were infected intraperitonealy with 1,400 blood trypomastigotes of *Trypanosoma cruzi* Y strain and were divided into groups: C.I.- infected animals, E.D. – Infected animals treated from the day 1 until the end of the experiment; 200dH S.D. – Infected animals treated on the day 1. Parasitological, clinical and immunological parameters were evaluated. The group of animals that received the medicine in a single dose presented higher value to total parasitaemia and lower value of pre patent period compared to control untreated group (p<0.05), as well as the number of amastigotes which was also higher for this group (S.D.) (p<0.05). Clinically, the S.D.group presented more stable temperature (p<0.05) but not presented another clinical difference among treatments. IL-6 and TNF-α presented similar dosage among treated groups as well as IL-4 and IL-10. IL-17A and INF-γ, presented highest values to S.D. group (p<0.05). All animals died until the 20th day of infection. The lack of improvement in clinical and parasitological parameters, the untimely death and the immunological imbalance display the harmful evolution of the experimental infection by *T. cruzi* using biotherapic 200dH. The results could be useful for homeopathic physicians. In human clinical use, the choice of dynamizations and treatment schedule should consider acute and chronic diseases to achieve the expected results.

Keywords: biotherapic, murine infection, Trypanosoma cruzi

INTRODUCTION

Medicines prepared from organic products, according to homeopathic pharmacotechnics are named biotherapics [1]. Biotherapics employed in the treatment of murine infection by *Trypanosoma cruzi* were carried out with encouraging results [2-5]. The experimental murine models of infection by *Trypanosoma cruzi* using Y strain was extensively studied [6] and its characteristics such as high parasitemia, peak of parasites on 8th day of infection, death of all untreated infected animals are well defined, being an excellent experimental model, which may be useful for testing medications that may be later used in humans [7]. Highly diluted medication has been extensively studied for years about its effects.
and mechanisms of action [8,9]. The most appropriate medication depends not only on the correct homeopathic selection, but also the dose and frequency considering that homeopathic medicines administered improperly can cause harm greater than the disease itself [8-10].

In this context the aim of this study was evaluated the effect of biotherapic of Trypanosoma cruzi in very high dynamization, in murine infection by the protozoan, by measuring of parasitological, clinical and immunological parameters using two different schedules of treatment.

MATERIAL AND METHODS

Experimental design
A blind, randomized, controlled trial was performed twice using Swiss male mice, aged 56 days-old. The experiment was approved by the Ethics Committee for Animal Experimentation (CEEA/ UEM) under protocol number 030/2008 following Brazilian Guidelines for Care and Animals Use (DBCA) elaborated by National Council of Animal Experimental Control (CONCEA) [11]. The animals were kept in controlled conditions for temperature (22.7±1.2°C), light/ dark cycles of 12 hours, and filtered water and food was offered ad libitum.

Animals
Forty-eight, 56 days old male Swiss mice, Mus musculus, from the Central Animal Facility of Universidade Estadual de Maringá, were used. The animals were infected intraperitoneally with 1,400 blood trypomastigotes of Trypanosoma cruzi Y strain [12].

Experimental groups
The mice were divided into three groups: C.I.- infected animals, treated with 7% ethanol-water solution, in water (10µL/mL), offered ad libitum in an amber bottle (n=16); 200dH E.D. – Infected animals treated with biotherapic T. cruzi 200 dH, in water (10µL/mL), offered ad libitum in an amber bottle, from the day of infection until the end of the experiment (n=16); 200dH S.D. – Infected animals treated with biotherapic T. cruzi 200 dH, in water (10µL/mL), offered ad libitum in an amber bottle, on the day of the infection, during 12 hours (n=16). The mice were divided into cages so that the mean initial weights of animals were statistically equal. The sample size considered statistical analysis [13] and the 3R’s emphasizing reduction, refinement and replacement of animal use [14].

Biotherapic T. cruzi 200dH
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 according Brazilian Homeopathic Pharmacopoeia [1]. The microbiological test and biological risk were negative, according to the regulations of the Brazilian Ministry of Health (RDC n°67) [15].

Parameters evaluated
Parasitological, clinical and immunological parameters were evaluated.

