Original Article

Evaluation the *In Vitro* Action of Ciprofloxacin Hydrochloride, Naproxen, and Folic Acid on *Trypanosoma cruzi*, using Benznidazole as a Gold Standard

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Abstract

Background: **Benznidazole is the only drug against Trypanosoma cruzi (T. cruzi) available in Brazil. However, it has limited efficacy and frequent side effects. Recent studies suggest that ciprofloxacin hydrochloride, naproxen, and folic acid may have beneficial effects in treating the Chagas disease.**

Objective: **To assess the in vitro parasiticidal action of ciprofloxacin, naproxen, and folic acid on the Y strain of T. cruzi, using benznidazole as a gold standard.**

Methods: **Tablets of benznidazole, ciprofloxacin hydrochloride, naproxen, and folic acid were macerated, weighed, and added to test tubes with a culture medium containing 100,000 forms per ml of T. cruzi trypomastigotes (strain Y). Samples from each tube were analyzed and counted in a Neubauer hemocytometer under an optical microscope for four days after adding the drugs. Thus, the minimum effective dose of each drug was determined.**

Results: **As compared with benznidazole, higher concentrations of ciprofloxacin and naproxen were effective as in vitro parasiticides. In contrast, folic acid showed no direct in vitro parasiticidal action. Hence, previous observations reporting its effectiveness are probably due to indirect action.**

Conclusion: **In our experiment, ciprofloxacin and naproxen showed a trypanosomicidal effect in vitro, but folic acid did not show this effect.**

Keywords: **Trypanosoma cruzi; In Vitro Techniques; Folic Acid; Naproxen.**

Introduction

Chagas disease, also known as American trypanosomiasis, was discovered and described by Carlos Chagas in 1909. The disease is caused by *Trypanosoma cruzi*, a unicellular flagellate parasite. Data from the World Health Organization estimate that there are about six to seven million contaminated people and a population of 70 million at risk of being infected.[1]

The disease transmission may be vectorial, by transfusion of blood and blood derivatives, organ transplantation, transplacental, and oral routes, among other mechanisms. Nowadays, the oral transmission related to the intake of açaí juice is the most critical of these mechanisms in Brazil, predominantly in the country's northern region.[2]

Chagas disease has two phases: acute and chronic. The acute phase follows infection and, usually, is not very expressive from a clinical point of view, with high parasitemia and inflammation. This phase lasts about eight to 10 weeks and after that, parasitemia and inflammation decrease substantially. The chronic phase begins at this point, lasting a variable period that can extend over a lifetime, with positive serology only and no clinical manifestations (indeterminate form). About 30 to 50% of the patients develop clinical signs 10 or 20 years after the infection, showing cardiac, digestive, or mixed manifestations, with increased morbidity and mortality.[3]

There are two drugs with parasiticidal effects on *T. cruzi* that have been used for more than 40 years: nifurtimox and benznidazole. However, these drugs have shown low efficacy when administered in the disease's chronic (late) phase.[4] Other pharmacological agents have been tested, for instance, amiodarone. This agent showed an effective *in vitro* parasiticidal action against the trypomastigotes of *T. cruzi*, requiring *in vivo* evaluations.[5]

Other tested compounds are ciprofloxacin, naproxen, and folic acid. Ciprofloxacin, a broad-spectrum antibiotic belonging to the quinolone group, has been tested as parasiticidal because it inhibits the trans-sialidase enzyme, which is vital for *T. cruzi*.[6,7] On the other hand, naproxen is an anti-inflammatory, antipyretic, and analgesic drug derived from propionic acid. This drug inhibits the isoforms I and II of the cyclooxygenase, related to the synthesis of prostaglandin, prostacyclin, and thromboxane

