PROGRESSIVE CHRONIC CHAGAS HEART DISEASE TEN YEARS AFTER TREATMENT WITH ANTI-TRYPANOSOMA CRUZI NITRODERIVATIVES

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Abstract. A randomized ten-year follow-up study involving 91 Chagas patients and 41 uninfected controls was undertaken to determine the effectiveness of nitroderivative therapy. Anti-Trypanosoma cruzi antibodies were consistently lower one year after treatment than 10 years thereafter (P < 0.001). The blood of all treated and 93.7% of untreated Chagas patients yielded polymerase chain reaction (PCR) product from probes annealing to T. cruzi nuclear DNA, indicating active infection. Competitive PCR showed means ± standard deviations of 20.1 ± 22.6 T. cruzi/ml of blood from untreated and 13.8 ± 14.9 from treated Chagas patients, but the differences between means were not statistically significant (P > 0.05). Electrocardiograms recorded a gamut of alterations several-fold more frequent in Chagas patients, regardless of treatment, than in uninfected controls (P < 0.001). These results show that nitroderivative therapy for T. cruzi infections is unsatisfactory and cannot be recommended since it fails to eradicate the parasite or change the progression of heart disease in chronic Chagas patients.

INTRODUCTION

Chronic Chagas disease is the main cause of heart insufficiency, arrhythmias, and sudden death in regions of the American continent where Trypanosoma cruzi infections are endemic.1–4 Late reports have shown that sterol biosynthesis inhibitors developed for treatment of fungal diseases may induce parasitological cure in 70–90% of mice infected with T. cruzi, and in 53% of chronic Chagas patients.5,6 Nevertheless, nitroderivative compounds with anti-trypansomal activity have been used to treat T. cruzi infections in humans.7–14 Benznidazole (N-benzyl-2-nitro-imidazoleacetamide) and nifurtimox (4-[5-nitro-3-methyl-furphurylideneamino]-tetrahydro-4-H-1, 4-thiazine-1-1-dioxide) curtail parasites and are indicated for treating acute Chagas disease.9–11 However, these drugs cannot be administered without the supervision of a physician because they provoke undesirable side effects.12,15–17 In addition, their efficacy for treating chronic Chagas disease has yet to be determined.

In the past 10 years, we have provided medical care for street cleaners of the Brasília Refuse Department. Brasília, Federal District, is an area free of T. cruzi transmission, in which street cleaners are not exposed to vectors of Chagas disease. Since this study population had migrated from rural regions endemic for Chagas disease in Brazil at least ten years before the beginning of the program, every serologically positive patient enrolled in this study had the chronic infection. We found that 18.5% of this randomized street cleaner population had at least two out of three positive immunological tests for T. cruzi infection.18 Interestingly, a cohort of these Chagas patients had already been treated with the nifurtimox or benznidazole nitroderivatives one year before they were enrolled in this health assistance program. In this follow-up study, we report clinical and laboratory data showing slow progression of heart disease in treated, as well as untreated Chagas patients. Furthermore, we used a molecular method and detected active T. cruzi infections in both groups of patients, regardless of treatment.

MATERIALS AND METHODS

Population. The clinical study was conducted at the Medical Offices of the Brasília Refuse Department. The University of Brasília Faculty Committee on the Conduct of Human Research approved the research protocol. Over the past ten years the physicians have provided health assistance to street cleaners in the program.

Immunological assays. Each individual with an epidemiological history of having lived in huts infested with triatomine bugs, was subjected to immunological evaluation.18 Specific antibodies to the parasite antigens were detected by enzyme-linked immunosorbent assay (ELISA), hemagglutination (IH), and immunofluorescence (IF) tests for Trypanosoma cruzi infection. Chronic infection was disclosed by seropositivity, using the criterion that a patient yielding two out of three positive tests is T. cruzi infected. In this study, each positive test was validated in parallel serological assays in a panel of sera from patients with parasitological confirmation of the infection.19

