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Severity of chronic Chagas disease is associated with cytokine/antioxidant imbalance in chronically infected individuals

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Abstract

Understanding the pathogenic mechanisms in chronic Chagas disease, a major cause of morbidity and mortality in Latin America, is essential for the design of rational therapeutic strategies. In this paper we show that the development of Chagas disease is a consequence of a long-term and complex relationship between parasite persistence and maladapted homeostatic mechanisms in the host which leads to pathologic changes. We performed a retrospective study on 50 patients with chronic Chagas disease and 50 healthy control individuals. The specific immune response was detected by ELISA and IHA tests using autochthonous antigens, inflammatory process with the cytokine tumour necrosis factor (TNF)-α and nitric oxide (NO), and antioxidant protection with glutathione peroxidase and superoxide dismutase (SOD) levels. We developed generalised linear modelling procedures to assess simultaneously which explanatory variables and/or their interactions better explained disease severity in patients. Our results show the existence of a strong relationship between anti-*Trypanosoma cruzi* levels and chronic Chagas disease ($P < 0.0001$). Taken together, the statistical data indicate both cumulative and complementary effects, where the increase in TNF-α ($P = 0.004$) and NO ($P = 0.005$) levels correlated with a reduction in glutathione peroxidase ($P = 0.0001$) and SOD ($P = 0.01$) levels drives the disease pathology in chronically infected patients. Our findings may have important implications for understanding host susceptibility to develop severe chronic infectious disease. In addition we show putative targets for the design of new therapeutic strategies to prevent disease progression, considering both specific treatment against the aetiological agent and modulation of the different immunopathological reactions in chronically infected individuals with chronic Chagas disease.

Keywords: Chronic infectious disease; Chagas disease severity; Pathogenesis; Cytokine–antioxidant imbalance; Tumour necrosis factor α; Nitric oxide; Superoxide dismutase; Glutathione peroxidase

1. Introduction

Chronic infectious diseases are still today, even in developed countries, a major cause of morbidity and mortality (Binder et al., 1999). Understanding the pathogenic mechanisms in such diseases is essential for the design of rational therapeutic strategies.

The question of whether chronic infection diseases are associated with the actual persistence of aetiologic agent in a human or evolve as an autoimmune process is still a matter of debate in health science. Whatever the basic assumption, either that the infectious agent causes the disease or that it is an autoimmune disease, no effective treatment has been found to stop the damage associated with chronic infections by aetiologic agents.

To date, Chagas disease, a suitable model of chronic infectious disease caused by the protozoan parasite *Trypanosoma cruzi*, is still a major public health problem in Latin America (Moncayo, 1997) for which effective human treatments are not available (Braga et al., 2000). Moreover the pathophysiology and clinical significance of

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chronic infection in Chagas disease are still incompletely understood. Currently, while some Chagas disease experts are postulating the crucial role of parasitic persistence in the development of clinical expression in infected people (Zhang and Tarleton, 1999), others argue that clinical variability observed across individuals might be explained by differential genetic properties of parasitic strains and/or host genetic tendency to disease susceptibility (Tibayrenc, 1998). All agree that the disease should be directly treated by acting on the aetiological agent responsible for the pathogenic impacts, although the activity of anti-chagasic drugs at the chronic stage is controversial, and many experts do not recommend their use at this stage (Tarleton and Zhang, 1999). Meanwhile, other experts proposed that an autoimmune mechanism, independent from the persistence of *T. cruzi*, is involved in the development of pathogenic processes in chronic Chagas disease (Viotti et al., 1994). Accordingly, Chagas disease should be viewed as an autoimmune disease and treated as such (Kalil and Cunha-Neto, 1996).

Infection by *T. cruzi* starts with an acute phase as observed in children for instance. It may be carried asymptomatically, and in most cases the infection disappears without the need of any specific treatment. However, once established in humans, *T. cruzi* persistence may result in a life-long chronic infection leading to serious damage and eventually causing death (Prata, 2001).

The evidence for the importance of the *T. cruzi* protozoan agent in disease is compelling, but its pathogenic effects are not unequivocal. As a matter of fact, the most severe cases of chronic Chagas disease are developed only in approximately 20–30% of the infected individuals. This suggests complex interactions between the unicellular parasitic protozoa and higher organisational levels and homeostatic mechanisms found in multicellular organisms, i.e. a human being. The continuing burden of people acquiring *T. cruzi* disease in Latin America highlights the importance of exploring more in depth the pathogenesis of chronic Chagas disease. Here we explore the working hypothesis that chronic Chagas disease is due to a long-term complex relationship between parasitic persistence and maladapted-host homeostatic mechanisms leading to a severe pathology in chronically infected individuals.

