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# Immunomodulatory properties and anti-apoptotic effects of zinc and melatonin in an experimental model of chronic Chagas disease

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ABSTRACT

The immunomodulatory effects of melatonin and zinc during chronic experimental Chagas' disease were studied. Early and late apoptosis by Annexin V-propidium iodide staining were evaluated. The expression of CD28, CD80, CD86, CD45RA and $CD4^+$T and $CD8^+$T cells were also evaluated by flow cytometry analysis. The combination of zinc and melatonin notably reduced the apoptotic ratios of splenic cells in the infected and treated animals when compared to untreated rats, during early and late stages of apoptosis. The percentages of $CD8^+$T cells in Zn, Mel or Zn and Mel treated rats were reduced when compared to infected and untreated animals. Higher percentages of CD28 expression in $CD4^+$ and $CD8^+$ T cell populations were observed in control and infected Zn-treated group as compared to untreated ones. Zn, Mel or the combination of both did not induce any statistically significant differences for B cells when comparing to treated control and infected groups. Zinc or Mel-treated animals presented a lower expression of CD86 when compared to untreated counterparts. According to our data, this work strongly suggest that the modulation of the immune system operated by zinc and melatonin administration affected the balance among T cell immune response, apoptosis and expression of co-stimulatory molecules during chronic *Trypanosoma cruzi* infection, inducing important changes in the host's immune response against the parasite. Future experiments in this field should be focused in improving our understanding of the key mechanisms underlying the involvement of melatonin and zinc in the immune response during chronic Chagas' disease.



## Introduction

Chagas disease, regarded as a neglected tropical disease by competent authorities as WHO- and NIH, is endemic in Latin America and an emerging infection in North America and Europe, since widespread emigration of *Trypanosoma cruzi* chronic carriers from endemic countries toward developed ones, the scope of Chagas disease threatens to expand exponentially to reach areas outside the traditional (Dias and Coura 1997).

Human *T. cruzi* infection evolves from a usually oligosymptomatic acute phase, with detectable parasitemia and acute myocarditis in 8% of the cases. The acute myocarditis is characterized by the presence of focal necrosis, severe inflammation and abundant amastigote nests (Rossi et al. 2010). In the early chronic phase, most infected individuals are asymptomatic, but over a period of years to decades, about 30–40% of the patients develop symptomatic Chagas disease (Rassi et al. 2000).

Although the precise mechanism associated with the pathogenesis of chronic chagasic cardiomyopathy remain incompletely understood, studies conducted by Tostes et al. (2005) have reported that apoptosis contributes to myocardial cell loss and suggest its contribution to the development of heart failure in Chagas' disease. The concept to exploit the apoptotic pathways for therapeutic purposes is attractive, because it has been demonstrated that cell death is a common strategy for survival and/or dissemination used

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by many intracellular organisms (Carmen and Sinai 2007). Apoptosis of infected cells seems to be a critical factor in the course of infection, since it can favor pathogen control. In contrast, pathogen-induced cell death leads to the elimination of key immune cells (Stahl et al. 2013).

Additionally, studies conducted by De Souza et al. (2003) demonstrated that cardiomyocytes become apoptotic after infection with different strains of *T. cruzi*. Besides this, Manque et al. (2011) also found that the two classical pro-apoptotic genes associated with activation of the intrinsic and extrinsic apoptotic pathways were up-regulated in murine cardiomyocytes during *T. cruzi* infection.

Most of our understanding of the pathogenic mechanisms involving *T. cruzi* infection comes from animal studies. Few works with humans describe the activation of T cells as a crucial event in the pathogenesis of Chagas' disease. Fonseca et al. (2005) isolated *T. cruzi*-specific $CD8^+$ T cells from endomyocardial biopsies of a patient with chronic Chagas cardiomyopathy, suggesting the involvement of these cells with the pathology. Based in these concepts, Reis et al. (1993) and Higuchi et al. (1997), described that in chronic chagasic cardiomyopathy patients, a predominance of $CD8^+$ T lymphocytes outweigh $CD4^+$ T cells in the focal and diffuse heart infiltrates. The same fact was also observed by other authors in chronically infected experimental models (Dos Santos et al. 2001; Medeiros et al. 2009).

Several studies have now firmly established that melatonin (N-acetyl-5-methoxytryptamine) and its metabolites, considered as endogenous antioxidant produced by pinealocytes (Reiter et al. 2007), suppress oxidative stress through several mechanisms (Carmen and Sinai 2007; Stahl et al. 2013; Reiter et al. 2007). It acts as a direct and indirect antioxidant, scavenging free radicals and stimulating antioxidant enzymes involved in the metabolism of free radicals (Reiter et al., 2003). Furthermore, melatonin enhances the activities of other antioxidants protecting other antioxidant enzymes from oxidative damage consequently, attenuating the production of free radicals (Manque et al. 2011; Klongpanichapak et al. 2007).