Parasitological parameters: Parasitemia was evaluated by daily counts of blood trypomastigotes from the day of infection, using the technique of Brener [12]. Others parasitological parameters were also analyzed: 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 parasites observed in each animal throughout the experiment.
the day of these peaks occurred was also considered (DPmax); prepatent period (PPP), which is the time from infection detection of the parasite in blood; patent period (PP), the period when the parasitemia can be detected. Tegument parasitism was evaluated in histological sections using hematoxylin eosin staining. Were collected heart, skeletal muscle, intestine and liver of euthanized mice before infection (time 0), and in the 4th, 8th and 12th days after infection (T4, T8 and T12). The number of nests of amastigotes and amastigotes per nest was evaluated by optical microscopy of histologically stained sections.

Clinical parameters: The animals were clinically evaluated for body temperature, body weight, food and water consumption, to measure the welfare of each animal.

Survival: The survival was computed during the course of the experiment and survival analysis and the curve of survival were obtained using statistical programs. The clinical assessments were performed based on Falkowski et al. protocol of clinical trial in mice infected by T. cruzi [16].

Immunological parameters: The cytokines (IL-2, IL-4, IL-6, INF-γ, TNF-α, IL-17A and IL-10) were measured using BD Cytometric Bead Array (CBA) Mouse Th1/Th2/Th17 Cytokine Kit in flow cytometry. The serum was collected from three animals per group. The animals were euthanized before infection (time 0), and in the 4th, 8th and 12th days after infection. The serum collected was stored at 20 °C at the time of collection until the time of dosing.

Statistical analysis
The data were compared statistically using Mann-Whitney test in the program 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 analysis.

RESULTS
Parasitological parameters
The parasitological data obtained in the evaluation of daily parasitemia of animals treated with biotherapic T. cruzi 200dH is shown in the figure and table 1. The parasitemia curve (figure 1) shows a non-significant increase to treated groups (E.D. and S.D.) in comparison with control group (p>0.05). Among treated groups E.D. and S.D. was also no difference in this parameter (p>0.05).

In the table 1 parasitological parameters obtained of parasitaemia curve shows no significant statistical difference for most of the evaluations. Only for the total parasitaemia and pre patent period parameters there was significant statistical difference. The group treated with a single dose of medicine was displayed highest values to total parasitaemia (p = 0.03), and lower for a pre patent period (p = 0.05) compared with the control group.
Figure 1: Curve of mean parasitemia of animals infected by *T. cruzi* and treated with biotherapic *T. cruzi* 200dH and control group.

Table 1: Mean and standard deviation of parasitological parameters, of animals treated with biotherapic *T. cruzi* 200dH and control group.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>P&lt;sub&gt;total&lt;/sub&gt;(x10&lt;sup&gt;-5&lt;/sup&gt;)</th>
<th>P&lt;sub&gt;max&lt;/sub&gt;(x10&lt;sup&gt;-5&lt;/sup&gt;)</th>
<th>DP&lt;sub&gt;max&lt;/sub&gt;(days)</th>
<th>PP(days)</th>
<th>PPP(days)</th>
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<tbody>
<tr>
<td>C.I.</td>
<td>6.67±5.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.29±2.49</td>
<td>8.33±0.58</td>
<td>11.33±2.31</td>
<td>4.66±0.58&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>E.D.</td>
<td>11.18±5.61</td>
<td>6.95±1.82</td>
<td>7.83±0.41</td>
<td>9.83±2.04</td>
<td>5.16±2.86</td>
</tr>
<tr>
<td>S.D.</td>
<td>15.12±5.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.29±2.81</td>
<td>7.89±0.33</td>
<td>10.66±2.23</td>
<td>4.11±0.33&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
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</table>

Groups: C.I. – control of infection; E.D. – animals treated with biotherapic *T. cruzi* 200dH since the day of infection by the end of the experiment; S.D. – animals treated with biotherapic *T. cruzi* 200dH on the day of the infection, during 12 hours. Data were obtained from daily parasitemia. 

P<sub>total</sub> - Total parasitemia; P<sub>max</sub> - Maximum peak of parasites; DP<sub>max</sub> - the day of these peaks occurred; PP - patent period; PPP – pre patent period. Equal letters indicate a statistically significant difference. (a) p=0.03; (b) p=0.05.

The evaluation of the number of amastigotes nests showed an increase on the 8<sup>th</sup> day of infection to animals treated with a single dose compared to animals treated every day and control group (p<0.05) in the heart. In the 12<sup>th</sup> day of infection, the differences were observed to animals treated with a single dose that presents higher number of amastigotes nests compared to control group in the skeletal muscle (p<0.01) and in the liver (p=0.05) (figure 2). Another comparison, among groups, to this parameter were no significative (p>0.05).

