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Effect of zinc supplementation on E-ADA activity, seric zinc, and cytokines levels of *Trypanosoma evansi* infected wistar rats

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ABSTRACT

The aim of this study was to evaluate the effect of zinc supplementation on the ecto-adenosine deaminase activity (E-ADA), zinc seric levels and cytokines (TNF-α, IL-1, IL-6, and IL -10) on rats experimentally infected by *Trypanosoma evansi*. Four groups with 10 rats each were used as negative controls (groups A and B), while the animals from the groups C and D were infected intraperitoneally with 0.1 mL of cryopreserved blood containing 1.4×10^4 of trypanosomes. Animals of groups B and D received two doses of Zinc (Zn) at 5 mg kg^{-1}, subcutaneously, on the 2nd and 7th day post-infection (PI). Blood samples were collected on days 5 ($n = 5$) and 15 PI ($n = 5$). Zn supplementation was able to increase the rat's longevity and to reduce their parasitemia. It was observed that seric Zn levels were increased on infected animals under Zn supplementation. Animals that were infected and supplemented with Zn showed changes in E-ADA activity and in cytokine levels ($P < 0.05$). Zn supplementation of healthy animals (Group B), increased the E-ADA activity, as well as reduced the concentration of cytokines. Infected animals from groups C and D showed increased levels of cytokines. Finally, we observed that Zn supplementation led to a modulation on cytokine's level in rats infected by *T. evansi*, as well as in E-ADA activity.

1. Introduction

Trypanosoma evansi is a flagellated protozoan of the family Trypanosomatidae that can be mechanically transmitted by blood-sucking insects causing disease in many animals [1]. *T. evansi* can be observed in tissues, blood and body fluids of infected animals leading to pathological disorders [2]. Pathological changes caused by this parasitic infection are multifactorial and several clinical signs are described in the host including fever, edema, weight loss, weakness associated with anemia and neurological signs [2–4]. In Brazil, the only available drug against trypanosomosis (diminazene aceturate) has shown low efficacy in many cases, i.e. recurrence of parasitemia. Therefore, new studies to test therapeutic compounds are necessary.

Studies with *Trypanosoma cruzi* and *T. evansi* using zinc (Zn) as a mineral supplementation showed the improvement of the immune status, as well as delay in the establishment of the disease [5–8]. Zn acts in many different ways, for example, in metabolic pathways of many enzymes, receptor's activation, and in the enhancement of the immune response [9,10]. It is also an important metal being a component of the enzyme ecto-adenosine deaminase (E-ADA), participating in E-ADA activation [9].

During *T. evansi* infection, changes in the concentration of adenosine and E-ADA activity in serum, cells, and tissues can occur [11,12] may appear associated with a reduction in seric levels of Zn [13,14]. E-ADA is part of the purine metabolism, and it is in charge

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of adenosine to inosine deamination contributing to the purinergic cascade [15]. This enzyme also acts as an endogenous regulator of the innate immunity, protecting the host tissue against excessive damage playing an important role on T lymphocytes differentiation and proliferation, also interacting with other cell surface proteins in order to create new immune-regulatory mechanisms [16–19].

Therefore, based on the hypothesis that *T. evansi* infection may have effects on seric zinc levels and consequently, on E-ADA activity, a situation that can generates interference on several physiological functions in *T. evansi* infected animals, we have designed this study to evaluate the effect of zinc supplementation on rats experimentally infected by *T. evansi* on E-ADA activity in serum and lymphocytes, and on levels of zinc and cytokines on serum samples.

2. Materials and methods

This experimental design was repeated only once, as approved by the Ethics Committee of our Institution. All biological tests (E-ADA activity, cytokine and zinc levels) were performed in triplicate in current study.

2.1. Trypanosoma evansi isolation

A cryopreserved strain obtained from a naturally *T. evansi* infected dog was used in this experiment [4].

2.2. Animal model

Forty 80-day-old Wistar male rats weighing in average 263 (±17) grams were kept in cages (five animals/cage), housed in an experimental room on a light/dark cycle of 12 h with controlled temperature and humidity (25 °C; 70%). They were fed with commercial feed and received water *ad libitum* throughout the experiment. These animals were submitted to an adaptation period of 10 days.

