Different Treatment Schemes with Canova Medication® Modify Differently the Course of Mice Infection by Trypanosoma cruzi

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Abstract

Objective: Purpose of this study was to evaluate the action of the Canova Medication (CM) in experimental infection of mice with Colombian strain belonging to genetic group T. cruzi in different treatment regimens.

Methods: In a blind, controlled, and randomized trial using 60 Swiss mice males, 28 days old. Swiss mice were inoculated with 10⁴ trypomastigotes/Colombian strain and treated with CM following different schemes: DI-20CM (treated with CM from the infection for 20 days); CI-9CM: treated with CM from the confirmation of infection for 9 days; AI-24CM: treated for 24 days beginning the treatment three days before infection; CONTROL: infected and untreated; NI: healthy uninfected. We evaluated: parasitaemia curve; mortality rate; histopathology; cytokines levels.

Results: The TNFα showed more concentration in all schemes of treatments. IL10 in AI-24CM group were statistically lower compared to the CONTROL (2.91 pg/mL, 26.38 pg/mL; p=0.046) can be related to the higher mortality rate, thus explaining the presence of diffuse inflammation in the heart of the animals of this group. The group CI-9 CM showed the best performance diminishing parasitological parameters and longer survival.

Conclusion: Administration of CM after infection confirmation was beneficial, however, it is still not still the ideal. When administered before the infection it promoted early death of animals. CM modulates the production of cytokines in experimental infection of mice by Trypanosoma cruzi. The modulation is dependent on the treatment scheme. The results alert to the indiscriminate use of ultra diluted medication, considering the animal species versus doses and treatment schemes.

Keywords: Trypanosoma cruzi; Chagas disease; Canova medication; Cytokine; Mice infection; Highly diluted medication

Abbreviations: NI: Non-Infected and Non-Treated Animals; CD: Chagas Disease; BZ: Benznidazole (N-Benzyl-2-Nitro-1-Imidazole Acetamide); CM: Canova Medication®; PPP: Prepatent Period; PP: Patent Period; MPD: Maximum Peak Day; MPP: Maximum Parasitemia Peak; TP: Total Parasitemia; Control: Infected Animals, Treated For 20 Days with a 1% Hydro Alcoholic Solution from Parasite Inoculation; G-16 CM: Infected Animals, Treated with CM for 16 Days from the 5th Day of Parasite Inoculation; AI-24 CM: Infected Animals, Treated with CM for 24 Days, Initiating the Treatment Three Days Before Parasite Inoculation; DI-20 CM: Infected Animals, Treated for 20 Days with CM from Parasite Inoculation; CI-9 CM: Infected Animals, Treated with CM for 9 Days After the 12th Day of Infection from the Infection Confirmation
Introduction

Chagas disease (CD), caused by Trypanosoma cruzi [1], is an anthroponosis vastly spread in most part of the American continent. It is considered neglected by the World Health Organization, being one of the most severe tropical diseases, in addition to being a great public health problem in Central and South America [2,3]. Currently the disease has been detected in other continents [4]. The host-parasite relationship in CD is complex, involving profound immune system reactions. It is characterized by intense and widespread inflammatory process, with severe immune suppression component, expressed, among others, by changes in the levels of several cytokines and their receptors [5]. Currently in Brazil, there is a single antiparasitic drug the benznidazole (N-benzyl-2-nitro-1-imidazole acetamide) (BZ), which is partially effective in the acute phase of the disease. However, it has low efficacy in the chronic phase and can cause significant side effects [6,7]. For this reason, searching for a more effective treatment that covers all the stages of the disease is the goal of many studies and investigations [8-11].

In recent years, there has been an increased interest from health professionals and the general population, in both Brazil and other countries, in the “Alternative or Complementary Practices” [12-14]. Several studies indicate that some natural products can be used to aid in the cure of diseases, as well as to maintain the resistance of the body against infections [15,16]. The modulation of the immunological system can be suggested as one of the way these agents exert their effects [15,17,18]. The new forms of immune modulatory therapy can be directed to specific cells or their products. Particularly, the use of immune modulatory medicinal products could be an alternative in CD treatment. The Canova Medication® (CM) is a complex composed of Aconitum napellus, Arsenicum album, Bryonia alba, Lachesis muta and Thuya occidentalis in dilution from 10-11 to 10-19 [19]. It is used as an immune modulator, and the literature describes it as having stimulating effect on the immune system by activating the macrophages [20-22]. The immune modulatory action of CM was observed in experimental infection, induced by Leishmania (L.) amazonensis [23], and also in the reduction of lesions caused by the Paracoccidoides brasiliensis, controlling the progression of the infection [24].

