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Curcumin pre-treatment modulate the activities of adenine nucleotide and nucleoside degradation enzymes in lymphocyte of rats infected with *Trypanosoma evansi*

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ABSTRACT

This study aimed to evaluate nucleoside triphosphate diphosphohydrolase (NTPDase) and adenosine deaminase (ADA) activities in lymphocytes from rats supplemented or not with curcumin 30 days prior to experimental infection with *Trypanosoma evansi*. Thirty-two adult male Wistar rats were divided in four groups. The pre-infection group 20 (PreI20) received orally 20 mg/kg of curcumin and pre-infection group 60 (PreI60) received orally 60 mg/kg of curcumin for 30 days prior inoculation with *T. evansi*. The infected e non-infected control groups received only oral vehicle for 30 days. *Trypanosoma evansi* infected groups were inoculated intraperitoneally with 0.2 ml of blood with 1×10^6 parasites. After inoculation the treatment of the groups continued until the day of euthanasia (15 days). The results showed that curcumin pre-treatment, with both doses, reduced ($P < .05$) NTPDase and increased ($P < .05$) ADA activity in lymphocytes of treated groups when compared to untreated and infected animals (control). The results of this study support the evidence that the regulation of ATP and adenosine levels by NTPDase and ADA activities appear to be important to modulate the immune response in *T. evansi* infection, once the treatment with curcumin maintained the NTPDase activity reduced and enhanced ADA activity in lymphocytes. It is possible to conclude that the use of curcumin prior to infection with *T. evansi* induces immunomodulatory effects, favoring the response against the parasite.

1. Introduction

Trypanosoma evansi is a protozoan belonging to the Salivaria group that infects a variety of mammals causing trypanosomiasis. In horses it causes the disease known as “Mal de Cadeiras” or “Surra” [1]. This disease was first described in horses and camels from India, in 1881 by Griffith Evans [2], and since then it has been described in tropical and subtropical regions of the world [3,4]. In infected hosts *T. evansi* can be found in tissues, blood and body cavities fluid, inducing an immune response [5,6].

The enzymatic changes related to immune response have been investigated [7–10], and seems to be a key point to understand the pathogenicity of the trypanosomiasis in animals. As part of these system, the adenine-nucleotide and -nucleoside converting ectoenzymes have a central role. The ecto-nucleoside triphosphate diphosphohydrolases (NTPDase) dephosphorylate the nucleotide ATP via ADP to AMP, whereas adenosine deaminase (ADA) catalyzes the conversion of adenosine to inosine [11]. NTPDase (CD39) was first described as a B lymphocyte activation marker, however this ectonucleotidase is also expressed in natural killer (NK) cells, monocytes and activated T cells. CD39 has substantial functions in the immune system including cytokine expression, cell–cell adhesion and cell proliferation and apoptosis via modulation of ATP levels [12]. Most importantly, alterations in the activity of this enzyme may be important in immune diseases [16].

These enzymes are extensively present in blood cells influencing several events such as platelet aggregation, inflammation and immune

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response [13]. The lymphocytes are cells responsible for recognition and destruction of invading antigens. The number of these cells can be affected by antigenic stimuli, or benign and malignant cellular proliferation. Adenine-nucleotide and –nucleoside modulate the lymphocytes function, mainly the extracellular ATP that regulates cell-cell interaction, important in activation processes, differentiation, development, proliferation, cell death and lymphocytes function [14]. Therefore, extracellular ATP and adenosine levels and the ensuing purinergic signaling can be dynamically controlled during inflammation by the action of enzymes expressed in immune cells. Moreover, adenosine is an anti-inflammatory molecule [15] and NTPDase plays a key role in lymphocyte functions, including antigen recognition and activation of cytotoxic T cells in addition to the capacity of cell-cell signal amplification [12].

Previous research from our group has already shown that the pretreatment with curcumin modulates the immune response in rats infected with *T. evansi* [16]. Was determined the levels of proinflammatory and anti-inflammatory cytokines. During the disease, lymphocytes releases interferon-gamma (IFN-γ) in response to parasite antigens, IFN-γ activates macrophages increasing their phagocytic capacity. Activated macrophages induces proinflammatory cytokines production, such as IL-1, IL-6 and TNF-α, participating in parasite replication and host immune response [6,17,18]. The anti-inflammatory property of curcumin is due to the phenols group incorporate in its molecule, which inhibit prostaglandins and leukotrienes release [19]. Curcumin is a substance present in food spices such as curry and Indian saffron [20].