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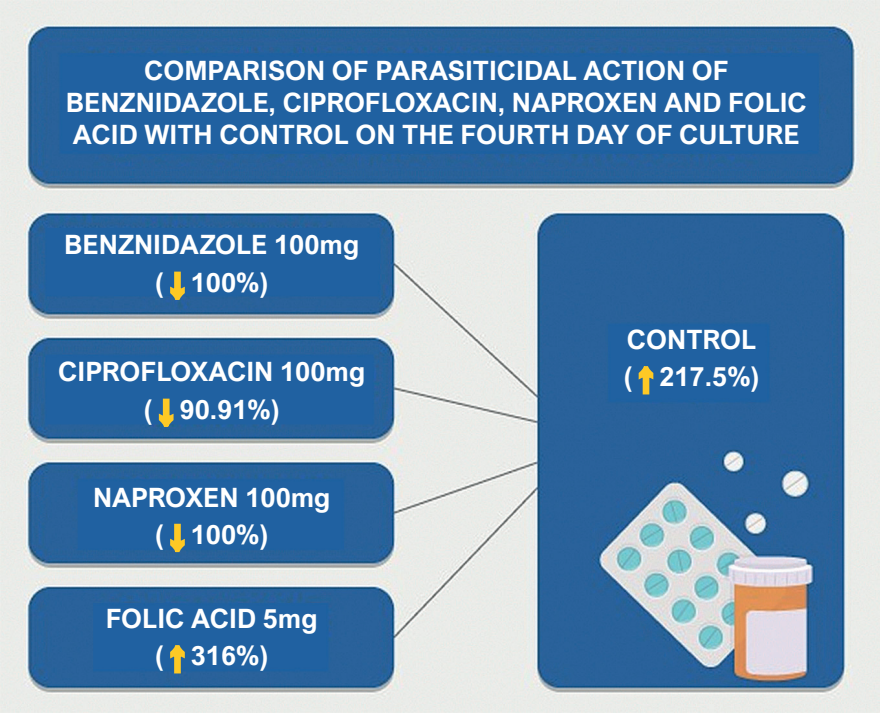
Central Illustration: Evaluation the *In Vitro* Action of Ciprofloxacin Hydrochloride, Naproxen, and Folic Acid on *Trypanosoma cruzi*, using Benznidazole as a Gold Standard



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from arachidonic acid. Naproxen has also been tested against *Trypanosoma* due to its inhibiting action on the synthesis of the farnesyl pyrophosphate enzyme, leading to cellular alterations in the parasite.[8,9] Folic acid is a manufactured form of folate used as a dietary supplement since it is not produced in the human body. The deficiency of folic acid is related to several diseases, such as cancer, Alzheimer's, and megaloblastic anemia. Although the *in vivo* action of this compound against *T. cruzi* has been demonstrated, its mechanism of action remains unclear, with uncertainties about whether the anti-parasitic effect of folic acid is direct or by stimulating the immune system.[10]

Adasme et al.[11] showed the parasiticidal action of ciprofloxacin, naproxen, and folic acid on the Mexican strains NINOA and INC-5 of *T. cruzi* using a murine model. Given the need for new drugs to treat Chagas disease, these encouraging data require further studies to confirm the results, since they have been below expected levels.[11,12] Therefore, our study aims to assess the *in vitro* parasiticidal action of ciprofloxacin, naproxen, and folic acid on the Y strain of *T. cruzi*.

Method

Parasites

The trypomastigotes of *T.cruzi* (strain Y) used in this study were derived from *in vivo* culture maintained in the laboratory by successive passages in mice of the A/Snell strains, each weighing 23g on average. This parasite culture is part of the routine of the Chagas Disease Laboratory of the Dante Pazzanese Institute of Cardiology. We used the red blood cell concentrate of blood samples collected from these mice on the seventh day after infection, which corresponds to the peak of parasitemia; this material is routinely discarded. This was an entirely sterile procedure performed in a laminar flow cabinet, and all instruments used had been sterilized in an autoclave, following the laboratory's standard operating procedures.

In vitro proliferation

The trypomastigote forms of the parasite were cultured in three test tubes containing 5 mL of liver infusion tryptose (LIT) medium supplemented with 10% inactivated fetal bovine serum.[13] Each tube received 1 mL of the infected red blood cell concentrate and was placed in a germination chamber at 28°C. After 10 days, fresh preparations were mounted using slides and 22x22mm coverslips and examined under an optical microscope following the blood culture method of Chiari et al. 1989.[14] In a laminar flow cabinet, 1 mL of culture was collected on each of the 10 days and transferred to tubes containing 5 mL of LIT medium. These tubes were kept in the germination chamber at 28°C for four days. The material was then mounted between slides and coverslips and examined as previously described. The parasites in the

samples were counted using a Neubauer hemocytometer to determine growth, and the tubes that contained 100,000 forms per mL were sorted to test the parasiticidal activity of the evaluated compounds.[15] For the experiment on drug effects, benznidazole was used as a gold standard for ciprofloxacin hydrochloride, naproxen, and folic acid.

