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INCREASING IN THE CASUISTRY OF ETIOLOGICAL TREATMENT BENEFITS IN CHRONIC CHAGAS DISEASE PATIENTS FROM EPIDEMIOLOGICAL SURVEILLANCE AREA IN SOUTHERN BRAZIL

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ABSTRACT

Currently there is no consensus to support the routine use of the etiological treatment in chronic phase of Chagas disease. The decrease of title in the conventional serologic methods and negativity in parasitological or molecular techniques in treated patients is useful to convince clinicians to perform the etiological treatment in this phase. This study aimed to evaluate changes in conventional serology and parasitological and molecular methods results of 36 chronic Chagas disease patients from epidemiological surveillance area in Southern Brazil, before and 10 years after treatment with benznidazole. Negative serologic conversion of indirect immunofluorescence (IIF) and enzyme-linked immunosorbent assay (ELISA) was not observed in patients studied. However, there was a significant relationship between treatment and the decrease of IIF titers (p=0.0095). Furthermore, mean antibody titers exhibited a significant reduction (p<0.0001) when compared before and 10 years after treatment. A decrease from 2 to 4 titers of IIF and negative hemoculture was observed in 44.4% patients and a decrease from 2 to 4 titers of IIF and negative polymerase chain reaction (PCR) was observed in 38.9% patients after treatment. This study collaborates with literature data and increases the casuistry towards benefits of the etiological treatment in the chronic phase of Chagas disease.

KEYWORDS: Chagas disease, chemotherapy, serologic diagnosis, hemoculture, polymerase chain reaction.

1. INTRODUCTION

Although Latin American countries have made enormous efforts to control the infection by *Trypanosoma cruzi* (etiological agent of Chagas disease), approximately 5 - 6 million people remain infected[1]. Moreover, due to infected individuals' migration to non-endemic countries this disease is becoming a global health problem[2].

Currently, there are only two drugs for the treatment of Chagas disease, nifurtimox and benznidazole, but neither of them is considered ideal[3].The etiological treatment with benznidazole in chronic Chagas disease, despite low cure rate and side effects, is recommended by several authors due to evidence in preventing or minimizing tissue lesions, improving clinical progression and prognosis of patients[4,5,6,7,8].

Parallel to the absence of a fully effective medicine, serological methods (indirect immunofluorescence, IIF; and enzyme-linked immunosorbent assay, ELISA) used for efficacy of etiological treatment evaluation have limitations, with results remaining positive years after treatment[9]. However, a significant decrease of the antibodies titers in IIF detected in long-term follow-up of patients treated etiologically suggest that eventually the titers will be negative, which is a sign of cure[5,9,10]. Furthermore, IIF titers of 160 or lower in treated patients can indicate a tendency to a cure, due of the low frequency of these titers in patients with Chagas disease who were not treated etiologically[11].

Parasitological methods (hemoculture and xenodiagnosis) are considered less sensitive, but positive results are unquestionably valuable to monitor therapeutic failure after etiological treatment[12]. Although the polymerase chain reaction (PCR) is highly sensitive for detecting *T. cruzi* DNA in samples from infected patients and animals[13,14,15,16], it is a complex method and is dependent of patient's parasitemia as well as the parasitological methods. PCR has been recommended only for alternative diagnostic support[17,18] or as a confirmatory proof in post-therapeutic monitoring of Chagas disease patients[19,20,21,22].

Although currently there is no consensus to support the

routine use of etiological treatment in the chronic phase of Chagas disease[23,24], association of the title decrease in conventional serologic methods and negativity in parasitological or molecular techniques in treated patients is useful to convince clinicians to perform the etiological treatment in chronic Chagas disease patients.

In this context, this study aimed to evaluate changes observed in conventional serology (IIF and ELISA), parasitological method (hemoculture), and molecular method (PCR) results in chronic Chagas disease patients from epidemiological surveillance area in Southern Brazil, before and ten years after benznidazole treatment.

2. MATERIAL AND METHODS

Patient Patients and ethics

Thirty-six patients with chronic Chagas disease from Southern Brazil were evaluated at the Chagas disease Laboratory at **State University of Maringa** (Universidade Estadual de Maringá - UEM). The patients had been treated with benznidazole (Rochagan-Roche) at doses from 5 to 7 mg/Kg/day, for 30 consecutive days, ten years previously. Of the participants, 20/36 (55.6%) were female and 16/36 (44.4%) male, ages between 32 and 70 years old (mean age of 47.2 ± 9.8 years).

After the purpose of the study was explained to patients, all signed a free and informed consent form approved by the Permanent Committee of Ethics in Research Involving Human Beings - UEM, protocol number 375/2007.

