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Major Article

Evaluation of *in vitro* anti-*Trypanosoma cruzi* activity of medications benznidazole, amiodarone hydrochloride, and their combination

***Angela Maria Lourenço*[1], *Cristiane Castro Faccini*[1], *Cristiane Aparecida de Jesus Costa*[1], *Gabrielle Balestrin Mendes*[1] *and Abilio Augusto Fragata Filho*[1]**

[1]. Laboratório de Doença de Chagas, Instituto de Cardiologia Dante Pazzanese, São Paulo, SP, Brasil.

Abstract

Introduction: Approximately seven to eight million people worldwide have Chagas disease. In Brazil, benznidazole is the most commonly used active drug against *Trypanosoma cruzi*; however, its efficacy is limited, and side effects are frequent. Recent studies suggest that amiodarone may be beneficial in the treatment of this disease, by exerting anti-*T. cruzi* action. This study evaluated changes in *T. cruzi* cell count in *in vitro* cultures subjected to different doses of benznidazole, amiodarone, and their combination. **Methods:** *T. cruzi* (Y strain) cultures containing approximately 100,000 cells were treated with either 100mg, 50mg, 25mg, 12.5mg, or 10mg of benznidazole, amiodarone, or their combination. On the 4th day, cell count was compared to the baseline data. **Results:** On the 4th day, no parasites were observed in any of the treated cultures. **Conclusions:** Benznidazole and amiodarone were equally effective in eliminating *T. cruzi* in culture. The combination of the two drugs was also equally effective, but our data cannot demonstrate synergism, as similar results were obtained when the drugs were tested individually or in combination. It is suggested that this study be repeated with other *T. cruzi* strains to determine whether similar results can be obtained again.

Keywords: Chagas disease. *Trypanosoma cruzi*. Trypanocidal activity. Benznidazole. Amiodarone hydrochloride.

INTRODUCTION

Chagas disease was described by Carlos Chagas in 1909. Its etiologic agent is the flagellate protozoan *Trypanosoma cruzi*[1]. *T. cruzi* infection in humans is one of the major endemic diseases in Latin America[2]. It is estimated that approximately seven to eight million people worldwide are infected[3]. Vector transmission occurs through the penetration of metacyclic trypomastigotes, present in the feces and urine of insects, through damaged skin or through healthy mucosa[2]. In Brazil, the principal method of transmission is the oral route, which is responsible for increased morbidity and mortality and is becoming one of the most common modes of transmission from the public health perspective[4]. According to the Brazilian Consensus of Chagas Disease, trypomastigote, epimastigote, and perhaps amastigote forms of *T. cruzi* may be transmitted orally[5]. Transmission can also occur through the transfusion of infected blood, from mother to fetus, through organ transplantation, or through laboratory accidents[3]. Many inhabitants of Latin America have migrated to other continents, carrying this disease and transmitting it, mainly through blood transfusions and organ transplantation, to the inhabitants of non-endemic countries that do not have triatomine vectors. Chagas disease is therefore present in North America, Europe, Asia, and Oceania, making it a worldwide public health problem[3,6].

The following developmental forms of *Trypanosoma* have been defined: epimastigote (found in axenic culture and in the digestive tract of the insect vector), trypomastigote (found in the insect vector, in cultured cells, and in the blood and intercellular space of the vertebrate host), and amastigote (found within vertebrate host cells or in cell cultures). *T. cruzi* can utilize a range of transmission mechanisms in the vertebrate host, and trypomastigotes are able to penetrate any type of cell (except neutrophils and eosinophils) in order to complete their life cycle. After penetration into the host cell, *T. cruzi* differentiates into the amastigote form and initiates the intracellular binary division process. The amastigotes transform into trypomastigotes, breaking open the host cell and moving into the bloodstream, spreading to penetrate cells of different organs, and then repeating the cycle[7-9]. In humans, the acute phase of the disease is characterized by intense parasitaemia and inflammation, but with low clinical expression and mortality. This phase lasts for approximately 8 to 10 weeks and is followed by a progressive decrease in the number of parasites

Corresponding author: Drª Angela Maria Lourenço.
e-mail: angela.lourenco@idpc.org.br
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in peripheral blood and initiation of the fibrotic process: a characteristic of the chronic phase[2,10]. When the insect feeds on human or contaminated animal blood, the circulating form of *T. cruzi* develops in its gut. A triatomine becomes infective 20 days after feeding with blood containing *T. cruzi* and can remain as such for its entire lifespan (approximately one year)[8].

The disease may have different clinical presentations in humans, varying from region to region. Different strains of the parasite are found in nature, circulating between man, vectors, domestic animals, and wild reservoirs. These strains behave differently with regards to parasitaemia curves, interaction with host cells, and immune response[7,9].