Tablets of each drug were individually macerated, weighed on a precision scale, and homogenized with 1 mL of LIT medium. Subsequently, the test tubes containing 5 mL of the solution with the parasites received the tested compounds at three concentrations (10, 20, and 100 mg) — except for folic acid (one dose of 5 mg) (Table 1).

The choice of doses was based on commercial presentations whose use has already been widely evaluated.

After completing the experiments, the minimum effective dose of each drug added to the culture was determined. This procedure was performed twice for each dose of the medication. Two control tubes without the addition of medication were used for comparison in all procedures. At the end of the fourth day of exposure to the tested drugs, the material of each tube was analyzed and counted in a Neubauer hemocytometer under an optical microscope.

Table 1 – Experimental design for testing different drug doses on culture of Trypanosoma cruzi trypomastigote (5mL-tubes)

Drugs tested	Treatment 1	Treatment 2	Treatment 3
Benznidazole	100mg	20mg	10mg
Ciprofloxacin hydrochloride	100mg	20mg	10mg
Naproxen	100mg	20mg	10mg
Folic acid	5mg		

Results

Table 2 shows the weight of each drug added to each test tube and the drug's final concentration in the 6mL solution (culture medium with parasites and drug). It also describes the absolute number of parasites in each tube on the first, second, third, and fourth days of treatment and the percentage of parasites in each vial on the fourth day in relation to the first day. These data represent the average values after two experiments with each tested compound and control (Central Illustration).

Control Group

The number of parasites in the control group increased by 217.5% on the fourth day of treatment.

Folic acid

There was an increase of 316% of parasites on the fourth day of treatment with folic acid at 0.83mg/mL, a result similar to that in the control group (Figure 1).

Ciprofloxacin

Treatment with 100mg of ciprofloxacin (16.66mg/mL) decreased the number of parasites to 9.09% on the fourth day. Despite less pronounced than the treatment with 100mg, the decreased concentration was still evident when applying 20mg (3.33mg/mL) and 10mg (1.66mg/mL) of ciprofloxacin, with 28.8% and 23.3% remaining parasites in relation to the first day, respectively (Figure 2).

Naproxen

The culture was negative for parasites with 100mg of naproxen (16.66mg/mL) on the fourth day of treatment. Although less significant than treatment with 100mg, the concentrations of parasites also decreased for the remaining treatments, as follows: 22.05% with 50mg (8.33mg/ml); 70.17% with 20mg (3.33mg/ml); 35.76% with 10mg (1.66mg/ml) (Figure 3).

Benznidazole

This drug represented the parasiticidal gold standard in the present study, as it resulted in negative culture for parasites with the treatment of 100mg (16.66mg/mL). In addition, the number of parasites decreased to 0.88% with 50mg (8.33mg/ml), 1.11% with 20mg (3.33mg/ml), and 1.5% with 10mg (1.66mg/ml) treatments (Figure 4).

Discussion

The only drugs with proven parasiticidal action currently used to treat Chagas disease are benznidazole and nifurtimox, although both have non-negligible side effects. Although their efficacy is recognized in the acute phase of the disease in children, their effects in the late chronic phase remain debatable.

The search for new compounds that can act on *T. cruzi* has led researchers to study compounds with potential action on parasite metabolism and its consequent destruction. Moreover, the *in vitro* parasiticidal activity of amiodarone has been recently shown to be similar to that of benznidazole.[16] All these studies have investigated the parasiticidal effects of drugs regularly used in various clinical situations based on their pharmacological properties. Thus, three drugs attracted attention — ciprofloxacin, folic acid, and naproxen[17] — and hence we developed the present *in vitro* study based on these findings.

Ciprofloxacin is a broad-spectrum synthetic antibiotic of the fluoroquinolone class; it has potential parasiticidal action by inhibiting trans-sialidase.[18] We showed an important parasiticidal action of ciprofloxacin at 16.66mg/mL (treatment with 100mg). Only 9.09% of the parasites remained alive at this concentration on the fourth day of culture growth. When using 20mg (3.33 mg/mL) and 10mg (1.66 mg/mL) of this antibiotic, parasite reduction in the culture was less pronounced though still important, with 28.8% and 23.3% of surviving parasites, respectively. These *in vitro* results are encouraging since ciprofloxacin has been used for a long time against several infectious conditions, with a well-known and safe handling.