A skin test against a T. cruzi subcellular fraction T12E antigen was performed in every subject enrolled in this study.19 An intradermal injection of 20 μg of protein in 100 μL of this antigen resulted in local inflammatory reaction, the diameters of which were registered after 48 hr. The area of induration was calculated according to the formula: a/2 × b/2 × π = cm². In a previous study it was shown that Chagas patients yielded indurated reactions > 1.5 cm², whereas controls yielded erythematous areas < 1 cm², at the site of T12E antigen injection.19

Xenodiagnosis. Parasitological demonstration of chronic T. cruzi infections was sought by xenodiagnosis. One diagnostic test consisted of 40 first-instar nymphs of the reduviid bug Dipetalogaster maxima, obtaining blood meals from one patient with positive immunological tests for the infection.20 Feces from each bug were examined twice (30 and 60 days post-exposure) for parasites.

Definition of study groups. One hundred and thirty-two street cleaners who volunteered to participate in this study were included in three groups. Among 91 street cleaners with serological evidence of T. cruzi infection, there were 45 chronic Chagas patients that had received specific nitroderivative therapy. In this group, 20 patients (45%) completed full treatment for 60 days, 14 (31%) ingested the drug for at least 30 days, and 11 patients (24%) received the nitroderivative for at least 20 days. We matched by age and gender.
those treated patients with 46 untreated chronic Chagas dis-
ease patients. Treated and untreated chronic Chagas disease
groups were matched with 41 uninfected control male street
cleaners with negative immunological tests for T. cruzi in-
fecion. Eighty-nine percent of treated, 91% of untreated
Chagas patients, and 92% of uninfected controls were 31 to
60 years of age.

**Electrocardiographic (ECG) and echocardiographic analyses.** A standard 12-lead ECG was obtained for every
patient on inclusion in the study and on other occasions, at
the physician’s discretion. Single patient multiple ECG al-
terations were cumulatively counted. Holter-type 24 hr re-
cordings were obtained on a single occasion. Also, two-dimen-
sional doppler echocardiograms (Esatec Echocardi-
ograph, model SIM-7000, Firenzi, Italy) were performed once
with standard views recorded on 0.5-inch VHS videotape.
Echocardiograms were considered positive if they showed
1) hypokinesia or akinesis of two or more contiguous seg-
ments, 2) abnormal wall motion, and 3) global hypokinesia
and systolic dysfunction. 23,24 Each of the above evaluation
studies of pathophysiological parameters of heart functions
was interpreted by two experienced specialists blinded to the
patient’s clinical status and outcome.

**DNA extraction and polymerase chain reaction (PCR).**
We drew 10 mL of peripheral blood and collected the buffy
coa to extract DNA from each subject in the study groups. 23,
DNA was analyzed by PCR with specific primers for the
constant kDNA minicircle region. 23,24 For and highly repeti-
tive sequence of nDNA of T. cruzi. 25 The kDNA primer set
(S35, 5′-ATATGTACGGG(T/G)GAGATGC-3′ and S36,
5′-GGTTCAGTTGGGTTGTTG-3′) annealing to the con-
stant minicircle region yields a 330 bp product and its ca-
tamer 660 bp. 25 The nDNA primer set (PON1, 5′-
TGCTTGAGGAGTTATGTG-3′ and PON2, 5′-AG-
GATGACGTTGATCATG-3′) amplifies a 250 bp frag-
ment. 25 The amplification of parasitic template DNA with
this nested set of primers would indicate an active T. cruzi
infection. A DNA thermal cycler (MJ Research, Watertown,
MA) was used for 30–32 cycles as follows: PON1/2, 94°C for
2 min, 58°C for 1 min, and 72°C for 1 min; S35/S36, 94°C for
2 min, 64°C for 1 min, and 72°C for 1 min. The reactions
were run with 100 ng of Chagas patient target template and
with 50 pg of DNA from T. cruzi culture forms. Each re-
action was done in 25 µL aliquots containing 2.5 U of Taq
polymerase (Perkin Elmer, Cetus Norwalk), 0.2 mM dNTPs,
50 mM TRIS-HCl (pH 8.0 at 20°C), 1.5 mM MgCl2, 200
mM ammonium sulfate, and 5 µM each primer. A 10 µL
aliquot from each PCR reaction was subjected to a 1% agar-
ose gel electrophoresis. Negative controls (water and
P388D1-IL1 DNA) and positive control (T. cruzi DNA)
were always included to detect DNA contamination and en-
sure that the PCR worked efficiently.