Nifurtimox and benznidazole are the only active drugs against *T. cruzi*; however, they have limited efficacy and cause frequent side effects[9,11].

Amiodarone (a Class III antiarrhythmic drug) is the most widely used drug for the treatment of patients with Chagas disease and cardiac arrhythmia. Its antifungal and antiprotozoal actions have been recently identified[12]. Amiodarone contains active components that target *T. cruzi*, both *in vitro* and in experimental animals. It acts through homeostatic disruption of Ca^{2+} and blocks oxidosqualene cyclase activity in *T. cruzi*, causing ultrastructural damage[13] and also blocking the biosynthesis of protozoan ergosterol[14]. Adesse et al. treated *T. cruzi*-infected cardiac myocytes with various concentrations of amiodarone and observed a different effect on the growth of the intracellular amastigote form of *T. cruzi*. They observed mitochondrial swelling and disorganization of reservosomes and the kinetoplast, as well as inhibition of the differentiation of amastigotes into trypomastigotes[14]. In a study using dronedarone (an amiodarone derivative), Benaim et al. showed that this new drug has the same effect on *T. cruzi*, using 50% of the concentration of amiodarone and observing the same mechanisms[15]. In a study using electron microscopy, Veiga-Santos et al. showed the synergic action of amiodarone and posaconazole (a potent antifungal drug) against *T. cruzi*, observing mechanisms that included wrinkling of the protozoan surface, swelling of the mitochondria, shedding of plasma membrane vesicles, alterations in the kinetoplast, disorganization of the Golgi complex, accumulation of lipid inclusion in the cytoplasm, and the formation of autophagic vacuoles[16].

Our study evaluates the ability of amiodarone hydrochloride (amiodarone), benznidazole (BZ), and a combination of the two, to prevent the proliferation and/or to eliminate *T. cruzi* (Y strain) *in vitro*, at doses of 100mg, 50mg, 25mg, 12.5mg, and 10mg/100,000 parasites.

METHODS

Parasites

The trypomastigote form of *T. cruzi* Y strain was obtained from *in vivo* cultures and maintained in the laboratory by successive passages in Swiss and A/Snell mice, weighing on average 23g each. Mice were each inoculated with approximately 100,000 *T. cruzi* cells. On the 8th day after inoculation (at the peak of parasitaemia), the mice were euthanized, and blood was obtained by cardiac puncture. The plasma was separated. The red blood cell concentrate that is routinely neglected was used for the *in vitro* proliferation of *T. cruzi*. The entire procedure was performed in a completely sterile environment, using laminar flow and autoclaved materials, as per standard operating procedures.

Proliferation *in vitro*

The trypomastigote form of the parasite was cultivated in three test tubes, each containing 5mL of liver infusion tryptose [(LIT), suitable for the cultivation of *T. cruzi*[17]] medium supplemented with 10% inactivated fetal bovine serum; each tube received 1mL of red blood cell concentrate, and was maintained in a growth chamber at 28°C. Fresh preparations were examined after 10 days between a slide and coverslip (22 × 22mm) through an optical microscope, according to a previously described blood culture method[18]. After this period, 1mL of the culture was transferred under laminar flow to another tube containing 5mL LIT medium. After four days in a growth chamber maintained at 28°C, the material was observed between a slide and coverslip, and parasite growth was determined by counting cells using a Neubauer hemocytometer, chosen for the study tubes with 100,000 *T. cruzi* cells per milliliter. On the 14th day, almost all trypomastigotes had differentiated into epimastigotes. Epimastigotes, were used in this study because according to the Brazilian Consensus, this form may also be infective[5]. BZ [obtained from the Pharmaceutical Laboratory of the State of Pernambuco (LAFEPE)] was used as the *gold standard* in comparison with amiodarone (obtained from Libbs Laboratory). The commercial presentation of the two drugs was used as this is how patients are medicated, and because there are no data available on the trypanosomicidal action of the excipients.

The BZ and amiodarone tablets were macerated, weighed on a precision scale, and homogenized, together with 1mL of complete LIT culture medium. The tablets were then added to test tubes containing 5mL of the parasite solution, at the following doses:

- **BZ**: 100mg, 50mg, 25mg, 12.5mg, and 10mg.
- **Amiodarone**: 100mg, 50mg, 25mg, 12.5mg, and 10mg.

This procedure was performed twice for each dose of medication. In all procedures, two control tubes were prepared, without the addition of the drugs: all tubes were maintained at 28°C to allow direct comparison.

At the end of the 4th day (the peak of parasite growth *in vitro*[17]), the contents of the tubes were observed using the Neubauer hemocytometer and the optical microscope. The results were compared with the numbers of parasites before treatment and expressed as percentages. This served to determine the lowest effective dose. Then, using this lowest effective dose, readings were taken from the 1st to the 4th day, using both the individual drugs and their combination.