2. Subjects and methods

2.1. Study design and subjects

We performed a retrospective study on 50 healthy control individuals (female/male ratio = 1.2 : 1, 18–65 years of age, mean ± SD age = 34.9 ± 17.8) and 50 patients with Chagas disease (female/male ratio = 1.2 : 1, 18–64 years of age, mean ± SD age = 35.3 ± 16.4). Healthy control individuals, from blood banks of the clinics and hospitals of Instituto Mexicano del Seguro Social (IMSS), Puebla, Mexico, were selected. The criteria included residents of Puebla state, aged between 18 and 65 years, > 50 kg, healthy clinically and seronegative to hepatitis B virus (HBC), hepatitis C Virus (HCV), *Brucella abortus* (BrA), hepatitis B surface antigen (HBsAg), Venereal Disease Research Laboratory (VDRL) and human immunodeficiency virus (HIV) and without history of immunisation, transplantation, menstruation, pregnancy or lactation, according to the technical Norm for Banks of Blood Protocols (TNBB). Chronic chagasic patients were recruited during clinical survey at the Medical Center Manuel Avila Camacho of IMSS, Puebla, Mexico. The diagnosis of Chagas disease was made in all patients by the detection of circulating antibodies against *T. cruzi* antigens by two standardised serological tests, according to WHO recommendations (World Health Organisation, 1991). Patients with other kinds of acute or chronic disorders suspected to be due to other diseases were not included in this study. None of the patients took exogenous dietary nitrates up to 48 h before blood sampling since it can contribute significantly to serum nitrate levels. Each subject gave fully informed consent under a protocol approved by the Hospital Research Committee from Puebla State.

2.2. Study groups

The individuals were stratified into five groups according to severity of Chagas disease, as assessed by medical interview and clinical studies including electrocardiography, echocardiography, X-rays of the chest, the gastrointestinal tract with barium swallow and oesophageal manometry: Group I included the 50 healthy control individuals, seronegatives to *T. cruzi* antigens and serological screening for blood donors, without evidence of abnormalities or alterations in clinical studies; Group II consisted of 24 asymptomatic individuals, seropositive to *T. cruzi* antigens without clinical evidence of abnormalities or indeterminate form; Group III was composed of eight seropositive patients with symptoms or gastrointestinal alterations; Group IV consisted of 10 seropositive patients with electrocardiography alterations, and finally Group V consisted of eight seropositive patients with both electrocardiography and echocardiography alterations and dilated cardiomyopathy.

2.3. Sample collection

In order to minimise the alteration of blood components and to standardise samples, venous blood was taken in the morning before subjects had consumed any meal, drugs or water. The blood samples were kept at room temperature for 2 h in order to clot. Then, the serum was frozen at −20 °C, and stored at −70 °C until use.

2.4. Serological characterisation

Serological response was estimated by two standardised immunological techniques, the immunoenzymatic ELISA and the immunohaemagglutination IHA tests employing autochthonous antigen of *T. cruzi*, performed in a previous work (Perez-Fuentes et al., 1998).

2.5. Trypanosoma cruzi antigen characterisation

Trypanosoma cruzi parasites, employing an autochthonous antigen were obtained by Xenodiagnostic from a patient with chronic Chagas disease in Puebla State, Mexico. The isolated parasites were characterised by Multilocus Enzyme Electrophoresis (MLEE), Random Amplified Polymorphic DNA (RAPD) and Biodeme like HUM/ME/1997/MEX/RyCH1 (*T. cruzi I*), according to recent taxonomy of *T. cruzi* (Momen, 1999).