Figure 2: Mean and standard deviation of the number of amastigotes nests in heart, skeletal muscle, intestine and liver of infected animals treated with biotherapic *T. cruzi* 200dH and control group, on the 12<sup>th</sup> day of infection.
Clinical parameters

The evaluation of clinical parameters shows no considerable differences among groups. The only parameter that showed differences among groups was the temperature between 4th and 12th days of infection (figure 3). For this parameter, animals treated with a single dose showed a more stable temperature, while animals treated every day showed a decrease with a subsequent increase of temperature in assessed time (figure 3). The statistical evaluation showed that animals treated with a single dose presents higher temperatures than control group (p<0.01) and E.D. group (p=0.02).

Figure 3: Temperature (°C) of infected animals treated with biotherapic T. cruzi 200dH and control group.

Groups: C.I. – control of infection; E.D. – animals treated with biotherapic T. cruzi 200dH since the day of infection by the end of the experiment; S.D. - animals treated with biotherapic T. cruzi 200dH on the day of the infection, during 12 hours. Equal letters indicate statistically significant difference p<0.05.
Survival
The survival was computed during the experiment, until the death of all animals. The survival analysis showed no difference among groups (figura 4).

Figure 4: Curve of survival of animals groups treated with biotherapic *T. cruzi* 200dH and control group.

Immunological parameters
The profile of cytokines evaluated in the 4th, 8th and 12th days of infection to different experimental groups was shown on the table 2.

The pro inflammatory cytokines IL-6 and TNF-α presented similar values between treated groups compared with control group in the 8th and 12th days of infection. The animals treated with a single dose of medicine presented highest dosage compared to animals treated every day in the evaluation of 8th and 12th day of infection to IL-6 (p<0.01 and p=0.02, respectively), and in the 4th and 8th days of infection to TNF-α (p<0.01 and p=0.03, respectively). In the 4th day of infection the group of animals treated with a single dose presented highest values than control group (p=0.01). Theses cytokines presented highest values in comparison with non infected animals in almost all dosages (p<0.05).

The anti-inflammatory cytokines IL-4 and IL-10 presented an increase in treated groups (S.D. and E.D.) compared to control group, mainly in the 12th day of infection (p<0.05).

The cytokine INF-γ presented highest values to control groups and to animals treated with a single dose in the 12th day of infection (p=0.05). The regulatory cytokine IL-17A presented highest values to animals treated with a single dose in the 8th day of infection in comparison with the control group (p<0.01). The same group presented similar values of cytokine dosage in the 12th day of infection in comparison with control group (p>0.05) and highest values compared to animals treated every day and non infected (p<0.01).
DISCUSSION
To the best of our knowledge this is the first study to present clinical and immunological alterations as a consequence of highly diluted biotherapeutic in 200dH employed in different schedules of treatment in animals infected with Trypanosoma cruzi. The group of animals that received the medicine in a single dose presented higher value to total parasitaemia and lower value of pre patent period compared to control untreated group (p<0.05), as well as the number of amastigotes which was also higher for this group (S.D.) (p<0.05). Clinically, the animals that received a single dose of medicine (S.D.) presented more stable temperature (p<0.05) but not presented another clinical difference among treatments. Pró inflammatory cytokines IL-6 and TNF-α presented similar dosage among treated groups as well as the anti-inflammatory IL-4 and IL-10. IL-17A presented highest values to S.D. group (p<0.05) and INF-γ, the cytokine related to the death of animals, also presented highest values (p<0.05). All animals died until the 20th day of infection. Considering parasitological parameters, more specifically parasitaemia and tissue parasitism, the literature show a direct relation with morbidity [6,17]. Using the same experimental model, studies have shown that T. cruzi biotherapeutic in 7dH, 17dH
and 30dH developing lower parasitemia levels and tissue parasitism compared with control group, with a better outcome of the infection [2,4,5], agreeing with the concept that morbidity and parasitism are directly related in mice infection by *T. cruzi*.