The CM has been clinically utilized, with benefits, in association with other medicines in the treatment of diseases wherein the immune system is impaired [25-27]. Considering that the infection caused by the T. cruzi presents immunosuppressive characteristic [5], and just like others highly diluted medications [28,29] the CM presents effect on the course of the experimental infection by this protozoan [30], studies evaluating the effect of different treatment schemes using Canova are welcome. In recent studies, Ferraz et al. [29], and Aleixo et al. [28] showed that different administration schemes of ultra diluted solutions cause different effects on the course of murine infection by T. cruzi. Parasitemia curve, the mortality rate, the tissue distribution of T. cruzi and its consequences are all-important tools to study the relationship parasite/host in this infection, being also used to evaluate the action of the medications tested [8,9,30-34]. Taking into account all the previous information, the objective of this study was to investigate whether different treatment schemes interfere in the development of the experimental infection by T. cruzi. In order to achieve the objective proposed here, it was used parasitological, histopathological and immunological parameters of infected mice with BZ-resistant strain and treated with CM.

Methods

Parasite and inoculum

It was used the Colombian strain, belonging to T. cruzi 1. The inoculum was determined according to Brener [35] for 10,000 blood trypomastigotes at 0.2mL/animal, intraperitoneal (ip) route. The parasites were obtained from mice previously inoculated, at the 14th day of the infection, with blood collected from the orbital plexus with heparinized Pasteur pipette.

Animals

Swiss male mice (23.7±1.2g) with 28 days of living, coming from the central animal house of UEM. The animals were acclimated for three days in the animal house of the sector, maintained in a controlled temperature environment (22.7±2.01 °C), a light/dark cycle of 12h, water and food offered ad libitum. All cages contained wood shavings. All behavioral experiments were conducted in the light phase, and under daily surveillance. Sixty Swiss male mice were divided in to standard cages so that the averages of the initial weights of mice in each group were statistically equal, with ten animals per cage [36]. The cages were numbered 1-6, and were randomized into treatment groups by picking numbers out of a hat.

Six treatment groups of 10 Swiss male mice each were studied:

i. **NI**: non-infected and non-treated animals.

ii. **Control**: infected animals, treated for 20 days with a 1% hydro alcoholic solution from parasite inoculation.

iii. **G-16 CM**: infected animals, treated with CM for 16 days from the 5th day of parasite inoculation.

iv. **AI-24 CM**: infected animals, treated with CM for 24 days, initiating the treatment three days before parasite inoculation.

v. **DI-20 CM**: infected animals, treated for 20 days with CM from parasite inoculation.

vi. **CI-9 CM**: infected animals, treated with CM for 9 days after the 12th day of infection from the infection confirmation.

Canova medication®

Canova Medication® (CM) [19] is a homeopathic complex, offered in the form of pills or solutions, for oral, intravenous or inhalant administration. It is produced in Brazil only by
T. cruzi infection and for 30 days. The blood was obtained from the tail to relieve stress. The treatment started on the 5th day from the induction of the infection, considering that most researchers to evaluate medicines in experimental infection with T. cruzi [37-40] use this procedure. One person who did not take part in assessment of the parasitological parameters gave the treatment. The treatment was randomized. For the treatment of the AI-24 CM, DI-20 CM, CI-9 CM groups, the CM was diluted in water (10µL/mL water), offered ad libitum in an amber bottle, and hygienized once every three days. According to Aleixo et al. [28], in veterinary medicine this is the most frequent form of administering highly diluted drugs. The treatment schemes were as follows:

1. **AI-24 CM group**: The treatment started three days before the induction of the infection. Considering that the CM is a modulator of the immunological response [20-22] and will promote the change in the host response after contact with the parasite.

2. **DI-20 CM Group**: The treatment started on the day of the inoculation of the parasites - based on the idea that the host response will be different considering that the organism has contact with the medicine and with the parasite at the same time [28].

3. **CI-9 CM Group**: The treatment initiated on the 12th day after the induction of the infection and after the confirmation of infection. The premise was that the mice would be already with the immune system activated by the presence of the parasite, and according to Aleixo et al. [28], the host would be more benefited.

**Parasitemia curve**

The parasitemia curve was evaluated after the 4th day of infection and for 30 days. The blood was obtained from the mouse-tails and the number of parasites was determined by the method of Brener [41]. The parasitemia of the mice of all groups was obtained blind: those who assessed the parasitemia did not aware of the treatment the animals had received. Based on the parasitemia curve, it was obtained information related to pre-patent period (PPP), patent period (PP), maximum peak of parasitemia (MP), day of maximum parasitemia (DMP) and total parasitemia (TP). Results were expressed as mean ± standard deviation for the studied groups.