2.6. Immunological characterisation

We estimated inflammatory processes with the proinflammatory cytokines TNF-α and the nitric oxide (NO) serum levels, and the antioxidant protection with two antioxidative enzymes, glutathione peroxidase and superoxide dismutase (SOD) levels, in serum samples. The TNF-α, glutathione peroxidase and SOD serum levels were analysed in threefold serial dilutions using a two-site sandwich enzyme-linked immunosorbent assay employing purified and biotinylated antibodies for TNF-α, glutathione peroxidase and SOD, respectively. After incubation with alkaline phosphatase coupled to streptavidin and developed with p-nitrophenyl phosphate, the absorbance was read on a microplate reader (Multiskan MS, Labsystems OY, Helsinki, Finland). Using a test wavelength of 405 nm and a reference wavelength of 495 nm, samples were compared to appropriate standard in threefold serial dilutions. The detection limits of TNF-α was 0.01 ng/ml, of glutathione peroxidase was 2.5 ng/ml and SOD was of 0.1 units/ml. The NO serum levels were evaluated by measuring nitrite in serum samples as previously reported (Perez-Fuentes et al., 1998).

2.7. Statistical analysis

We used generalised linear modelling to assess simultaneously which explanatory variables and/or their interaction terms better accounted for the present attribution to any one of the five categories of patients above defined. The introduction of different explanatory variables into models may permit to keep constant one parameter when interpreting the effect of another variable under investigation. Stepwise procedures were used to retain the best minimal models as described in S-Plus 2000 guide to statistics (S-PLUS. Programmer's Guide 2000. Data Analysis Products Division, Mathsoft Inc, Seattle, WA, USA).

When the order of entry of the different retained predictors altered residual deviance and partial testing, we decided to select variables according to their Akaike criterion based on calculation of the *Cp* parameter (from the lowest to the highest) (Crawley, 1993). We used the tolerance option at the 0.05 level, which avoids constructing highly multi-collinear models in a stepwise procedure. Single predictors and their two-way and three-way interaction terms were first introduced in the null model, and then in the minimal model we opted for entering single corresponding terms when their corresponding interactions were significant (Crawley, 1993). During the stepwise elimination procedure, terms with a *Cp* value higher than the *Cp* value for the null model were kept into final models even if not significant (Venables and Ripley, 1994). The response data, i.e. clinical variants, corresponded to proportions, and it was found to be significantly underdispersed with five levels ($n = 50, 24, 8, 10, 8$), respectively. A series of analyses revealed that both Poissonian and Quasi-likelihood error structures provided the most appropriate models. We first used a Poissonian error structure (with a log link), and then we developed a Quasi-likelihood estimation with an identity function (McCullagh and Nelder, 1989; Venables and Ripley, 1994), which indeed represented the most appropriate statistical tools for analysing our data. The variances for terms in the models were compared using χ^2 statistics for Poissonian model and *F*-tests for Quasi-likelihood approximation. In addition, a separate analysis was performed on a contingency-table derived from the raw data (Crawley, 1993), which yielded similar results (data not illustrated). In each case, minimal models retained were compared to other models with other kinds of error structure. When the data suggested that there were no linear trends, the explanatory variables were transformed and fitted again to try to improve their contribution to the model.

3. Results

Table 1 shows the data (mean ± SD) for the parameters used in the model for each clinical group. Our findings show that the best fits for assignment of patients to any one of the five categories (four different Chagas disease clinical symptoms and one control group) were obtained with two minimal models as illustrated in models A and B (see Table 2). In model A, the ELISA parameter was the best predictor of Chagas disease severity degree (56.70% of the total deviance explained) ($P < 0.0001$). Then, glutathione peroxidase explained up to 29.02% ($P < 0.0001$) of total deviance. No other parameters and/or their interaction terms were introduced in the final model. Then in model B (Table 2), we obtained similar but more detailed results as shown in model A. ELISA always was the best predictor of disease severity (43.48%) ($P < 0.0001$) then followed by glutathione peroxidase (19.23%) ($P < 0.0001$), TNF-α (8.56%)

Table 1
Immunological parameters

Clinical group	ELISA[a]	TNF-α (ng/ml)	NO (mM/ml)	GPx (ng/ml)	SOD (units/ml)
I	0.014 ± 0.008	4.24 ± 1.17	0.074 ± 0.01	138.11 ± 14.7	1.680 ± 0.6
II	0.072 ± 0.025	6.94 ± 1.29	0.106 ± 0.02	115.37 ± 18.9	8.083 ± 2.8
III	0.090 ± 0.045	7.39 ± 1.24	0.110 ± 0.03	101.66 ± 12.9	7.117 ± 2.4
IV	0.109 ± 0.047	9.49 ± 1.52	0.132 ± 0.04	86.21 ± 13.6	5.365 ± 1.4
V	0.133 ± 0.03	9.70 ± 0.74	0.151 ± 0.03	75.03 ± 12.9	2.867 ± 1.3

Data for immunological parameters in clinical groups are presented as mean ± standard deviation of the mean. Group I, healthy controls; Group II, seropositive asymptomatic individuals in indeterminate form; Group III, seropositive patients with gastrointestinal alterations; Group IV, seropositive patients with ECG alterations; Group V, seropositive patients with dilated cardiomyopathy.