To clinical parameters, once, treated group no presents significantly benefits in comparison to control group for most evaluated parameters. The difference was only observed in temperature evaluation between 4th and 10th day of infection. The animlas treated with a single dose of medicine group presented higher and more stable temperature and the animals treated every day presented a decrease with a subsequent reestablishment. Maintaining a stable body temperature is the main aspect of homeostasis [18]. In acute diseases, the survival improves when the temperatures increase [19], and in some cases is observed a hypothermia before death, as well as in our experimental model [20-22]. In the animals treated every day, the increase of the temperature shows an amendment of the body which can be regarded as a response to the medication in order to achieve homeostasis. In this acute murine model of infection by *T. cruzi*, is not allowed to be observed 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 200dH dynamization provide no positive effect in whole considering the health of animals. The lack of improvement in clinical and parasitological parameters of animals treated with 200dH led to the early mortality of treated animals, in which although do not show statistical difference in comparison among groups, survived at least one day less.

All of these evaluations to parasitological, clinical and survival parameters can be considered as an outcome of the immune balance. The cytokines are important prosecuters of this process. Some of them act as pro-inflammatory as well as TNF-α, IL-1, IL-2, IL-6 and IL-7 and others act as anti-inflammatory II-4, IL-10, IL-13, TGF-β. Simultaneously, IL-17A act as a regulatory substance as just as the IL-10. The increase of TNF-α and IL-6 and the decrease of IL-4, IL-10 and IL-17A in this experiment showed us that the treatment using 200dH, mainly in the schedule using a single dose, promotes a stimulus in the organism [6].

Acute infection of mice by *T. cruzi* is characterized by parasitemia and inflammatory reactions in tissues [6], and may be lethal for them. The overproduction of TNF-α, INF-γ and IL-10 are involved with susceptibility and resistance to infection [6]. In our experiment, the parasite load increases and give rise to an higher production of pro (INF-γ and TNF-α), and anti-inflammatory (mainly IL-10) cytokines that compete for improving or dampening the control of the acute phase, as well as the literature presents to susceptible animals infected by *T. cruzi*, showing to us that 200dH is not a beneficial medication to this model os infection considering cytokines dosage. Generally, we can state that the use of highly diluted medicine *T. cruzi* 200dH increased total parasitemia and tissue parasitism, showed no clinical improvement, displaying only a reaction trend in the organism between 4th and 10th day of infection, when the treatment promoted a more stable temperature in the animals treated with a single dose of 200dH and a reestablishment in the animals treated every day with 200dH biotherapic. The evaluation of cytokines...
profile, the balance of cytokines showed that the treatment using biotherapic *T. cruzi* 200dH promotes an increase of pro-inflammatory and a decrease of anti-inflammatory cytokines. The regulatory cytokines IL-10 and IL-17A not be able to restore the organic homeostasis and the result was a trend of improvement, without success, as shown in the parasitological and the clinical parameters, which culminated with the death of the animals, as well as occurred with the control group.

In homeopathic principles, a worse prognosis can be understood like a temporary intensification of new symptoms, following a dose of a homeopathic medication [9] with a 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 [9,23]. Once, is possible to observe that highly diluted medication promotes a stimulus in the organism, however. In our experiment was possible observed the stimulus promote by highly diluted medication, however because we employed an acute model of infection was not allowed to observe a health reestablishment of animals, which died untimely.

The murine infection model using Swiss mice infected with *T. cruzi* strain Y is an acute infection model, whose morbidity and mortality causes almost all animals died in the first 20 days after infection. The use of high dynamization, such as 200dH in homeopathy, is indicated in chronic cases with very interesting results. Our study used a high dynamization, in acute infection model in order to know what would be the response of this intervention. Proving the literature, the dynamization used did not provide any benefit in acute model studied. Although various treatment regimens have shown different results, none of them had good enough results to justify their clinical application in practice. What can we get with our results was to reinforce what we see in the medical clinic where high dynamizations, in single doses are harmful to acute diseases. Knowledge of the effect of diluted drugs, the adequacy of dynamizations and the treatment schedules is a indispensable factor for the use of these medicines. Thus, more studies are needed for the correct use of drugs diluted in homeopathic practice.

**CONCLUSION**

The lack of improvement in clinical and parasitological parameters, the untimely death and the immunological imbalance with an increase of pro-inflammatory and decrease of anti-inflammatory and regulatory cytokines display the harmful evolution of the experimental infection by *T. cruzi* using highly diluted biotherapic 200dH. Considering that mouse model reproduces a great number of features of Chagas disease, even if not totally correlated to the human disease, this results could be useful to help homeopathic physicians. In human clinical use, the choice of dynamizations and treatment schedule should consider acute and chronic diseases to achieve the expected results.

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