**Hybridization.** The PCR amplification products were
transferred by capillarity to a nylon membrane. Prehybrid-
ization and hybridization were performed in 6× SSC, 5×
Denhardt’s, 0.5% SDS and 100 µg/mL salmon DNA solu-
tion. The membranes were prehybridized for 4 hr and were
then hybridized for 12 hr with internal oligonucleotide se-
quencies for different amplified fragments. For S35/36 prod-
ucts, the sequence was 5′-GGTTCAGTTGGGTTGTTG(G/C)-
(T/G)TC-3′. 23 For PON1/2 products, the sequence was
PON3 5′-CCGGCCCTGTGTCTGCGGC-3′. 25 These oligo-
nucleotides were radiolabelled with [γ-32P]-dATP (3000
Cimol) using the polynucleotide kinase method following
the manufacturer’s recommendation (Life Technologies). Af-
ter the membrane was washed once for 5 min with 1× SSC
and 0.1% SDS and twice for 30 min each with 0.2× SSC and
0.1% SDS, it was autoradiographed for variable periods of
time.

**Competition assays.** Competitive PCR assays were con-
ducted with S35/36 primers set to quantify the number of parasites in the blood samples that had previously yielded
amplification products of expected size with T. cruzi kDNA
and nDNA probes. These assays consisted of a mixture of an
unknown quantity of template DNA (T. cruzi minicircle
DNA) with serial dilutions of a known quantity of competi-
tor DNA. This 280 bp competitor DNA fragment, with
binding sites for S35/S36 primers, resulted from digestion
of a 330 bp kDNA product of PCR. 26 The competitor frag-
ment, purified and cloned in pT7 Blue vector (Novogen,
Madison, WI), yielded a PCR product distinguishable from
the template (280 versus 330 bp). The determination of
equivalency points was made by visual comparison in 1.5%
wide range agarose/0.5% standard agarose gel. In PCR-pos-
itive samples, using the known amount of the competitor
DNA in the reaction, the unknown quantity of the template
was calculated, under the assumption that there were 10,000
minicircles/parasite and thus 15 fg of 330 bp template/par-
asite. 27

**Statistical analyses.** Results are presented as mean ±
standard deviation. Differences between ECG alterations
were determined by Fisher’s exact test. Categorical data
showing ECG alterations recorded in two occasions were
compared by Mantel-Haenszel chi-square. 28 P < 0.05 was
considered significant, and P ≤ 0.1 was considered indica-
tive of a trend. The SAS program was used for computer
analysis.

**RESULTS**

**Sampling analysis and parasitological investigation.**
Performing this longitudinal study in the street cleaner
population who had an epidemiological background of exposure
to insect-vectors of the T. cruzi infection was successful be-
cause these employees have job stability. Absenteism was
avoided and long-term health assistance either at work or at
home was secured. The statistical sampling analysis revealed
adequate proportions between groups of Chagas patients, re-
gardless of treatment, and of control subjects. After 10 years,
75.5% of treated, 78% of untreated Chagas patients, and all
control subjects continued to participate in this program.
Chagas disease patient drop-out was due to retirement with
subsequent change of address, or death; 4 (9%) treated and
3 (6.5%) untreated Chagas disease patients died during the
10-year period of clinical observation. Neither retirement nor
death was registered among the uninfected control group
consisting of 41 street cleaners showing negative immunolo-
gical tests for the infection.

Xenodiagnoses that were performed in every Chagas pa-
tient, one year after treatment, demonstrated the presence
of blood forms of T. cruzi in 3 (6.6%) treated patients and in
5 (10.9%) untreated Chagas patients. We used serum and
DNA from these eight patients showing consistently positive results to validate immunologic and PCR assays.