**Mortality rate**

The mortality rate was evaluated along the course of the experiment, resulting in the following parameters: days of survival and percentage of mortality expressed by the number of animals died within a group. The results were expressed as percentage of cumulated deaths during the period evaluated. The animals that survived to the patent period of infection remained under observation for a period of 120 days after inoculation. The animals used in the survival study died as a direct result of infection.

**Histopathology**

For histopathological analysis, three animals from each experimental group were euthanized on the 21st day of infection. They were selected randomly by picking numbers out of a hat. The day of euthanasia was determined in previous studies, being the day of peak parasitemia since the tissue distribution and the cytokine levels are a direct reflection of levels of parasite in the blood [34-37,42].

The method of euthanasia was exsanguination by cardiac puncture after general anesthesia. All efforts were made to minimize suffering. Fragments of the heart, skeletal muscle, liver, spleen and large intestine were collected, fixed in 10% buffered formalin, and paraffin-embedded. For each animal, four sections of each organ were made, with 5µm each, and separated by 20µm intervals. The sections were stained with hematoxylin-eosin (H&E). In the cuts, tissues were evaluated for the presence of parasites and inflammation, and this assessment is expressed in numbers of amastigotes nests per field, number of amastigotes per nest, number of inflammatory foci per field in the case of focal inflammation, and number of the fields with the presence of diffuse inflammation. Ten random fields from each cut were researched, with a 40X objective. The values shown for each organ in each group are the mean ± standard deviation of four slides for each animal, being three animals per group.

**Cytokines dosing**

The same animals used in histopathological studies was used for cytokine analysis, the serum was collected. The assay was performed using the CBA KIT for the B&D flow cytometer. The cytokines IL-2, IL-4, IL-6, IL-10, IL-17A, IFNy, TNFa were dosed. The technique was developed in accordance to the instructions of the manufacturer. It was used the FACS Calibur flow cytometer (Becton-Dickinson, Rutherford, NJ, USA), equipped with Cell Quest software (Joseph Trotter, Scripps Research Institute, La Jolla, CA, USA). The result was expressed in mean ± standard deviation of the values achieved from each animal.

**Statistical analysis**

It was used the Kolmogorov-Smirnov Test to assess the normality of the data. For comparing parasitism tissue and parasitological parameters among the groups, the Kruskal-
Wallis ANOVA, the Median, and the LSD tests were employed. The analysis considered 5% significance level for all comparisons.

**Ethics**

All procedures involving mice followed the Ethical Principles of Animal Experimentation established by the National Council for the Control of Animal Experimentation (CONCEA) [43], Brazilian College of Animal Experimentation (COBEA) and Brazilian Society of Laboratory Animal Science (SBCAL). Were performed according to protocols approved by the Committee for Ethical Conduct in Animal Experimentation (CEUA) of the State University of Maringa (report 032/2005 on 21/Jun/2005).

**Results**

**Parasitological evaluation**

![Figure 1: Parasitemia curve](image1)

*Figure 1: Parasitemia curve:* Average parasitemia curve obtained in male Swiss mice, 28 days old, inoculated with 10^4 trypomastigotes blood of the Colombian strain of T. cruzi, in the different treatment schemes, with Canova medication (CM), or with 1% hydroalcoholic solution (Control). G-16 CM: infected and treated animals with CM for 16 days from the 5th day of the induction of the infection. AI-24 CM: infected and treated animals for 24 days, starting treatment three days before the induction of the infection. DI-20 CM: infected and treated animals, using CM for 20 days from the inoculation of parasites. CI-9 CM: infected and treated animals with CM for nine days from the 12th day of infection.

![Figure 2: Survival rate](image2)

*Figure 2: Survival rate:* Male Swiss mice, 28 days old, inoculated with 10^4 blood trypomastigotes of the Colombian strain of T. cruzi, in the different treatment schemes with Canova medication (CM), or with 1% hydroalcoholic solution (Control). G-16 CM: infected and treated animals with CM for 16 days from the 5th day of infection induction. AI-24 CM: infected and treated animals for 24 days, starting the treatment three days before the induction of the infection. DI-20 CM: infected and treated animals with CM for 20 days from the inoculation of parasites. CI-9 CM: infected and treated animals with CM for nine days from the 12th day of infection.
Table 1: Prepatent period (PPP), patent period (PP) and the maximum peak day (MPD) of animals in the different treatment schemes with Canova medication (CM).