Ethical considerations

This research project was approved by the Ethics Committee for the Use of Animals (CEUA) of the Dante Pazzanese Institute of Cardiology, under the Project Registration No.: 008/2015.

RESULTS

The results are shown in **Figure 1** and **Figure 2**.

DISCUSSION

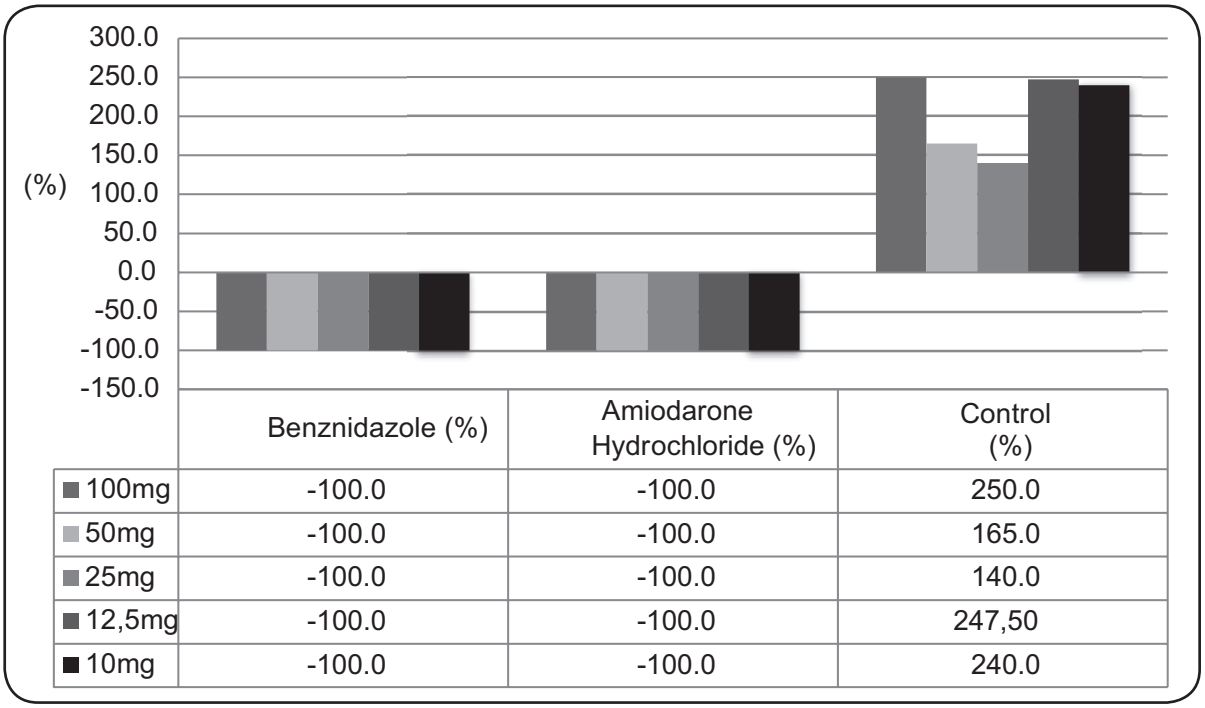
This study was conducted to determine whether other drugs such as amiodarone have comparable *in vitro* trypanocidal action to BZ, the most widely used trypanocidal drug in Brazil. The efficiency of BZ treatment is unclear, and the literature is not consensual.

The analysis of randomized patients treated with either BZ or placebo (BENEFIT study[19]) showed that the drug was not effective in patients with established heart disease. Although BZ decreased the number of circulating parasites in blood, it did not significantly reduce clinical worsening. It was observed in this study that only one subset of patients, who had also been treated with amiodarone as well as BZ, seemed to benefit from the treatment[19]. A retrospective study evaluated patients with normal electrocardiograms, who were either treated with BZ or left untreated. After following these patients for two decades, a significant decrease was observed in the appearance of electrocardiographic changes in treated patients[20].

Adesse et al.[14] demonstrated that amiodarone induces drastic morphological changes in intracellular amastigotes *in vitro*, including mitochondrial swelling and disruption of reservosomes and the kinetoplast. The drug decreased intracellular amastigote count and trypomastigote release after completion of the intracellular parasite cycle, and blocked amastigote differentiation into trypomastigotes. It also promoted the recovery of cellular physiology concomitant with the elimination of intracellular parasites. Treatment with amiodarone of animals infected with *T. cruzi* reduced parasitaemia and increased survival. Although the antiparasitic activity of amiodarone has been shown before, there is a lack of data on the effect of this compound on *T. cruzi* structure and host cells, and the recovery of these cells after antiparasitic treatment. In the heart, the effects of this drug include inhibition of Na^+/Ca^{2+} channels. It was found that the *in vitro* and *in vivo* activity against *T. cruzi* take place through Ca^{2+} homeostasis disruption and inhibition of ergosterol biosynthesis[14].