[a] Reference value for the antibody anti-*T. cruzi* levels is 0.044 ± 0.009 OD.

($P < 0.004$), SOD (8.38%) ($P < 0.01$), NO (8.19%) ($P < 0.005$), and finally the two-way interaction term between NO and TNF-α (3.31%) ($P < 0.04$). No other independent variables and/or their two-way or three-way interactions term were significant in model B.

4. Discussion

Interestingly, these patterns answer some questions on the origin of pathogenesis in chronic Chagas disease. This is the first time, at least to our knowledge, that it is statistically demonstrated on a sample of patients that the disease severity degree is strongly associated not simply to a specific factor but to a combination of different parameters, thus giving strong support to the concept of complex mechanisms regulating disease pathogenesis (see Fig. 1).

First, our findings show a strong but expected relationship (43.48–56.70% of the deviance explained) between antibody anti-*T. cruzi* levels, determined by ELISA using autochthonous antigens, and disease severity (Fig. 1a). Many previous studies have attempted to clearly differentiate between asymptomatic infection and chronic Chagas disease using serologic methods, with crude antigens (Cervetta et al., 1997) and more recently with several recombinant antigens (Umezawa et al., 1999). Contrary to other studies (Bucio et al., 1999), the specificity of our analysis was to use autochthonous antigens obtained from local *T. cruzi* strains isolated from Puebla state, and characterised as *T. cruzi* I (Momen, 1999). Consistently,

Table 2
Summaries of generalised linear models

	df	Deviance	Residual df	Residual dev.		Pr(χ^2)
MODEL A						
Null model			49	31.378		
ELISA (Log)	1	9.734	48	21.644		**0.00091**
Glutathione peroxidase	1	4.983	47	16.661		**0.01280**
	df	Deviance	Residual df	Residual dev.	*F*	Pr(*F*)
MODEL B						
Null model			49	65.920		
ELISA (Log)	1	20.442	48	45.478	37.835	**0.00000**
Glutathione peroxidase	1	9.044	47	36.434	16.740	**0.00012**
NO	1	3.849	46	32.585	7.124	**0.00572**
TNF-α	1	4.023	45	28.562	7.446	**0.00494**
Poly(SOD,2)#1						
	2	3.939	43	24.623	3.645	**0.01823**
Poly(SOD,2)#2						
ELISA × GPx	1	1.138	40	21.304	2.107	0.07776
NO × GPx	1	0.020	39	21.284	0.037	0.42465
NO × TNF-α	1	1.555	38	19.729	2.878	**0.04933**
NO × Poly(SOD,2)#1						
	2	0.485	36	19.244	0.449	0.32097
NO × Poly(SOD,2)#2						

Model A is the minimal model (Poisson error, log link) retained after a backward stepwise selection procedure using the Akaike criterion of deletion ($\Phi = 1$, residual deviance = 14.209, df = 46) for explaining the control group and the four Chagas disease clinical variants with the significant independent parameters and their interactions terms. Model B (Quasi-likelihood estimation, identity link) was generated according to the same previous procedure ($\Phi = 0.541$, residual deviance = 18.910, df = 35). ELISA values were logarithmically transformed (log naperian) and SOD parameter was transformed into a polynomial function order two [(Poly(SOD,2)#1 as a first order term and Poly(SOD,2)#2 as the second order term] to fit the data. Significant explanatory variables are in bold characters. See text for abbreviations used. df is the degree of freedom, and Pr(χ^2) and Pr(*F*) the probabilities associated with statistics.