| Group     | PPP(days) | PP (days) | MPD       |
|-----------|-----------|-----------|-----------|
| Control   | 5.7±1.16  | 20.1±3.31 | 23.2±3.33 |
| G-16 CM   | 7.5±2.59  | 18.7±3.38 | 24.1±4.01 |
| AI-24CM   | 7.1±2.13  | 14.8±2.25 | 20.1±0.74 |
| DI-20CM   | 7.1±1.92  | 17.2±3.79 | 22.3±3.95 |
| CI-9CM    | 7.0±2.98  | 15.8±4.87 | 19.7±3.34 |

Table 2: Maximum parasitemia Peak (MPP) and total parasitemia (TP) of animals in the different treatment schemes with Canova medication (CM).

| Group     | MPP (Trypanomastigote/ mL) | TP (Trypanomastigote/ mL) |
|-----------|-----------------------------|---------------------------|
| Control   | (2.03±1.66)x10⁴             | (5.99±2.86)x10⁶           |
| G-16 CM   | (2.09±1.6)x10⁴              | (9.82±9.82)x10⁶           |
| AI-24CM   | (1.82±1.31)x10⁴             | (4.85±2.99)x10⁶**         |
| DI-20CM   | (2.35±1.83)x10⁴             | (6.39±5.51)x10⁶           |
| CI-9CM    | (0.83±1.21)x10⁶*            | (4.47±7.83)x10⁶*          |

Figures (1&2), and Tables (1&2) present the parasitological data in detail. Parasitemia curve observed for the control group followed the graphic standards described in the literature for the Colombian strain [35-39]. Parasitemia curves were distinct for each treatment scheme. After the 22nd day of infection, the differentiation between the curves became clearer (Figure 1); the effect of each treatment scheme can be easily individualized. In the G-16 CM group, the parasitemia increases until the death of the animals, and it is higher than in the Control group (p=0.0248). In the DI-20 CM group, the curve follows the same pattern observed for the Colombian strain, however with delayed parasitemia peak, and parasitemia levels higher than those observed in the Control group. In the AI-24 CM group, the parasitemia observed (Figure 1) is only from two days, considering that all the animals died by the 23rd day of infection; the parasitemias observed are higher (p=0.0014) than in the Control group. Parasitemia curve of the CI-9CM group is higher than in the Control group, but more stable and with downward trend.

Average parasitemia curve obtained in male Swiss mice, 28 days old, inoculated with 104 blood trypanomastigotes of the Colombian strain of T. cruzi, in the different treatment schemes with Canova medication (CM), or with 1% hydroalcoholic solution (Control). G-16 CM: infected and treated animals with CM for 16 days from the 5th day of the induction of the infection. AI-24 CM: infected and treated animals for 24 days starting treatment three days before the induction of the infection. DI-20 CM: infected and treated animals, using CM for 20 days from the inoculation of parasites. CI-9 CM: infected and treated animals with CM for nine days from the 12th day of infection. Prepatent period (PPP) was significantly higher (p = 0.0000) for the treated groups compared to the Control group (Table 1). Likewise, all treated groups showed significantly lower patent period (PP) (G-16 CM = 18.7 days/p = 0.0232; AI-24 CM = 14 days/p = 0.0000; CM-20 DI = 17 days/p = 0.0000 and CI-9CM = 15 days/p = 0.0000) compared to that observed for the Control group (20 days) (Table 1). Excepting the G-16 CM group, all the other groups significantly advanced the maximum peak day (MPD) of parasitemia, as it can be seen in Table 1.

Statistical significance (p<0.000* and p<0.05**) compared to control group. Control: infected animals treated with 1% hydroalcoholic solution. G-16 CM: infected animals treated using CM for 16 days from the 5th day of the induction of infection. AI-24 CM: infected animals and treated for 24 days starting treatment three days before the induction of the infection. DI-20 CM: infected animals and treated, using CM for 20 days from the inoculation of parasites. CI-9 CM: infected animals and treated using CM for nine days from the 12th day of infection. Maximum parasitemia peak (MPP) and the total parasitemia (TP) were significantly lower in the CI-9 CM group (8.33x10⁵ trypanomastigotes/mL and 4.47x10⁶ trypanomastigotes/mL, respectively) than in the Control group (2.03x10⁶ trypanomastigotes/mL and 5.99x10⁶ trypanomastigotes/mL) (p<0.0000 and p=0.0000) (Table 2). Statistical significance (p<0.000* and p<0.05**) compared to control group. Control: infected animals treated with 1% hydroalcoholic solution. G-16 CM: infected animals treated using CM for 16 days from the 5th day of the induction of infection. AI-24 CM: infected animals and treated for 24 days starting treatment three days before the induction of the infection. DI-20 CM: infected animals and treated, using CM for 20 days from the inoculation of parasites. CI-9 CM: infected animals and treated using CM for nine days from the 12th day of infection. Survival time was significantly different between groups (p=0.0001) (Figure 2). The CI-9 CM group presented longer survival time, with an animal surviving indefinitely.