2.3. Experimental design and parasitemia estimation

The animals were divided into four groups named A to D, and these composed eight subgroups of our experimental design. Groups A [A_1 ($n = 5$); A_2 ($n = 5$)] and B [B_1 ($n = 5$); B_2 ($n = 5$)] represented the uninfected animals (or negative control), while the rats of groups C [C_1 ($n = 5$); C_2 ($n = 5$)] and D [D_1 ($n = 5$); D_2 ($n = 5$)] were inoculated intraperitoneally with 0.1 mL of cryopreserved blood (containing *T. evansi*) at 1.4×10^4 of *T. evansi* on day 0. Thus, groups A and C represented the negative and positive controls of infection, respectively, while rats from groups B and D received one (B_1 and D_1, on the 2nd day post-infection - PI) or two (B_2 and D_2, on the 7th day PI) doses of zinc (Zn) of 5 mg kg^1 (Biogénesis – Bagó®). Zinc was administered subcutaneously according to an adaptation of the technique described by Dalla-Rosa and cols [8]. The adaptation consisted in a date change, setting the administration of the first dose on day 2 PI, in order to supplement the animals with Zn before the regular onset of parasitemia, thus, reducing the infective parasite load, retarding the prepatent period and, consequently, increasing the longevity of animals.

In order to lead the infected animals to a chronic infection, the protocol recommended by Tavares and cols [20] was followed. The evolution of parasitemia and treatment was monitored daily by blood smears that were microscopically examined throughout the experiment.

2.4. Sample collection

Blood collection was performed on days 5 (subgroups A_1, B_1, C_1, and D_1) and 15 PI (subgroups A_2, B_2, C_2, and D_2). Due to our experimental design, the animals of groups B1 and D1 received only one dose of Zn. After this procedure, the rats were humanely euthanized by decapitation as recommended by the Ethics Committee. An aliquot of blood (±4 mL) was stored in tubes with anticoagulant for lymphocyte separation in order to analyze E-ADA activity on these cells; and another blood aliquot (±3 mL) was used for serum separation, which was used for assessment of seric Zn, E-ADA activity, and cytokines levels.

2.5. E-ADA activity on lymphocytes and serum

Lymphocytes were obtained from whole blood by gradient separation using Ficoll-Histopaque™ plus according to the technique described by Böyum [21]. Protein concentration on lymphocytes was measured by a method described by Peterson [22]. E-ADA activity was measured by spectrophotometry in serum and lymphocytes by the method standardized by Giusti and Gakis [23] using adenosine substrate. The procedure was performed in triplicate with the mean value used as the base for calculation. Briefly, the reaction was started by addition of the substrate (adenosine) to a final concentration of 21 mmol/L with 1 h incubation at 37 °C. The reaction was stopped by adding 106 mmol/L/0.16 mmol/L phenol-nitroprusside/mL of solution. The reaction was immediately mixed to 125 mmol/L/11 mmol/L of alkaline hypochlorite (sodium hypochlorite) and vortexed. Ammonium sulfate (75 μmol/L) was used as ammonium standard. The ammonia concentration is directly proportional to the absorption of indophenol at 650 nm. The value of E-ADA activity in lymphocytes and serum were expressed as U/mg of protein and U/L, respectively.

2.6. Levels of seric zinc

The procedure used was described in a previous work [24]. Briefly, 100 μL of serum and 2 mL of HNO_3 (14 mol L^{-1}) were mixed in a 15 mL polypropylene tube. The mixture was heated in a water bath (70 °C) during 10 min. After sample mineralization deionized (18.2 MΩ cm) water was added to the tube on a final volume of 10 mL right before reading by inductively coupled plasma optical emission spectrometry (ICP OES). Zn emission line selected was at 213,856 nm and the results were expressed in μg L^{-1} [24].

2.7. Seric levels of cytokines

Tumor necrosis factor alpha (TNF-α), and interleukins 1, 6, and 10 (IL-1, IL-6, and IL-10) were measured by ELISA assay using Quantikine Immunoassay kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions and as described by Paim and cols [25]. Briefly, 96 well microplates were sensitized with the primary antibody at room temperature (RT) for 30 min; then the sample was added and incubated (37 °C) for 30 min. After washing, the secondary antibody conjugated with peroxidase was added and incubated. Cytokine concentration was determined by the intensity of the color measured by spectrometry in a micro ELISA reader.

2.8. Molecular analysis

The presence of *T. evansi* DNA was investigated on brain tissue by PCR (five samples representative of day 15 PI – group D). For preparation of DNA templates, a small section (0.4 × 0.4 mm) of each brain was washed three times (5 min) in bi-distillated water on a shaker system. Then, brain tissues were cut into small fragments, incubated with lysis buffer (1% SDS, 100 mM EDTA, pH 8.0, 20 mM Tris–HCl, pH 8.0, and 350 mg/mL of proteinase K) at 37 °C for 18 h; centrifuged at 14,000 g for 5 min; and the DNA was