Taking the following information together: NTPDase and ADA enzimes control levels of immunomodulatory molecules: the activity of these enzymes are altered on the surface of lymphocytes of rats infected with *T. evansi:* and curcumin has an important immunomodulatory effect against infection: the aim of this study was to determine the in vivo effect of different doses of curcumin pre-treatment on NTPDase and ADA enzymatic activity in lymphocytes of rats experimentally infected with *T. evansi*. Herein, we tested that hypothesis that curcumin treatment can improve immunomodulatory response mediated by ectonucleotidases enzymes in parasitemic diseases.

2. Material and methods

2.1. Reagents

Adenosine 5′-triphosphate disodium salt (ATP), adenosine 5′-diphosphate sodium salt (ADP), and curcumin (curcumin > 80%; curcuminoid content > 94%) were purchased from Sigma Chemical Co (St. Louis, MO, USA). All other reagents used in the experiments were of analytical grade and of highest purity.

2.2. Animals

Thirty-two male Wistar rats (90–110 days) were used in this experiment. They were housed in cages (five per cage) on a natural day/night cycle at a temperature of 21 °C, with free access to water and standard ration ad libitum. This study was approved by the Ethics and Animal Welfare Committee of the Rural Science Center of the Federal University of Santa Maria (CCR/UFSM), No. 017/2012 in accordance with existing legislation and the Ethical Principles published by the Brazilian College of Animal Experiments (COBEA).

2.3. Experimental design and curcumin treatment

The animals were randomly divided in four groups (8 rats in each group: control (C; non-infected rats); *T. evansi* infected control (IC); *T. evansi* infected and pretreated with curcumin 20 mg/kg of body weight for 30 days (PreI20); *T. evansi* infected and pretreated with curcumin 60 mg/kg of body weight for 30 days (PreI60). The choice of curcumin dosages was made based in previous studies that described beneficial results [16]. Curcumin was diluted with corn oil and administered by oral gavage, daily, not exceeding 0.1 ml/kg of body weight. Treatments were administrated once a day (8–9 h a.m.). The treatment of the groups continued for 15 days after inoculation. Both control groups (C and IC) received orally only vehicle (corn oil).

2.4. Etiological agent and inoculation

The rats were inoculated intraperitoneally with 0.2 ml of blood containing 10^6 parasites. All rats were infected by *T. evansi* at the same day. The control group (C) received 0.2 ml of physiological solution by the same route. The etiological agent isolate used was obtained from a naturally infected dog and maintained in liquid nitrogen at the laboratory of Dr. Silvia G. Monteiro (Brazil).

2.5. Parasitemia estimation

The presence and degree of parasitemia were determined daily by blood smear examination of each animal. The blood smear were made from a drop of blood from the caudal vein of each rat, stained with Romanowsky (Diff-Quick) and visualized under optical microscope (1000×) determining the average number of trypanosomes in 10 homogeneous random fields (considering erythrocytes).

2.6. Collection and preparation of blood samples

Fifteen days post inoculation (pi) the animals were anesthetized with isoflurane for blood collection by cardiac puncture and then euthanized. Blood samples were stored in tubes with ethylenediaminetetraacetic acid (EDTA 10%). The peripheral lymphocytes were isolated using Ficoll Hypaque density gradient as described by BÖYUM [21]. After separation, only samples with at least 95% of lymphocytes, as verified in the coulter STKS (Miami-USA), were used. Lymphocyte viability and integrity were confirmed by determining the percentage of cells excluding 0.1% Trypan blue and measuring lactate dehydrogenase (LDH) activity [22]. Enzymatic activity was measured immediately after obtaining the lymphocytes.

2.7. NTPDase activity in lymphocytes

NTPDase activity was determined according to the method described by LEAL et al. [23]. Briefly, proteins of all samples were adjusted to 0.1–0.2 mg/ml and 20 µl of intact cells (2–4 µg protein) were added to a reaction medium containing 0.5 mM CaCl2, 120 mM NaCl, 5.0 mM KCl, 60 mM glucose and 50 mM Tris-HCl buffer, pH 8.0, in a final volume of 200 µl, and preincubated for 10 min at 37 °C. The reaction was started by adding ATP or ADP as substrate at a final concentration of 2.0 mM and was stopped with 5% trichloroacetic acid (TCA). All the samples were analyzed in triplicate, and enzymes (intact lymphocytes) were added to the control after the addition of TCA in order to correct the non-enzymatic hydrolysis of the substrate. The inorganic phosphate (Pi) released was measured by the method of CHAN et al. (1986) and enzymatic activity was reported as nmol of Pi released/min/mg protein.