Male Swiss mice, 28 days old, inoculated with 104 blood trypanomastigotes of the Colombian strain of T. cruzi, in the different treatment schemes with Canova medication (CM), or with 1% hydroalcoholic solution (Control). G-16 CM: infected and treated animals with CM for 16 days from the 5th day of infection induction. AI-24 CM: infected and treated animals for 24 days, starting the treatment three days before the induction of the infection. DI-20 CM: infected and treated animals with CM for 20 days from the inoculation of parasites. CI-9 CM: infected and treated animals with CM for nine days from the 12th day of infection. Mortality rate of animals from the Control group, G-16 CM, AI-24 CM and DI-20 CM was 100%. In the group CI-9 CM, mortality rate was 90%. All the animals in the AI-24 CM group died faster (between 20th and 24th dpi) than the ones in the Control group (between 21st and 78th dpi).
Histopathological evaluation

Figure 3: Microphotographs of cardiac tissue sections: Cardiac tissue sections stained with HE, obtained from male Swiss mice, infected with the Colombian strain of *T. cruzi* in various therapeutic treatments. A: Control: several nests of amastigotes and diffuse inflammatory process can be seen (40x magnification). B: G-16 CM the presence of several nests of amastigotes, as well as focal and diffuse inflammation (40x magnification) can be observed. C: AI-24 CM: only a nest of amastigotes, can be seen, but with marked focal and diffuse inflammation (40x magnification). D: DI-20 CM: presence of several nests of amastigotes (20x magnification). E: CI-9 CM: it can be observed the presence of several nests of amastigotes, and moderate diffuse inflammatory process (40x magnification).

Table 3: Nests of amastigotes in different tissues of animals in the different treatment schemes with Canova medication (CM).

| Group      | Heart       | Spleen | Liver      | Muscle     | Intestine   |
|------------|-------------|--------|------------|------------|-------------|
| Control    | 20.63±1.36  | 0      | 0.81±0.24  | 6.19±1.18  | 6.37±1.61   |
| G-16 CM    | 40±8.37**   | 1.25±0.5| 0.25±0.5   | 11±3.92*   | 6.75±4.65   |
| AI-24CM    | 10.5±3.66*  | 0      | 0.25±0.16  | 4.17±2.01  | 2.08±0.57*  |
| DI-20CM    | 42.67±9.05**| 0      | 0.16±0.19  | 12.08±3.93*| 8.92±0.83   |
| CI-9CM     | 31.92±4.84* | 3.08±0.88*| 0.66±0.47  | 1.59±0.42* | 3.35±1.26   |

Table 4: Amastigotes per nest in different tissue of animals in the different treatment schemes with Canova medication (CM).

| Group      | Heart       | Spleen | Liver    | Muscle    | Intestine   |
|------------|-------------|--------|----------|-----------|-------------|
| Control    | 616.75±81.22| 0      | 27.13±9.36| 368.31±83.32| 61.06±7.18  |
| G-16 CM    | 725±17.43   | 13.5±16.54| 13.25±26.5| 843±26.47* | 74.25±55.51 |
| AI-24CM    | 331.75±110.65*| 0   | 13.41±9.36| 270.5±114.8 | 17.5±4.44*  |
| DI-20CM    | 1195.84±226.18**| 0  | 12.5±15   | 820.33±357.2*| 87.67±16.74 |
| CI-9CM     | 734±96.02   | 45.84±10.56*| 18.6±9.68 | 88.75±440.25| 28.59±11.31 |

Table 5: Amastigote nests and amastigotes per nest in sections of tissues of animals in the different treatment schemes with Canova medication (CM).

| Group      | Total Nests | Total Amastigotes |
|------------|-------------|-------------------|
| Control    | 34±1.58     | 1073.25±149.37    |
| G-16 CM    | 59.25±7.27* | 1669±193.92*      |
| AI-24CM    | 17±3.99*    | 633±138.66**      |
| DI-20CM    | 63.83±10.17*| 2116.34±368.37*   |
| CI-9CM     | 40.5±4.44   | 915.34±105.17     |