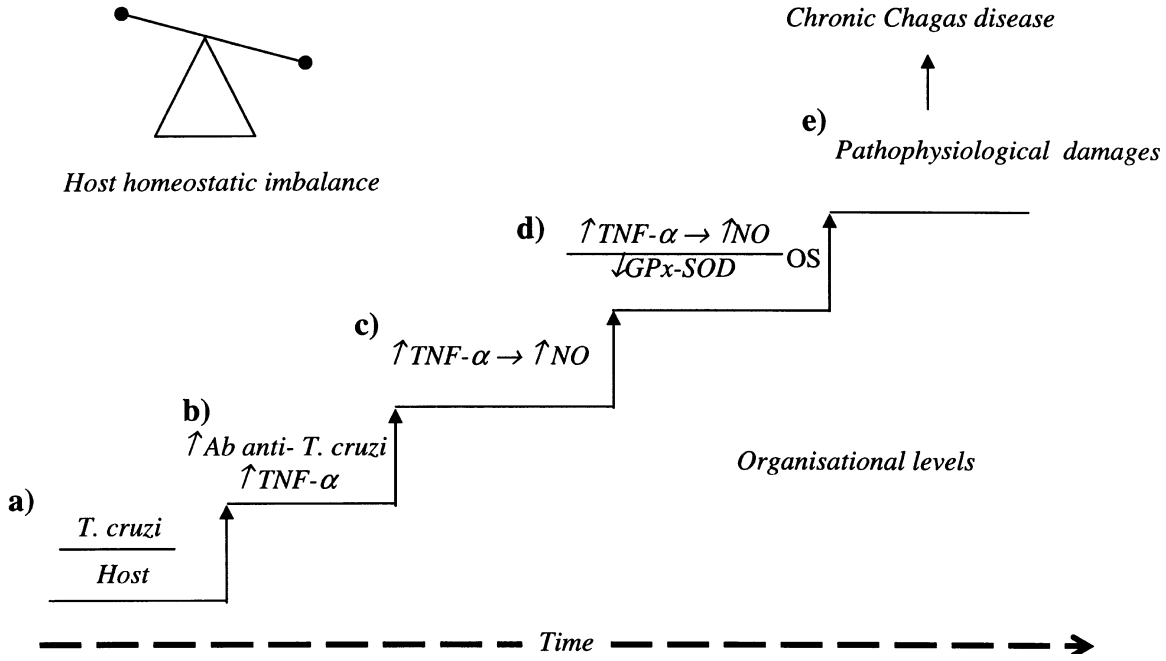

Fig. 1. Schematic illustration of the continuous multilevel process, and the possible pathogenic mechanisms involved in the development of chronic Chagas disease. (a) In the human infection by *Trypanosoma cruzi* protozoan parasite, the relationship between infected individuals and different *T. cruzi* strains induces parasite persistence in a chronic infection. (b) This then ensures a constant stimulus for the host immune response in producing high antibody (Ab) anti-*T. cruzi* levels and in releasing specific cytokines, e.g. TNF-α. (c) Increase of these pro-inflammatory cytokines induce cytokine imbalance. (d) Chronic exposure to high TNF-α, which activates the inducible form of nitric oxide synthase (iNOS) to produce high NO levels, yields in turn a depletion of antioxidant systems, e.g. GPx and SOD, which leads to oxidant–antioxidant imbalance thus producing oxidative stress (OS); OS may cause pathological consequences associated with its cytotoxic effect on infected and non infected cells. (e) Progressive damage in a long-term process, associated to non-adaptive homeostatic mechanisms, lead to physiological and organic abnormalities favouring the development of severity degree in chronic Chagas disease.

the strong correlation obtained between autochthonous antibody levels and chronic Chagas disease severity in patients from Puebla state might naturally reflect locally adapted host immune responses against *T. cruzi* natural clones circulating in the area (Tibayrenc et al., 1986). Such a host immune reaction as observed in this work would not have been so notable in previous studies probably due to the fact that they used allochthonous antigens from *T. cruzi* strains to test for local host reaction (Cetron et al., 1993).