2.8. ADA activity in lymphocytes

ADA activity in lymphocytes was measured in spectrophotometer by the method of GIUSTI and GALANTI [24]. The reaction was started by adding substrate (adenosine) to a final concentration of 21 mmol/l and incubations were carried out for 1 h at 37 °C. The reaction was stopped by adding 106 mmol/l/0.16 mmol/l phenol-nitroprusside/ml solution. The reaction mixtures were immediately mixed to 125 mmol/l/11 mmol/l alkaline hypochlorite (sodium hypochlorite) and vortexed. Ammonium sulfate of 75 µmol/l was used as ammonium standard. The

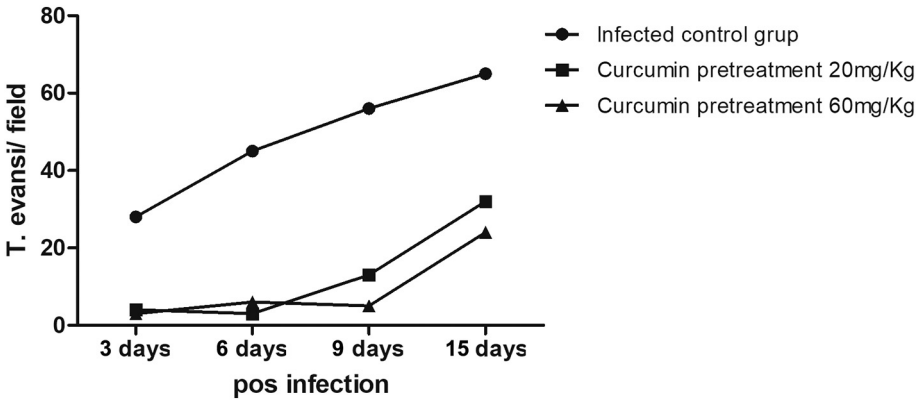

Fig. 1. Effect of oral curcumin pre-treatment and treatment on the parasitemia of Trypanosoma evansi infected rats. Curcumin was administered daily by oral gavage. All rats were infected by *T. evansi* at same day. (IC) *T. evansi* infected control group; (PreI20) *T. evansi* infected and pretreated with curcumin 20 mg/kg body weight for 30 days; (PreI60) *T. evansi* infected and pretreated with curcumin 60 mg/kg body weight for 30 days. The treatment of the groups continued until the day of euthanasia (15 days after inoculation). Trypanosoma/ fild at 1000× magnification.

ammonia concentration is directly proportional to the absorption of indophenol at 620 nm. The specific activity is reported as U/L in lymphocytes.

2.9. Statistical analysis

Data was analyzed statistically by one-way ANOVA followed by Duncan's multiple tests. Differences were considered significant when the probability was $P < .05$. The values were represented as mean ± standard deviation.

3. Results

3.1. Parasitemia

Trypanosoma evansi could be detected in the blood of all infected rats from 24 to 48 h after inoculation (1–2 parasites/field at 1000×). Independent of the dose used (20 or 60 mg/Kg), throughout the entire experimental period, the parasites circulation levels were significantly lower in curcumin pretreated groups ($P < .05$) compared to *T. evansi* infected control group (IC) (Fig. 1).

During the 30 days of oral pretreatment of curcumin by gavage, no animals showed altered clinical signs or death due to its use in both doses. Similar data have already been published in previous studies by our group [16].

3.2. NTPDase activity

Our results showed that curcumin pre-treatment could affect NTPDase activity in rats infected by *T. evansi* ($P < .05$). In the ATP (Fig. 2) and ADP (Fig. 3) hydrolysis, a significant increase ($P < .05$) was observed in rats infected by *T. evansi* compared to control group (C). The pre-treatments with curcumin (PreI20 and PreI60) prevented the increase in ATP and ADP hydrolysis when compared to the infected control group (IC) ($P < .05$).

3.3. Adenosine deaminase activity

Results obtained for the ADA activity (Fig. 4) showed a significant decrease ($P < .05$) in the *T. evansi* infected group (IC) when compared to the not infected control group (C). The pre-treatment with curcumin in the doses of 20 and 60 mg/kg was able to prevent the decrease in the ADA activity when compared to the infected control group (IC) ($P < .05$).