Serological Tests

Venous blood sample (5mL) was collected from each patient, and the anti - *T. cruzi* IgG-class antibodies were assessed by IIF and ELISA according to manufacturers' recommendations. For IIF we used the Imunocruzi antigen (Biolab®, Rio de Janeiro, Brazil) and anti-human immunoglobulin G (IgG)-fluorescein conjugate (Biolab®, Rio de Janeiro, Brazil), and for ELISA the Chagatest-ELISA recombination v.3.0 diagnosis kits (Wiener®, Argentina) and Chagas Test Elisa III (Bioschile® Ingenieria Genética S.A, Chile). For IIF, titers ≥ 40 were considered positive, and for ELISA, sera with equal or higher than the cutoff plus 10% absorbency were considered reagent. The indeterminate zone was defined by the values of absorbency found between the cutoff ± 10%, and results in this zone were considered doubtful. The samples were tested in duplicate and in case of doubtful results or when there was a disagreement between the two ELISA diagnosis kits or between ELISA and IIF, the samples were repeated in duplicate. Results that remained discordant between IIF and ELISA and/or for both ELISA diagnosis kits were considered inconclusive. These procedures were executed for all 36 patients, before and ten years after treatment. For both serological methods, positive and negative controls for Chagas disease were included.

Hemoculture

Venous blood sample (30mL) was collected from each patient in vacuum tubes (BD Vacutainer®, USA) containing sodic heparin. Blood was distributed in Falcon tubes (Labcon®, USA), and hemocultures were processed immediately in LIT (liver infusion tryptose) medium and incubated at 28°C, according to Chiari *et al.* (1989)[25] with modifications. The samples were homogenized twice a week and examined after 30, 60, 90, and 120 days. Hemoculture was performed for all patients before and ten years after treatment.

Polymerase Chain Reaction (PCR)

Ten milliliters of blood was collected from each patient in an equal volume of Guanidine-EDTA (6 M Guanidine-HCl; 0.2 M EDTA; Sigma Chemical Company®, USA) pH 8.0. DNA extraction, conditions of PCR reaction and amplified products revelation of were performed as described by Gomes *et al.* (1998)[13].

To control contamination, PCR steps were carried out in separate rooms with exclusive reagents, materials and equipment for each working space. In the DNA extraction step and PCR step, negative controls with uninfected individuals blood samples, and positive controls of patients with Chagas disease were used. To exclude the possibility that negative PCR results were due to presence of reaction inhibitors, 10 pg of total DNA extracted from *T. cruzi* culture was added to the negative samples and a new amplification was executed. PCR was realized in duplicate for all the patients ten years after etiological treatment and in 14/36 (38.8%) of these patients before treatment, because during this period, PCR method was being implemented in the laboratory and evaluated as a tool for post-therapy monitoring.

Statistical Analysis.

The statistical analysis of the relationship between the antibodies titers in IIF (titers of 160 or lower) and the treatment (before and ten years after etiological treatment), was performed by the McNemar Chi-square test. For the comparison of mean antibody titers of the patients before and after etiological treatment, the values were transformed by applying the formula $\log_2 T/10$ (T = titers of antibodies in IIF) and analyzed by Mann-Whitney test. Data were compared using Statistica 8.0 Software, at a significance level of 5%.

3. RESULTS

Conventional serology revealed absence of negative serologic conversion in all chronic Chagas disease patients. However, 15/36 (41.7%) patients demonstrated before etiological treatment IIF titers of 160 or lower and ten years after etiological treatment 33/36 (91.7%) patients showed these titers values (Table 1), i.e., there was a significant relationship between treatment and decrease of IIF titers (p=0.0095). Furthermore, the mean of IIF titers ex-

hibited a significant reduction (p<0.0001) when compared before (258.84) and ten years after treatment (98.90) (Figure 1).

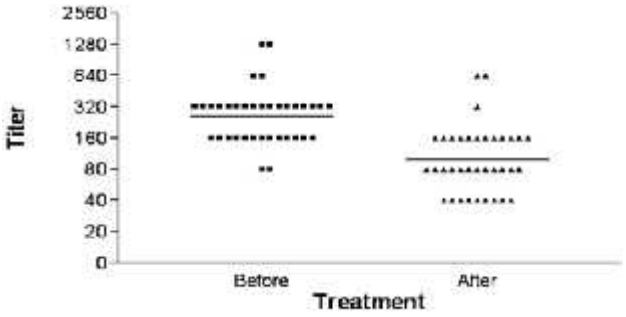

Figure 1 - Chronic Chagas disease patients (n=36) and indirect immunofluorescence (IIF) titers before and ten years after etiological treatment with benznidazole. Each symbol (■ and ▲) represents a serum sample from one patient. The solid horizontal lines represent the mean antibody titers before treatment (258.84), and after treatment (98.90) (p<0.0001).