**Phenotype and genotype markers of the T. cruzi infections.** There was a significant statistical difference in proportions of positive results from IF, IH, and ELISA tests between Chagas patients and uninfected controls (P < 0.001). These statistically significant serological differences were not observed between groups of treated and untreated Chagas patients (Table 1). Furthermore, quantitative results of IF and IH tests conducted one year after treatment showed mean serological titers that were not statistically different among treated or untreated Chagas disease patients. Interestingly, IF and IH tests conducted in parallel on sera of Chagas patients, regardless of treatment, showed antibody titers consistently lower one year after treatment than 10 years thereafter, and the differences between these means were statistically significant (P < 0.001). Moreover, mean antibody titers detected on a single occasion in treated and untreated groups of Chagas patients, either by IF or by IH tests, were not statistically different (data not shown).

In this series, the diagnosis of Chagas disease was covalidated in every case by skin testing with the T12E T. cruzi antigen one year after treatment with nitroderivatives. An intradermal injection of 20 μg of parasite protein in 100 μL of saline in the forearm of Chagas patients resulted in an indurated inflammatory reaction. The mean area of induration among treated Chagas patients (8.05 ± 6.9 cm²) was not statistically different from that obtained from untreated Chagas patients (8.13 ± 5.8 cm²). The mean of skin reactions in the control group was 0.72 ± 0.3 cm². The differences between means of skin reactions elicited by T. cruzi T12E antigen in Chagas patients and among control street cleaners are statistically highly significant (P < 0.001).

In addition, the serological diagnosis of chronic Chagas disease was covalidated by a panel of 94 standard sera from patients with parasitological demonstration of T. cruzi.20 These results were used further to covalidate results of PCR in DNA samples extracted from human blood and tested with T. cruzi nuclear DNA primers PON1/2. Every treated patient and 93.7% of untreated Chagas patients yielded a specific PCR amplification product with primers that annealed to T. cruzi nDNA (Figure 1A). This product hybridized with its complementary internal sequence (Figure 1B), thus confirming nested PCR specificity.

Polymerase chain reaction products amplified with T. cruzi nuclear DNA primers yielded positive results in proportions statistically different between Chagas patients and uninfected controls (P < 0.001, Table 1). Results of this genotype marker of the T. cruzi infection correlate with the results of specific antibodies, and were not statistically different between treated and untreated Chagas patients. Altogether, these findings showed a consistent association of phenotypic and genotypic markers of T. cruzi infections in Chagas disease patients.

**Quantitation of parasitemia by competitive PCR.** Quantification of parasitemia was then performed in samples of DNA from Chagas patients yielding a PCR amplification product of 250 bp with PON1/PON2 primers, and a specific 330 bp product with T. cruzi kDNA primers S35/S36. The competitive PCR assays showed T. cruzi kDNA bands at various points of equivalency with the competitor DNA bands, regardless of whether the template DNA originated from treated or untreated Chagas patients. The competitive PCR quantification of T. cruzi in treated and untreated Chagas patients is shown in Figure 2 and Table 2. Twenty-seven untreated Chagas patients showed bands whose intensity was compared to that of DNA from a known quantity of T. cruzi showing equivalency in a range of < 0.4 to 75 parasites/mL of blood. The average means of T. cruzi/mL in this group was 20.1 ± 22.6. In addition, 24 treated Chagas patients showed bands whose equivalency ranged from < 0.5 to 3 × 10⁶. After excluding the treated patient with a dangerously high parasitemia, the average mean in treated Chagas disease group was 13.8 ± 14.9 parasites/mL of blood. The differ-

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**TABLE 1**
Validation of clinical diagnosis of chronic Chagas disease with phenotypic and genotypic markers of Trypanosoma cruzi infection