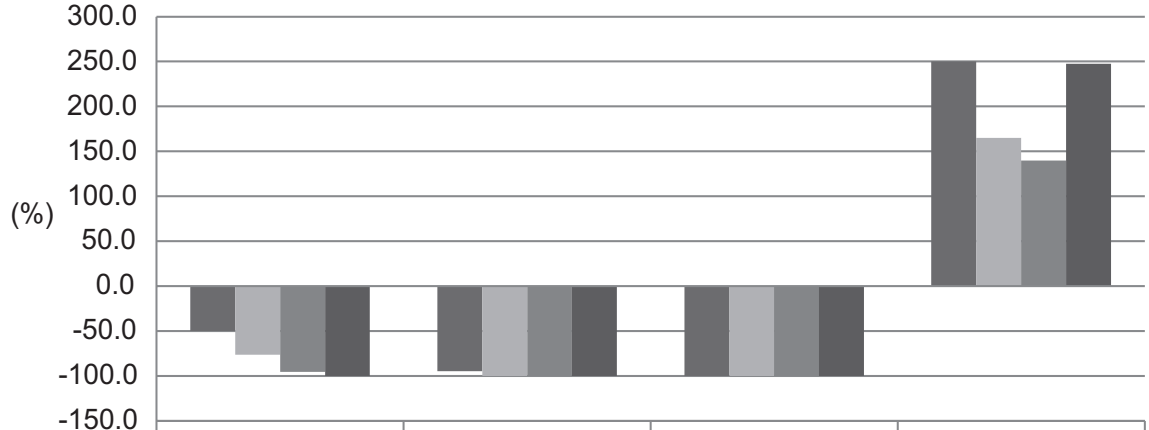
We cannot compare the drug concentrations studied here in culture with those observed in human plasma at the usual therapeutic doses, as the parasite concentrations are very different in both cases, and many factors interfere with the absorption of drugs.

Amiodarone treatment in mice infected with trypomastigotes has previously been seen to reduce parasitaemia and increase animal survival. When administered in combination with posaconazole, there was a delay in the progression of parasitaemia. Most of the animals treated with this combination presented a negative blood culture, xenodiagnosis, and blood polymerase chain reaction (PCR) for the nuclear deoxyribonucleic acid (DNA), indicating very low parasitic loads. These results demonstrate that amiodarone has anti-



	Benznidazole (%)	Amiodarone Hydrochloride (%)	Control (%)
100mg	-100.0	-100.0	250.0
50mg	-100.0	-100.0	165.0
25mg	-100.0	-100.0	140.0
12,5mg	-100.0	-100.0	247,50
10mg	-100.0	-100.0	240.0

FIGURE 1: Variation in *Trypanosoma cruzi in vitro* culture cell count after treatment with different dosages of BZ and amiodarone, along with control culture counts. Readings were taken on the 4th day after the addition of drugs. **BZ:** benznidazole.



	Benznidazole (%)	Amiodarone Hydrochloride (%)	Benznidazole + Amiodarone (%)	Control (%)
1st day	-50.0	-94,50	-100.0	250.0
2nd day	-76,50	-100.0	-100.0	165.0
3rd day	-95,50	-100.0	-100.0	140.0
4th day	-100.0	-100.0	-100.0	247,50

FIGURE 2: Variation in *Trypanosoma cruzi in vitro* culture cell count after treatment with 10mg BZ, 10mg amiodarone, or 10mg of both drugs in combination, along with control culture counts. Readings were taken from the 1st to the 4th day after the addition of drugs. **BZ:** benznidazole.

T. cruzi activity both *in vivo* and *in vitro*, used independently or in combination with other drugs[12].

Oral transmission of Chagas disease is currently the most important route for *T. cruzi* transmission in Brazil, and can also be utilized by the epimastigote form of the parasite: in our cultures, almost all forms of *T. cruzi* were epimastigotes

We noted that *in vitro*, amiodarone had equivalent trypanocidal action to BZ at all doses studied; that is, all parasites (*T. cruzi* epimastigotes) were dead on the 4th day. When we added both drugs in combination to cultures, the same result was observed. It was therefore not possible to say whether these drugs had a synergistic effect, as there was no difference between the parasite counts in cultures treated with individual or combined medications on the 4th day.

Limitation of the study

In this study, we used only the Y strain of *T. cruzi*, and were not aware whether amiodarone would have the same effect on other *T. cruzi* strains. We suggest that this study be repeated with the use of other *T. cruzi* strains, in order to determine whether the same results will again be obtained.

Conflict of interest

The authors declare that there is no conflict of interest.

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