Naproxen is a propionic acid derivative with analgesic, antipyretic, and anti-inflammatory properties, classified as a non-hormonal anti-inflammatory compound. Its action is based on the inhibition of cyclooxygenase (Cox I and II), which

Table 2 – Absolute number of parasites per treatment in each tube on the first, second, third, and fourth days of treatment and the percentage of parasites in each vial on the fourth day

	Control	Folic acid	Cipro. Hydrochloride	Cipro. Hydrochloride	Cipro. Hydrochloride	Naproxen	Naproxen	Naproxen	Naproxen	BenZ	BenZ	BenZ	BenZ
		5mg	100mg	20mg	10mg	100mg	50 mg	20mg	10mg	100mg	50 mg	20mg	10mg
1st day	188.5	200	66	125	189	46.5	34	85.5	288	164	56.5	180	298.5
2nd day	282.5	610	23.5	100	120	107	29	77	417.5	125	7	134.5	189
3rd day	335	611	10.5	56.5	40.5	0	12	81	157	11.5	3.5	32	51.5
4th day	**410**	**632**	**6**	**36**	**44**	**0**	**7.5**	**60**	**103**	**0**	**0.5**	**2**	**4.5**
Percentage of parasites remaining on the 4th day	217.50%	316	9.09%	28.80%	23:28	0%	22.05%	70.17%	35.76%	0%	0.88%	1.11%	1.50%
Concentration per tube in mg/ml		0.83	16.66	3.33	1.66	16.66	8.33	3.33	1.66	16.66	8.33	3.33	1.66

**6 ml of total volume in each culture tube – two experiments per sample. Cipro: ciprofloxacin; BenZ: benznidazole.*

interferes with the synthesis of prostaglandins, prostacyclins, and thromboxane, from arachidonic acid.[19]

Naproxen at 100mg *in vitro* (16.66 mg/mL) eliminated the parasites from the culture. However, this action was much less expressive at 50mg (8.33 mg/mL), 20mg (3.33 mg/ml), and 10mg (1.66 mg/ml), resulting in concentrations of parasites of 22.05%, 70.17%, and 35.76%, respectively. Naproxen has been used in clinical practice for many years, with well-established mechanism of actions and contraindications.

Folic acid has been used as a dietary supplement for a long time, especially in patients with various types of anemia. Folate deficiency is related to several diseases, such as certain types of cancer, Alzheimer's disease, hypertension, and some maternal-fetal complications.[17] It is also known that cellular immunity is significantly compromised in folate deficiency.[20]

In the present study, folic acid did not inhibit parasitic growth at 5 mg (0.83 mg/ml), resulting in an increase of 316% in the *T. cruzi* in culture in relation to the initial number of parasites.

Benznidazole was used as a gold standard due to its indisputable parasiticidal action, as observed for the doses of 100mg (16.6 mg/mL; 0%), 50mg (8.3 mg/mL; 0.88%), 20mg (3.3 mg/mL; 1.11%) and 10mg (1.7 mg/mL; 1.5%).

Ciprofloxacin and naproxen were also effective *in vitro* as parasiticides at higher concentrations compared to this gold standard. In contrast, folic acid showed no direct *in vitro* parasiticidal action. Thus, previous observations about its effectiveness are probably due to indirect action by improving the immune profile.[17,20] Further studies are needed to confirm this hypothesis.

Conclusion

The use of ciprofloxacin hydrochloride, naproxen, and folic acid is well established and safely controlled. Therefore, in vivo studies, including human tests, are needed to determine whether the parasiticidal effect of these drugs on the parasite's life cycle is direct or indirect. This could bring new perspectives to the treatment of Chagas disease.

In our experiment, ciprofloxacin and naproxen showed a trypanosomicidal effect in vitro, but folic acid did not show this effect.

Limitations of the study

Our study examined the ability of these drugs to eliminate or to prevent the proliferation of the *T. cruzi* Y strain and in an *in vitro* culture medium only.