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Table 6: Focal inflammation present in sections of tissues of animals in the different treatment schemes with Canova medication (CM).  

| Group     | Heart   | Muscle  | Intestine | Liver   |
|-----------|---------|---------|-----------|---------|
| Control   | 3.8±0.24| 3.1±0.25| 3±0.35    | 0.8±0.24|
| G-16 CM   | 1±0.82**| 3.25±1.71*| 4±2.94   | ±0      |
| AI-24CM   | 6.25±1.45**| 4.5±1.29**| 3.68±0.98 | 0.3±0.67|
| DI-20CM   | 2.5±0.92 | 4.75±0.78*| 6.25±0.57| 0.33±0.27|
| CI-9CM    | 0.8±0.58**| 1.67±0.47 | 3.3±1.19 | 0.25±0.32|

Table 7: Diffuse inflammation present in sections of tissues of animals in the different treatment schemes with Canova medication (CM).  

| Group     | Heart   | Muscle  | Intestine | Liver   |
|-----------|---------|---------|-----------|---------|
| Control   | 5.19±0.72| 2.93±0.83| 7.75±0.68 | 1.81±0.37|
| G-16 CM   | 5.75±0.5 | 5.25±1.89| 10±*     | 0.5±1.0 |
| AI-24CM   | 9.25±0.84**| 6.25±1.34 | 9.83±0.34*| 0.75±0.5 |
| DI-20CM   | 6.34±0.61 | 4.92±0.69*| 9.24±0.17 | 1.84±0.58|
| CI-9CM    | 3.5±1.11* | 1.9±1.09 | 9.0±0.79 | ±0      |

Table 3-7 & Figure 3 show details of the histopathological evaluation for the different experimental groups. In Table 3 & Figure 3, it can be observed that in the heart, excepting the AI-24 CM group (p = 0.0349), all the treated groups showed significantly larger number of “amastigotes nests” compared to the Control group (GI-16CM-p=0.0005; DI-20CM-p=0.0001; CI-9CM-p=0.0206). Average number of nests of amastigotes in different tissue. Statistical significance (p <0.05* and p <0.005**) compared to control group. Control: infected animals treated with 1% hydrosolcohol solution. G-16CM: infected animals treated using CM for 16 days from the 5th day of the induction of infection. AI-24CM: infected animals and treated for 24 days starting treatment three days before the induction of the infection. DI-20 CM: infected animals and treated, using CM for 20 days from the inoculation of parasites. CI-9CM: infected animals and treated using CM for nine days from the 12th day of infection.

Cardiac tissue sections stained with HE, obtained from male Swiss mice, infected with the Colombian strain of T. cruzi in various therapeutic treatments. A: Control: several nests of amastigotes and diffuse inflammation process can be seen (40x magnification). B: G-16CM the presence of several nests of amastigotes, as well as focal and diffuse inflammation (40x magnification) can be observed. C: AI-24CM: only a nest of amastigotes, can be seen, but with marked focal and diffuse inflammation (40x magnification). D: DI-20CM: presence of several nests of amastigotes (20x magnification). E: CI-9CM: D: DI-20CM: it can be observed the presence of several nests of amastigotes, and moderate diffuse inflammatory process (40x magnification).

Considering the parameter “number of amastigotes per nest” in the heart (Table 4), while the AI-24 CM group presented less than the Control group (p = 0.0153), the DI-20 CM group had the greatest “number of amastigotes per nest” compared to the Control group (p=0.0001). In the skeletal muscle, the “number of amastigotes nests” are significantly greater in the G-16 CM and DI-20 CM groups (p=0.0235 and p=0.0075, respectively), and lower in the CI-9 CM group (p=0.0291) compared to the Control group. The G-16 CM and DI-20 CM groups had higher (p=0.0059 and p=0.0081) “number of amastigotes per nest” than the Control group. In the intestine, the AI-24 CM group showed lower parasitism tissue than the Control group (p=0.0192) for both the “number of nests” and the “number of amastigotes per nest” (p=0.0356).

In the spleen, the CI-9 CM group showed significantly more “nests of amastigotes” and “amastigotes per nest” than the Control group (p=0.0412 and p=0.0497). In the liver, there was no statistical difference between the different groups. Average number of amastigote per nest in different tissue Statistical significance (p <0.05* and p <0.005**) compared to control group. Control: infected animals treated with 1% hydrosolcohol solution. G-16 CM: infected animals treated using CM for 16 days from the 5th day of the induction of infection. AI-24CM: infected animals and treated for 24 days starting treatment three days before the induction of the infection. DI-20 CM: infected animals and treated, using CM for 20 days from the inoculation of parasites. CI-9CM: infected animals and treated using CM for nine days from the 12th day of infection.