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Chagas patients</th>
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<tbody>
<tr>
<td>IF</td>
<td>Treated</td>
<td>Untreated</td>
</tr>
<tr>
<td>Positive</td>
<td>44</td>
<td>43</td>
</tr>
<tr>
<td>Negative</td>
<td>1</td>
<td>2</td>
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**Figure 1.** (A) Polymerase chain reaction (PCR) amplification of sequences of nDNA from Trypanosoma cruzi with specific PON1/2 primers. Lane 1, 100 base pair (bp) molecular weight marker; Lane 2, water (negative control); Lane 3, P388D1-IL1 DNA; Lanes 4 and 5, untreated and treated Chagas patient DNA, respectively; and Lane 6, T. cruzi DNA. (B) Southern hybridization with an internal fragment of DNA complementary to the PCR amplification product. Note the 250 bp bands in Lanes 4, 5, and 6 (see Methods).
The assays consisted of mixing an unknown quantity of T. cruzi Chagas patient by competitive polymerase chain reaction (PCR). The competitive PCR was performed using PCR primers amplifying products distinguishable from the parasite DNA (280 versus 330 base pairs [bp]). Lane 1, molecular weight standard; Lane 2, water (negative control); Lane 3 P388D1-IL1 DNA; Lane 4, T. cruzi DNA equivalent to 1 parasite; Lanes 5 to 8, 100 ng of DNA from a Chagas patient and various quantities of competitor DNA: Lane 5, 0.15 fg; Lane 6, 1.5 fg; Lane 7, 15 fg; and Lane 8, 150 fg. The point of equivalency between the 330 and the 280 bp bands was at 15 fg (Lane 7) of competitor DNA, which is approximately the amount of DNA in 1 parasite. The parasitemia was calculated considering that 10,000 minicircles/parasite contains 15 fg of the 330 bp/parasite.

Clinical study. Twelve-lead ECG recordings were obtained from chronic Chagas disease patients one and 10 years after anti-T. cruzi chemotherapy, from untreated Chagas patients, and from uninfected control subjects on the same occasions. Ventricular premature beats, bundle branch blocks, intraventricular conduction disturbances, and changes in ventricular repolarization were recorded several-fold more frequently in groups of Chagas patients, whether or not they had been treated, than among uninfected control subjects (Figure 3). It should be stressed that the differences among these proportions were statistically significant (P < 0.001). Furthermore, ECG recordings 10 years later revealed the same gamut of alterations in higher proportions in Chagas patients than in uninfected controls, and those differences in proportions were also statistically highly significant (P < 0.001). Interestingly, the Mantel-Haenszel statistical analysis of ECG alterations, recorded one and ten years thereafter, showed highly significant differences in treated (P < 0.0001) as well as in untreated Chagas patients (P < 0.025) in relationship to uninfected controls.

The cumulative recordings of ECG alterations are summarized in Table 3. One year after treatment, ECG recordings showed alterations in 79% (34 of 43) of treated and 67.4% (31 of 46) of untreated Chagas patients. Ten years thereafter the ECGs recorded showed such alterations in 80.5% (29 of 36) of treated Chagas patients, and in 75% (27 of 36) of untreated patients. In contrast, ECG alterations were recorded in 24.3% (10 of 41) and in 22.5% (9 of 40) of uninfected control subjects, respectively, one and ten years thereafter. The frequencies of ECG alterations in treated and untreated Chagas patients were not statistically different (P > 0.05). Moreover, those frequencies were subjected to general association test and revealed lack of statistical significance (P = 0.19). However, the frequency of ECG alterations between both groups of Chagas patients and in the uninfected control group was statistically highly significant (P < 0.001), regardless of whether the ECG recordings were taken one or ten years apart. Furthermore, an increasing frequency of ECG alterations was recorded in untreated Chagas patients, ranging from 67.4% to 75% over a ten-year period, not being statistically different (P = 0.5) from those registered among treated Chagas patients (79% to 80.5%), and among uninfected control subjects (24.3% to 22.5%), over the same time span.