Table 1. Laboratorial data of chronic Chagas disease patients before and ten years after treatment with benznidazole.

	Tests before treatment				Tests ten years after treatment				
Patient	IIF	ELISA	HC	PCR	IIF	ELISA Wiener® kit	ELISA Bios-Chile® kit	HC	PCR
1	160	R	P	P	40	R	R	N	P
2	320	R	P	P	80	R	R	N	N
3	640	R	N	N	40	R	R	N	N
4	320	R	N	-	640	R	R	N	N
5	1280	R	N	-	640	R	R	N	N
6	320	R	N	-	40	R	R	N	N
7	160	R	N	-	80	R	R	N	N
8	320	R	N	-	40	R	R	N	N
9	320	R	N	-	320	R	R	P	P
10	160	R	N	P	40	R	R	N	N
11	320	R	N	-	80	R	R	N	N
12	320	R	N	-	80	R	R	N	N
13	160	R	N	P	160	R	R	N	N
14	160	R	N	-	80	R	R	N	N
15	160	R	P	P	160	N/N[a]	WR/WR[a]	N	N
16	1280	R	N	-	160	R	R	N	N
17	320	R	N	-	160	R	R	N	N
18	320	R	P	-	40	R	R	N	P
19	160	R	N	P	160	R	R	N	N
20	80	R	N	-	40	N/I[a]	WR/WR[a]	N	N
21	320	R	P	-	160	R	R	N	N
22	320	R	P	P	160	R	R	N	P
23	80	R	N	P	80	R	R	N	N
24	320	R	P	P	40	R	R	N	N
25	320	R	N	P	80	R	R	N	N
26	640	R	P	-	160	R	R	N	N
27	320	R	N	N	40	R	R	N	N
28	160	R	N	-	160	R	R	N	P
29	160	R	N	-	160	R	R	N	P
30	160	R	N	-	160	R	R	N	P
31	160	R	P	-	80	R	R	N	N
32	320	R	N	P	80	R	R	N	N
33	320	R	N	-	80	R	R	N	N
34	160	R	P	-	80	R	R	N	N
35	160	R	N	P	80	R	R	N	N
36	320	R	N	-	160	R	R	N	N

ELISA - enzyme-linked immunosorbent assay; IIF - indirect immunofluorescence; HC -hemoculture; PCR - polymerase chain reaction; R - reagent; P - positive; N - negative; I - inconclusive; WR - weak reagent; – not realized. [a] Results that remained discordant between IIF and ELISA and/or for both ELISA diagnosis kits were considered inconclusive.

In 34/36 (94.4%) patients, IIF and ELISA were positive and in 2/36 (5.6%) patients these results were inconclusive after treatment. Of these cases, one patient (patient 15, Table 1) showed positive IIF, negative ELISA in two reactions by Wiener® diagnostic kit and weakly reagent in two reactions by Bioschile® diagnostic kit. The other patient (patient 20, Table 1) showed positive IIF and three different results for ELISA, negative and inconclusive in reactions by Wiener® diagnostic kit and weakly reagent in two reactions by Bioschile® diagnostic kit. These patients presented negative results in hemoculture and PCR after etiological treatment, and one of them (patient 15, Table 1) showed positive hemoculture and PCR before treatment.

Hemoculture was negative for 35/36 (97.2%) patients, including 10/36 (27.8%) who showed positive hemoculture before treatment. However, 1/36 (2.8%) patient with negative hemoculture before treatment presented a positive result after treatment (Patient 9, Table 1). PCR was negative for 29/36 (80.5%) patients and positive in 7/36 (19.5%). Furthermore, 10 (83.3%) individuals of 12 that presented PCR positive before treatment showed negative result ten years after treatment (Table 1).

The relationship between decrease of antibodies titers in IIF and results of hemoculture and PCR in chronic Chagas disease patients ten years after etiological treatment with benznidazole was demonstrated in Table 2. A decrease from 2 to 4 titers of IIF and negative hemoculture was observed in 16/36 (44.4%) patients and a decrease from 2 to 4 titers of IIF and negative PCR was observed in 14/36 (38.9%) patients.

Table 2. Relationship between decrease of indirect immunofluorescence (IIF) antibody titers and results of hemoculture and polymerase chain reaction (PCR) in chronic Chagas disease patients, ten years after etiological treatment with benznidazole.