Table 6 & 7 shows, in detail, the inflammatory process in the heart, the skeletal muscle, the intestine and the liver in the different treatment schemes studied. In the heart, it shows the presence of focal inflammation in G-16 CM (p=0.0005) and CI-9 CM (p=0.0003) groups, and diffuse inflammation in CI-9 CM (p=0.0099) group, being lower than in the Control group. In the intestine, it shows that the presence of diffuse inflammation was statistically lower in the CI-9 CM group (p=0.0453) than in the Control group.

Average per group of the total number of amastigote nests and amastigotes per nest. Statistical significance (p <0.001* and p <0.005**) compared to control group. Control: infected animals treated with 1% hydrosolcohol solution. G-16 CM: infected animals treated using CM for 16 days from the 5th the induction.
of infection. Al-24CM: and treated for 24 days starting treatment three days before the induction of the infection. DI-20 CM: infected animals and treated, using CM for 20 days from the inoculation of parasites. CI-9 CM: infected animals and treated using CM for nine days from the 12th day of infection. Average of total number of diffuse inflammation present in sections of tissue. Statistical significance (p <0.05* and p <0.005**) compared to control group. Control: infected animals treated with 1% hydroalcoholic solution. G-16 CM: infected animals treated using CM for 16 days from the 5th day of the induction of infection. AI-24CM: infected animals and treated for 24 days starting treatment three days before the induction of the infection. DI-20 CM: infected animals and treated, using CM for 20 days from the inoculation of parasites. CI-9 CM: infected animals and treated using CM for nine days from the 12th day of infection.

Cytokines dosage

Cytokines values for IL-2, IL-4, IL-6, IL-10, IL-17A, IFNγ, and TNFα evaluated for different treatment regimens are shown in Table 8. The IL-2 was only detected in the NI group. It was observed lower production of IL-17A in the Cl-9 CM group (0.02 pg/mL, p=0.0463) compared to the Control group (0.85 pg/mL). In the same way, the serum levels of IL-17A were significantly higher in the Control group (0.85 pg/mL) compared to the other groups treated with CM water diluted (DI-20 CM-0.003 pg/mL, p=0.049, Al-24 CM - 0.02 pg/mL, p=0.049).

The IL-10 levels were significantly higher in the Control group compared to the AI-24-CM group (p=0.046). The TNFα and IFNγ serum levels were significantly higher in the treated groups (p=0.0495) compared to the NI group, however without significant difference compared to the Control group.

Mean and standard cytokines levels (pg/mL) in the serum of mice. Statistical significance: p <0.05* compared to NI and p <0.005** compared to control group. Control: infected animals treated with 1% hydroalcoholic solution. G-16CM: infected animals treated using CM for 16 days from the 5th day of the induction of infection. AI-24CM: and treated for 24 days starting treatment three days before the induction of the infection. DI-20CM: infected animals and treated, using CM for 20 days from the inoculation of parasites. CI-9CM: infected animals and treated using CM for nine days from the 12th day of infection.

Discussion

In the present study, the different treatment schemes exerted different effects in the course of the experimental mice infection, expressed by changes in the parasitological, histopathological and immunological parameters evaluated, compared to the Control animals infected with the Colombian strain of T. cruzi that showed the classic pattern for this strain infection [35-39]. While for some parameters, it was not possible to identify differentiation between the treatment schemes, for others it was clear that the different schemes of the CM utilization could be beneficial, or harmful, to the animals infected and treated.

The treatment scheme of the CI-9CM group, with the animals being treated from the 12th day of infection and confirmation of their infection showed better results considering the parasitological parameters. In this group, the PPP was significantly higher; both the parasitemia peak and the total parasitemia were lower than that observed in the Control group. All these alterations indicate a more balanced host-parasite relationship [28,29]. In the same way, the CI-9CM group resulted in longer survival, with an animal surviving indefinitely, reinforcing the parasitological data that showed a better performance for this group. This change in the infection course, in this studied model, reflects a trend of lower mortality rate that, indisputably, is an observed benefit for such treatment scheme.