In this study, Holter-type 24 hr ECG and echocardiographic recordings were obtained on a single occasion, ten years after treatment. The Holter recordings revealed ventricular arrhythmias in 69.6% (23 of 33) of treated Chagas patients, in 58.3% (21 of 36) of untreated Chagas patients, and in 23.5% (8 of 34) of uninfected control subjects (Table 3). The differences between proportions observed in treated and untreated Chagas patients are not statistically significant (P > 0.5). However, the differences in proportions between the former groups and the control group are highly significant (P < 0.001). The echocardiogram revealed alterations in proportions that were not statistically different between Chagas patients and control subjects.

The parasitemia was calculated considering that 10 parasites/mL, high untreated (39.4 ± 0.4); 2) Moderate, and High parasitemias. One treated patient showing very high parasitemia (3 × 10⁸ T. cruzi/mL) was not included in the statistical analysis.

![Figure 2. Quantitation of Trypanosoma cruzi DNA in a treated Chagas patient by competitive polymerase chain reaction (PCR).](image)

![Figure 3. Progressive electrocardiographic alterations recorded on two occasions, ten years apart, in nitroderivative treated and untreated Chagas patients. VRC = ventricular premature contractions; BRB = bundle branch block; CBB = combined branch block; ICD = intraventricular conduction disturbance; VRC = ventricular repolarization change; NIL = absence of alteration. Note that bars representative of electrocardiogram (ECG) alterations, recorded on the second occasion, shifted to the right of the scale.](image)

<table>
<thead>
<tr>
<th>T. cruzi/mL*</th>
<th>Uninfected</th>
<th>Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>0.6 ± 0.2 (n = 6)</td>
<td>1.0 ± 0.4 (n = 6)</td>
</tr>
<tr>
<td>Moderate</td>
<td>3.5 ± 1.3 (n = 7)</td>
<td>4.9 ± 2.2 (n = 7)</td>
</tr>
<tr>
<td>High</td>
<td>39.4 ± 18 (n = 14)</td>
<td>30.1 ± 10 (n = 10)</td>
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* Parasitemias detected by competitive PCR were considered Low (<2 parasites/mL); Moderate (<10 parasites/mL), and High (>10 parasites/mL). The assays consisted of mixing an unknown quantity of T. cruzi template DNA with serial dilutions of a known quantity of competitor DNA, as described in Methods. Untreated versus Treated, P > 0.05 for Low, Moderate, and High parasitemias. One treated patient showing very high parasitemia (3 × 10⁸ T. cruzi/mL) was not included in the statistical analysis.
Frequency of electrocardiogram (ECG), 24 hr Holter monitoring, and echocardiographic (Echo) alterations in treated and untreated Chagas disease patients and in uninfected control subjects over a ten-year period

<table>
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<th>Recordings</th>
<th>1988</th>
<th>1998</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>ECG</td>
<td>Holter</td>
</tr>
<tr>
<td>Chagas treated</td>
<td>34/43 (79.0%)</td>
<td>29/36 (80.5%)</td>
</tr>
<tr>
<td>Chagas untreated</td>
<td>31/46 (67.4%)</td>
<td>27/36 (75.0%)</td>
</tr>
<tr>
<td>Uninfected controls</td>
<td>10/41 (24.3%)</td>
<td>9/40 (22.5%)</td>
</tr>
</tbody>
</table>

*Number of patients (with alteration)/number tested. Alterations of ECG, Holter, and Echo were scored as indicated in the text.

### Discussion

Records in the files of Chagas disease patients in the study groups have shown that nifurtimox (10 mg/kg/day) was prescribed for 62% (28 of 45) of Chagas patients. Nitroimidazole benznidazole (10 mg/kg/day) was prescribed to treat 38% (17 of 45) of the remaining Chagas patients. The files registered various side effects such as headache, anorexia, gastritis, and visual and mental disturbances, loss of libido, dermatitis, peripheral neuropathy, seizures, and weight loss. Probably as a consequence of the severe side effects, 35.7% of those patients took the dose of nifurtimox for less than 30 days. Benznidazole produced less frequent side effects and 70% of the patients took the drug dose for 60 days.