Decrease of IIF titers in relation to beginning of treatment	Hemoculture		PCR	
	Negative n/%	Positive n/%	Negative n/%	Positive n/%
2 to 4	16/44.4	0/0.0	14/38.9	2/5.6
≤1	19/52.8	1/2.8	15/41.6	5/13.9
Total	35/97.2	1/2.8	29/80.5	7/19.5

n - number of patients; % - percentage.

4. DISCUSSION

In view of the diagnosis methods limitations for chronic Chagas disease patients post-treatment monitoring and complexity of the disease progress, in this study 36 patients were evaluated before and ten years after etiological treatment by the laboratory methods: conventional serology (IIF and ELISA), parasitological (hemoculture), and molecular (PCR).

Although chronic Chagas disease patients assessed presented positive or inconclusive results in conventional serology (IIF and ELISA) ten years after treatment it was observed a significant relationship between etiological treatment and decrease of IIF titers (titers of 160 or lower). Luquetti *et al.* (2008)[12] reported that titers of 160 or lower in treated patients can indicate a tendency toward cure, since these titer levels occur infrequently in untreated patients with Chagas disease. Moreover, the comparison of

mean antibody titers detected by IIF before treatment was significantly higher than ten years after treatment etiological. Other studies[26,27] have also observed a lower mean of antibody titers detected by IIF when groups of treated patients in relationship to untreated patients were compared, and the titers decline is accepted and recommended by other authors as a sign of cure[11,12].

The evaluation of serology methods showed two samples of patients with inconclusive results, due to results disagreement between IIF and ELISA or/and between Wiener® and Bioschile® ELISA diagnostic kits. The results difference may be related to, the kind of antigen used in each technique (different kind of soluble antigens in ELISA test and the entire parasite in IIF); *T. cruzi* genetic diversity (existence of various proteins in this parasite which cause differences in its immunogenicity)[17,28,29], and host's immunological response[30]. This disagreement in results among the serological methods corroborates with other studies[5,31], which demonstrate the challenge of using only these diagnostic techniques to follow patients etiologically treated[31].

Differences in hemoculture sensitivity have been reported[25,33]. Such differences may be related to distinct levels of parasitemia that depend on the disease phase, the parasite strain and the host immune response[21]. In the present study, ten years after treatment it was observed negative hemoculture in 35/36 (97.2%) patients, including those who showed positive results before treatment 10/36 (27.8%). Negative results in most of the hemocultures indicate that this technique has low sensitivity to monitor cure in chronic Chagas disease[34]. However, these negative hemoculture results could be related to parasitemia decrease in treated patients who previously had positive hemoculture results.

Due the long persistence of anti-*T.cruzi* antibodies after chemotherapy and the low sensitivity of most parasitological methods, PCR has been used to be a higher sensitivity method than hemoculture and a very useful tool for treated patients follow-up[35,36]. According to PCR results, therapeutic failure were observed in 7/36 (19.5%) patients evaluated ten years after etiological treatment. However, PCR results was negative in 29/36 (80.5%) patients and in 14/36 (38.9%) patients hemoculture and PCR were negative associated to decrease of 2 to 4 titers in IIF, indicating etiologic treatment benefits.

One patient showed negative hemoculture before treatment and, positive hemoculture and PCR after treatment. These different results may be related to the intermittent parasitemia that occurs in the chronic phase of Chagas disease, which can influence parasitological and molecular methods results. Due to positive hemoculture and PCR, the patient received a second treatment with benznidazole (doses from 5 to 7 mg/Kg/day, for 30 consecutive days), which resulted in persistent positive serology and PCR six months after the end of treatment. Although, the period of time between the second treatment and the post-therapeutic evaluation was short, the persistence of positive results could be explained by the presence of strain resistant to the drug, several investigations reported the existence of *T. cruzi* strains that are naturally resistant to chemotherapeutic agents[37,38]. Moreover, some investigators continue to emphasize the host-parasite interaction importance for success or failure of therapy[20].

5. CONCLUSION

In the present study, despite the absence of an untreated control group, important results were obtained when samples of patients were compared before and ten years after etiological treatment. There was decrease of IIF titers in a high percentage of patients, and PCR and hemoculture negative were associated to decreasing of 2 to 4 titers in IIF, indicating etiologic treatment benefits. In conclusion, this study collaborates with literature data and increases casuistry towards benefits of etiological treatment in chronic phase of Chagas disease[4,5,6,21,39]. Furthermore, according to Viotti *et al.* (2014)[7] greatest challenge now is changing the mindset and habits of health professionals, biased by the old paradigm in which most doctors prescribe for Chagas disease patients' only symptomatic treatment of cardiomyopathy and digestive symptoms, avoiding antiparasitic drugs.

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