This result is supported by the findings of Aleixo et al. [28], showing that the administration of medication highly diluted in water and four days after the detection of the infection, in mice inoculated with the strain Y of T. cruzi, promoted better clinical and parasitological evolution, with lower parasitemia and mortality, tending to higher animal survival. Aleixo et al. [28] also demonstrated that the administration of highly diluted medication offered by gavage produced harmful changes in the course of the infection in mice infected by the Y strain of T. cruzi. Pupulin et al. [30] also showed that the administration of highly diluted medication offered by gavage, altered the evolution course of the experimental infection by the strain Y of T. cruzi, a strain partially sensitive to conventional allopatic treatment.

The authors say that administered for 20 consecutive days, daily from the 5th day of the infection, the CM was harmful, causing 100% of death in the infected animals, in a short period. This result was also observed in this study, for the GI-16CM group treated by gavage. It was interesting to notice that, along with the results described above, the animals in the GI-16CM group, as well as all the others that received the CM showed increase in PPP, and anticipation in the parasitemia peak, as observed in the CI-9CM group, which showed the best performance [44]. This result leads to the discussion that the CM really alters host-parasite relationship in experimental infection. This conclusion may suggest that in clinical approach using CM, the practitioner must be careful in relation to the best treatment scheme for the patient.

Pupulin et al. [30] using the CM treatment by gavage and the murine infection with the Y strain of T. cruzi, observed an increase in parasitemia, and a clear change in the patterns of tissue tropism for the treated group. In this work, different administration schemes of the CM interfered in the infection evolution, with differences in parasitological and histopathological parameters, in relation to the Control group. The treatment initiated at different periods of the infection, resulting in different effects, and suggesting alterations of the host-parasite relationship. The CM is considered an immunomodulator, and the infection by T. cruzi is described as intense and widespread inflammatory process, with strong immunosuppression component, expressed by changes in the levels of several cytokines and their receptors.
The best performance was observed with the CI-9CM group where animals were only treated after the detection of the infection. For the AI-24CM group, the treatment started 3 days before the infection. The animals in this group showed a more intense reaction when in contact with the parasite, probably by heart failure, because as observed in the AI-24CM group, the presence of inflammation in the heart was significantly higher compared to the Control group, a fact also observed in the liver. In the present article, the wide variation of IL-17A calls attention. In the Control group, it is observed greater concentration compared to that in the treated groups. In the CI-9CM group, which presented the best performance, its concentration is equal to the group that presented early mortality and worse performance (AI-24CM), being seven times higher than in the DI-20CM group that presented ascending parasitemia. Shortage of IL-17 response results in higher mice susceptibility to T. cruzi infection [46]. Deficiency on this cytokine response leads to a severe inflammatory response, increasing the IFNγ and TNFα production, with liver damage and death [47]. According to Guedes et al. [48], the IL-17 controls the resistance to the T. cruzi infection modulates the differentiation of Th1 cells, having a strong correlation between low levels of IL-17 and high levels of IFNγ and TNFα, with severe myocarditis in humans. According to Miyazaki et al. [46], the dosage of IL-17A in splenocytes of infected animals with T. cruzi showed maximum peak on the 21st day of infection. In the present study, in the group of animals with the worst performance (AI-24CM) the dosage of IL-17A in the serum was low on the 21st day of infection.

Accented heart inflammation was also observed with premature death of the animals, as reported by Guedes et al. [49]. For the best performance group (CI-9CM), it was also observed a low production of IL-17A, but with the presence of statistically lower inflammation in the heart compared to the Control group, and with an animal surviving indefinitely. This difference between the groups AI-24CM and CI-9CM can be explained by the modulation of the immune system by the CM with the same direction to results for IL-17A, INFγ and TNFα but with IL-10 in the opposite direction, with significantly reduced concentration in AI-24CM compared to Control group. Comparison between CI-9CM and AI-24CM was not significant. The intra-group variation was very high so the standard deviation was so big. Others experiments with more animals in groups will be necessary to confirm these data. In the murine infection with the Colombian strain of T. cruzi, the control of the parasitemia, even for a period and with some survival can be considered extremely relevant data [36,37] suggesting deeper studies related to the treatment schemes with Canova.

Conclusion

The CM modulates the production of cytokines in experimental infection of mice by T. cruzi, the modulation is dependent on the treatment scheme. Its a fact that the treatment scheme used determined the behavior of each group. The concurrent administration of drugs with the infection promoted increase of the second peak of parasites (DI-20CM). The administration of CM before the infection (AI-24CM) promoted early death of animals; when administered after confirmation of the infection (CI-9CM) it was beneficial, however, it is still not the ideal. Although the CM is used successfully in human diseases involving infection and immune suppression, our results alert to the indiscriminate use of ultra diluted medication and to the need for more studies, considering the animal species versus doses and treatment schemes.

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