A clinical and laboratory follow-up study of 45 Chagas patients subjected to treatment with trypanocidal nifurtimox or benznidazole nitroderivatives over a period of ten years, showed that specific chemotherapy for chronic T. cruzi infections should not be implemented. Certainly, it may be argued that the efficacy of the drug was underestimated because chemotherapy was not completed in some Chagas patients to whom nifurtimox was prescribed. However, Chagas patients who completed full treatment with benznidazole failed to achieve parasitological cure, as shown by consistently positive results of serology and PCR assays.

Chagas patients were treated with trypanocidal nitroarenes because physicians thought that treatment of T. cruzi infections would prevent progression of severe ECG alterations they already presented, considered to represent a high risk of heart trouble and death. However, results after 10 years of clinical and laboratory observations showed that no benefit was achieved. Phenotypic and genotypic markers of T. cruzi strongly suggested that the chronic infections persisted after treatment, and progressive ECG alterations were recorded in treated, as well as untreated Chagas patients, the proportions not being statistically different. The controversial findings reported in the literature concerning the efficacy of Chagas disease treatment with either nifurtimox or benznidazole may be considered to be a consequence of a lack of systematic randomization and an adequate methodological approach for unravelling critical features of the chronic infections such as: 1) use of low sensitivity xenodiagnostic method to detect a low level of parasitemia in a chronic patient; 2) requirement of follow-up study for time periods longer than those during which the patients were under clinical and laboratory observations; and 3) lack of improvement of a single clinical finding which could not associate a clear benefit of chemotherapy.

In this study, detection of persistent T. cruzi infections in treated Chagas patients by highly sensitive PCR amplification of parasite DNA, which indicates persistence of the infection in every nitroderivative-treated patient, casts doubt on the efficacy of specific chemotherapy. Using this molecular method, we revealed levels of parasitemias that were not statistically different among treated and untreated Chagas patients. This finding and slow progressive clinical alterations observed in both groups of patients discussed below explain why treatment of chronic infection appeared to have no benefit.

We conducted PCR with T. cruzi nuclear DNA specific set of primers to monitor drug efficacy in treatment of chronic infections because previous studies showed that sequences of kDNA minicircle of T. cruzi may integrate in the DNA of the host cell. In this study, PCR amplification products were obtained with nuclear DNA primers PON1/PON2 and template DNA derived from 100% of treated and from 93.7% of untreated Chagas patients, thus showing persistence of the infection, regardless of whether the patient was treated. Since the parasite nuclear DNA does not integrate in the host DNA, the presence of PCR amplification product with T. cruzi nuclear DNA PON1/PON2 was considered evidence of a living infection. Furthermore, the evidence of ongoing infections is in keeping with immunological test results, which were indistinguishable in treated and untreated groups of patients.

Anti-T. cruzi antibodies that were present in treated as well as untreated Chagas patients showed consistently increasing IF and IH titers, probably a consequence of chronicity of the infections. Further, skin testing with a parasite subcellular antigen showed a delayed-type indurated reaction in every Chagas patient, regardless of whether they were treated. These findings show that humoral and cell-mediated immune responses cannot be distinguished, either qualitatively or quantitatively, in either treated or untreated Chagas patients. However, an acquired cell-mediated immunosuppression against the parasite antigen was described in the course of administration of benznidazole, and nitroderivative-related compounds are immunosuppressive drugs used to prevent homograft rejection.

Chronic Chagas heart disease is a leading cause of arrhythmias and sudden death. ECG recordings showing ventricular premature contractions, single or combined bundle branch blocks, intraventricular conducting disturbances, and changes of ventricular repolarization were present in significantly higher proportions of Chagas patients, regardless of treatment, than in uninfected control subjects. ECG alterations were also recorded 10 years later in significantly higher proportions among Chagas patients than among age-
matched controls. A tendency to progression of ECG lesions was determined by statistical analysis showing a variable increment of those alterations recorded in a time-lapse period of 10 years. Furthermore, increment of those ECG alterations was statistically validated for both groups of treated and untreated Chagas disease patients. Chagas disease patients that received nitroderivatives demonstrated no improvement in any clinical parameter ten years after treatment. The ECG recordings showed more frequent alterations in the group of treated Chagas patients than in the uninfected control group. Those alterations showed progressive features; while their proportions in treated and untreated Chagas patients remained unchanged 10 years after treatment. Also, Holter-type 24 hr ECG confirmed those alterations in proportions that were significantly different between Chagas patients and uninfected controls, and lack of differences in proportions between Chagas patients, regardless of treatment. Furthermore, four treated and three untreated patients died over a period of 10 years of clinical observations.