Zinc is required in a variety of complex mechanisms including DNA synthesis, RNA transcription, cell division, cell activation, gene expression, apoptosis, hormone regulation and others (MacDonald 2000; Cousins 2006; Maret 2009). The wide involvement of this nutritionally important oligoelement in the immune system (Kruse-Jarres 1989) includes its ability to influence the production and signaling of numerous inflammatory cytokines in a variety of cell types (Abbas and Lichtman 2005; Zhou et al. 2010).

Planelles et al. (2003) describe that the APC ability to deliver the second co-stimulatory signal is mainly inducible by infectious agents, but some other works report that the protozoan parasites could also negatively modulate the APC function. Van Overtvelt et al. (1999), describe that the *in vitro* infection of human dendritic cells by *T. cruzi* induces an impaired maturation process reducing both the secretion of cytokines and the up-regulation of co-stimulatory molecules.

Understanding how *T. cruzi* tricks the immune system is important to design effective immunotherapies against Chagas' disease. In this context, this work deals with *T. cruzi* chronically infected *Wistar* rats trying to evaluate the development, the progression of infection and the immunopathology of this disease. For this purpose, the immunomodulatory effects of melatonin and zinc during the chronic experimental Chagas' disease were studied. The expression of CD28, CD80, CD86 and the percentages of B cells, $CD4^+$T and $CD8^+$T cells were evaluated by flow cytometry analysis as well as early and late apoptosis process in spleen cells.

## Materials and methods

### *Animals*

Male Wistar rats (40 animals) four weeks old, weighing 90–100 g were used. Rats were obtained from the Facility House of the Universitary Campus of Ribeirão Preto. Animals were randomized into the following groups: control (C), zinc control (ZC), melatonin control (MC), zinc and melatonin control (ZMC), infected (I), zinc infected (ZI), melatonin infected (MI), zinc and melatonin infected (ZMI). A total of 5 animals were used per group and per day of experiment. Animals were separated into groups of 5 in plastic cages. The cages were kept under controlled temperature (25°), in specific pathogen-free and standard environmental conditions with a 12 h light/dark cycle with commercial rodent diet and water available *ad libitum*. Rat pads were changed 3 times per week to avoid an accumulation of ammonia from urine. The protocol of this study was approved by the local Ethics Committee (protocol number 08.1.835.53.5).

### *Parasites and experimental infection*

Rats were intraperitoneally (i.p.) inoculated with $1 \times 10^5$ blood tripomastigotes of the Y strain of *T. cruzi*. Parasitemia was determined by Brener's method (Brener 1969). The assays were performed on 60 days after infection. It is important to emphasize that the inherent resistance of Wistar rats to most *T. cruzi* strains, we found it necessary to use relatively high inoculums ($1 \times 10^5$ blood trypomastigotes).

### *Parasite extracts*

Parasite antigens of *T. cruzi* were purified by following the procedure described by Camargo et al. (1997), with some modifications. Bloodstream trypomastigote forms obtained after heart puncture of infected Balb/c mice, at the peak of the parasitemia, were used for the infection of LLC-MK2 cells (monkey kidney epithelial cells) in culture to establish the intracellular cycle, and maintained at 37 °C in RPMI-1640 medium with fetal calf serum (5%) in an incubator (5% $CO_2$ atmosphere). After 5 a 9 days, Tissue culture-derived trypomastigotes (TCTs) of the Y strain of *T. cruzi* were harvested from the supernatants of LLC-MK2 cultures, previously infected, and purified by centrifugation, for cellular debris separation. After another centrifugation, the resulting pelleted was washed in cold PBS, and subjected to greater than five rounds of freeze-thawing followed by 5 sonication rounds (30 s, 20 kHz, 30 W). Cellular debris were removed by centrifugation at $20{,}000 \times g$. Protein concentrations were determined using a Bio-Rad protein assay, and the samples were stored at −70 °C until use. Then, the effects of *T. cruzi* antigens in the induction of apoptosis in splenocytes were evaluated.

### *Treatment scheme*

Both treatments (zinc and melatonin) were given orally. Rats were supplied with zinc sulphate (Sigma Chemical Co. MO, USA), at a dose of 20 mg/kg body weight, dissolved in 0.1 mL of distilled water. Melatonin (Sigma Chemical Co., St Louis, MO, USA) was administered 0.1 mL daily by gavage, at 5 mg/kg of body weight dissolved in polyethylene glycol 400 (PEG 400) (Brazão et al. 2011). The treatment of the infected group started 48 h after infection. Control groups were treated as prior described.

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