What appears more interesting here is the importance taken by other factors in explaining chronic Chagas disease severity in patients (see Fig. 1c,d). Experimental infestation of mice with *T. cruzi* generally induces cytokine production, which modulates host resistance against parasite. TNF-α is a proinflammatory cytokine that mediates specific resistance to *T. cruzi* infection (Silva et al., 1995). However, some authors showed that an alteration in quantity and/or quality of cytokine production might be strongly associated to the development of chronic Chagas disease as well (Laucella et al., 1996). For instance, excessive TNF-α production contributes to the immunopathology and severity of some human infectious diseases such as AIDS (Barbaro et al., 2001), tuberculosis (Zhou et al., 2000), malaria (Grau et al., 1989), human African trypanosomiasis (Okomo-Assoumou et al., 1995), and has also been implicated in the cachexia occurring in the acute *T. cruzi* infection in mice (Truyens et al., 1995). Moreover, TNF-α has been demonstrated to modulate the expression of adhesion molecules participating in inflammatory processes by recruitment of lymphocytes into inflammatory sites, thus contributing to the progression of the local inflammatory reaction in chagasic cardiomyopathy (Bachmaier et al., 1997). In the present study, the high TNF-α levels observed in patients with severe myocardiopathy (group V) would then suggest that the abnormalities in the cytokine TNF-α balance could participate in the evolution of pathogenesis of chronic Chagas disease (Fig. 1b).

Then, proinflammatory cytokines, e.g. TNF-α, are involved in generating inducible form of nitric oxide synthase (iNOS), which produces a continuous and potentially large supply of nitric oxide (NO) in tissues that normally experience only low and tightly controlled levels of this ubiquitous molecule (Gazzinelli et al., 1996). It has been shown that NO plays a pivotal role in the immune response as a first line of defence against *T. cruzi* (Vespa et al., 1994). However, there is evidence that excessive NO may then cause host pathologic consequences (Farrell et al., 1992; Miller et al., 1993), and some examples showed that NO is associated with the severity of cerebral malaria (Grau et al., 1989). Concerning Chagas disease, high NO levels have been identified in seropositive individuals to *T. cruzi* (Perez-Fuentes et al., 1998). Further, NO has been involved in the lesions of the peripheral autonomic neurons observed in the acute phase of experimentally *T. cruzi* infection in rats

(Garcia et al., 1999). Experimental evidence exists which identified that proinflammatory cytokines generated by activated immune cells induce an increase in NO in rat isolated myocytes (Tsujino et al., 1994), or which suggested that NO issued from cardiac iNOS might participate in the pathogenesis of murine chagasic heart disease (Huang et al., 1999). More recently, it was demonstrated that NO derived from inducible nitric oxide synthase (NOS2) plays an important role in the development and progression of ventricular dilatation and systolic dysfunction in acute murine chagasic myocarditis (Chandra et al., 2002). Our findings confirm the potential importance of both TNF-α and NO levels in dilated cardiomyopathy (Habib et al., 1996), thus suggesting that high NO levels induced by appropriate TNF-α levels may induce damages, mainly by oxidative processes, in parasitic infected cells (Fig. 1c).

Usually, cells respond to diverse inflammatory attacks using a cascade of different events in order to repair, protect or degrade damaged proteins as a defensive strategy to ensure cell reproduction and survival. Oxidative stress (OS) is the main mechanism of immune response against parasitic infection (Schirmer et al., 1987). OS produces multiple changes and may eventually lead to cell death because of an increase in oxidant generation or a decrease in antioxidant protection (Fiers et al., 1999). Previous studies have demonstrated that the Glutathione peroxidase is a key enzyme acting on the antioxidant metabolism of the human host. Glutathione peroxidase protects against OS in reducing glutathione-like molecules to catabolise hydrogen peroxide and protect cells from the oxidant molecules which increase in turn as a result of chronic immune activation (Hayes and McLellan, 1999). Furthermore, glutathione peroxidase is an essential protective component against NO cytotoxicity for both host and parasite cells (Romao et al., 1999). In addition, SOD has been shown to be implicated as an inflammatory mediator during *T. cruzi* infection (Cardoni et al., 1990), and induction of SOD protects cells against oxidative damages associated to proinflammatory cytokines like IL-1 and TNF-α (Hirose et al., 1990). Interestingly, TNF-α has been shown to induce the expression of the mitochondrial MnSOD (Wong and Goeddel, 1988). We effectively demonstrated in this work an importance of SOD in explaining Chagas disease pathogenesis evolution (Fig. 1d).

Taken together, the statistical data suggest both cumulative and complementary effects of glutathione peroxidase, TNF-α, NO and SOD as factors responsible for Chagas disease severity. This chain of events is summarised in Fig. 1 where reduction in glutathione peroxidase and SOD levels associated with increase in TNF-α and NO levels allows antioxidant-imbalance and oxidative damage, thus favouring the evolution of disease (Fig. 1d,e). Further research should then focus on such complex mechanisms involved in chronic disease severity as suggested in this work.

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