Effective chemotherapy of *T. cruzi* infections is needed because of the enormous burden chronic infections impose on human populations living in endemic areas where active insect-vector transmission of parasites occurs. *Trypanosoma cruzi* infections can be transmitted by several species of reduviid bugs, but those species of Triatominae with peridomestic habits are mainly responsible for the endemcity of Chagas disease. In this study, we observed that over one-third of the chronic Chagas disease population had high parasitemias (average 30.1 ± 10 treated and 39.4 ± 18, untreated); one Chagas patient presented with approximately 3 × 10^9 T. cruzi/mL of blood, demonstrated by molecular method. This patient can be considered a reservoir facilitating peridomestic insect-vector dissemination of the infection in endemic areas. This human case suggests that an efficient anti-trypanosomal agent that curtails the parasitemia would be of great assistance in controlling this endemic disease. Immune responses for this patient would ensure diagnosis and preclude dissemination of infection by blood donation. Furthermore, an effective chemotherapy for the infection is also highly desirable as it would help determine the role of the parasite in the pathogenesis of multiple pathological manifestations of Chagas disease.

The administration of nitroderivatives in this series of Chagas patients produced severe side effects, such as peripheral neuropathy, encephalopathy, and seizures that precluded some patients taking the drug for 60 days. Under experimental conditions, apathy, ataxia, spastic tetraplegia with hyperreflexia of stretching reflexes, balance disorders, asymmetrical gait, and electroencephalographic alterations were described in mongrel dogs treated with doses of benznidazole ranging from 5 to 40 mg/kg/day. Histopathologically, those alterations correlated with various types of lesions affecting the meninges, cerebral cortex, hemisphere white matter and subcortical gray matter, brain stem, cerebellum, and the spinal cord.

Determining long-term toxicity of nitroderivative chemotherapy requires monitoring for several years. Its chronic toxicity in terms of tumor growths should be measured epidemiologically, because nifurtimox and of benznidazole administered to rabbits resulted in lymphoma growth in 36.5% of treated animals; untreated controls showed no growth. The potency of the drug, rather than the duration of its administration, was associated with lymphoma in female Swiss mice injected with 2-nitroimidazole, 5-nitroimidazole, or with a 5-nitrophuran derivative. Furthermore, the survival rate among 16 patients subjected to benznidazole, for treatment of Chagas disease reactivation, was 57% after 2 years, compared to 82.4% among 75 non-Chagas disease heart transplant patients. Interestingly, 37.5% of heart-transplant- ed Chagas patients developed malignant tumors after a mean follow-up of 25.3 ± 2.1 months in contrast to only 2.7% of patients in the untreated control group after 34.6 ± 3.6 months.

Chemotherapy of chronic *T. cruzi* infections with nitroderivative compounds should not be recommended because observation results discussed here show: 1) severe side effects precluded patients older than 20 years of age from complying with full treatment prescribed by physicians; 2) nitroderivative therapy may release parasitemia as a consequence of drug-induced immunosuppression and parasite resistance, probably through the selection of highly virulent clones; 3) lack of clinical findings showing a benefit of treatment with nitroderivatives; 4) lymphoproliferative tumors, such as those described in Chagas patients undergoing heart transplant and benznidazole therapy, can be produced experimentally in rabbits and mice upon injection of either nifurtimox or benznidazole at the dose used to treat human Chagas disease patients; and 5) eradication of the infection appears to be required, in view that decreasing parasitemias did not abrogate humoral and cell-mediated immune responses associated with autoimmunity and pathogenesis in Chagas disease patients, but this result cannot be achieved by administration of nitroderivatives.

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