Author Contributions

Conception and design of the research, Acquisition of data, Analysis and interpretation of the data and Critical revision of the manuscript for content: Faccini CC, Lourenço AM, Fragata Filho AA; Statistical analysis: Fragata Filho AA; Writing of the manuscript: Faccini CC.

Potential conflict of interest

No potential conflict of interest relevant to this article was reported.

Sources of funding

There were no external funding sources for this study.

Study association

This study is not associated with any thesis or dissertation work.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Instituto Dante Pazzanese de Cardiologia under the protocol number 026/2022. All the procedures in this study were in accordance with the 1975 Helsinki Declaration, updated in 2013.

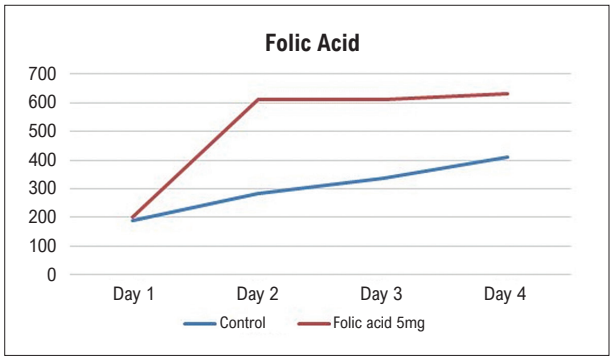

Figure 1 – *Variation in Trypanosoma cruzi in vitro culture cell count after treatment with 5mg folic acid.*

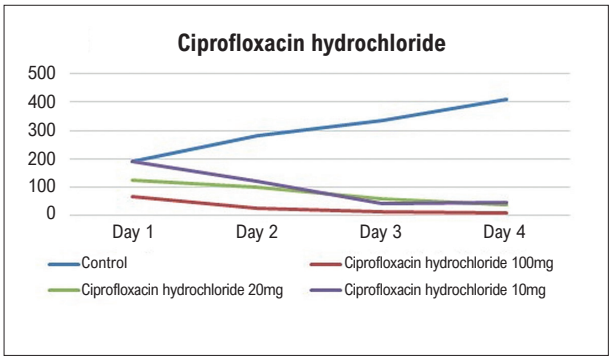

Figure 2 – *Variation in Trypanosoma cruzi in vitro culture cell count after treatment with 10mg, 20mg and 100mg ciprofloxacin hydrochloride, along with control culture counts.*

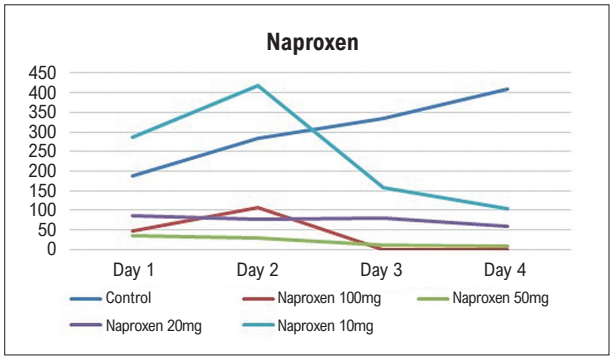

Figure 3 – *Variation in Trypanosoma cruzi in vitro culture cell count after treatment with 10mg, 20mg, 50mg and 100mg naproxen, along with control culture counts.*

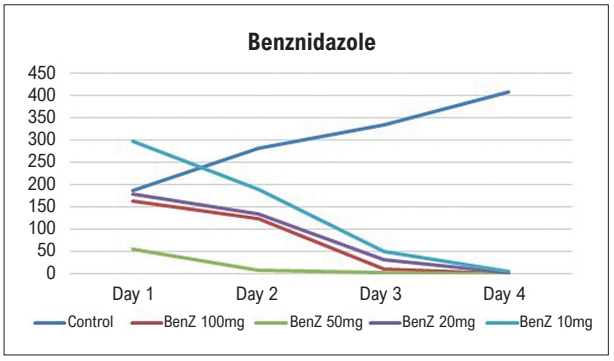

Figure 4 – *Variation in Trypanosoma cruzi in vitro culture cell count after treatment with 10mg, 20mg, 50mg and 100mg benznidazole, along with control culture counts.*

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