4. Discussion

T. evansi infection alters enzymatic activity of the purinergic system in rats [8]. The changes occcur during the host immune response to the parasite. These enzymes are part of a complex system wich regulates

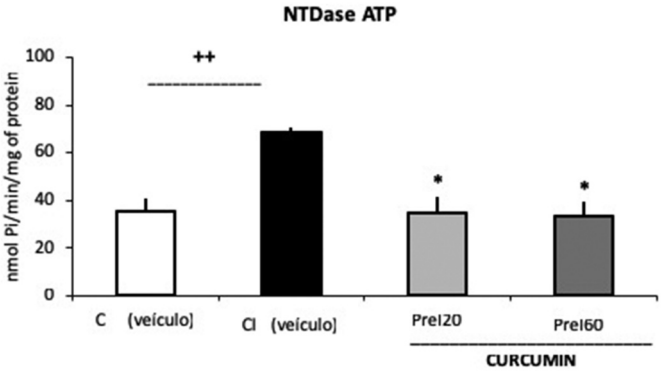

Fig. 2. Effect of oral curcumin pre-treatment for 30 days in the NTPDase (ATP) activity in lymphocytes on the parasitemia of *Trypanosoma evansi* infected rats. Curcumin was administered daily by oral gavage. All rats were infected by *T. evansi* at same day. The treatment of the groups continued until the day of euthanasia (15 days after inoculation). (C) control group - non-infected; (IC) *T. evansi* infected control group; (PreI20) *T. evansi* infected and pretreated with curcumin 20 mg/kg body weight for 30 days; (PreI60) *T. evansi* infected and pretreated with curcumin 60 mg/kg body weight for 30 days; Values represent mean ± SE, ($n = 8$ per group); at 1000× magnification.

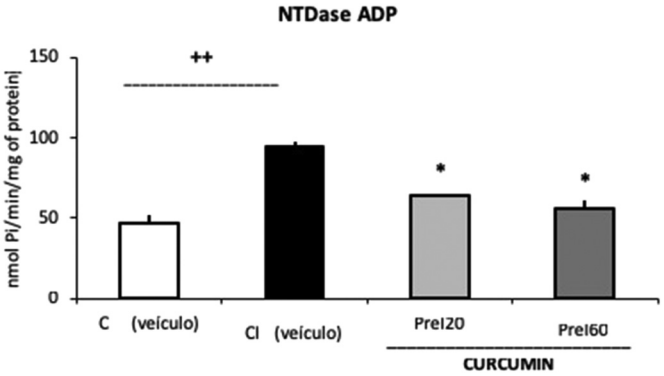

Fig. 3. Effect of pre-treatment for 30 days with curcumin in the NTPDase (ADP) activity in lymphocytes from rats infected with Trypanosoma evansi (C) control group - non-infected; (IC) *T. evansi* infected control group; (PreI20) *T. evansi* infected and pretreated with curcumin 20 mg/kg body weight for 30 days; (PreI60) *T. evansi* infected and pretreated with curcumin 60 mg/kg body weight for 30 days. Values represent mean ± SEM, ++ represents statistical difference between C and IC groups ($P < .001$); * represents statistical difference between IC and groups that receive curcumin ($P < .05$, $n = 8$ per group).

the signaling mediated by adenine-nucleotide and –nucleoside, controling their degradation and formation [23]. Therefore, depending on the ATP and ADP concentration, they function as proinflammatory molecules, affecting the stimulation and proliferation of lymphocytes

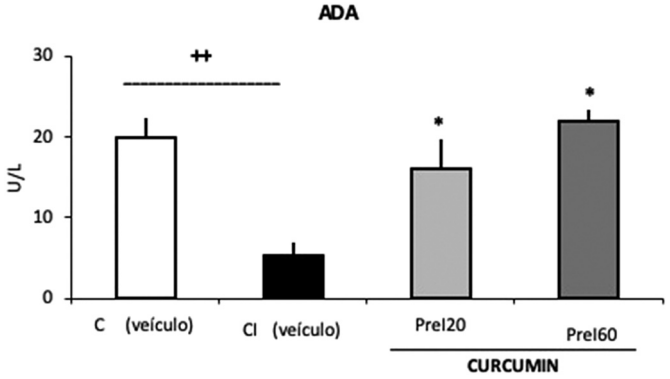

Fig. 4. Effect of pre-treatment for 30 days with curcumin in the ADA activity in lymphocytes from rats infected with Trypanosoma evansi (C) control group - non-infected; (IC) *T. evansi* infected control group; (PreI20) *T. evansi* infected and pretreated with curcumin 20 mg/kg body weight for 30 days; (PreI60) *T. evansi* infected and and pretreated with curcumin 60 mg/kg body weight for 30 days. Values represent mean ± SEM, ++ represents statistical difference between C and IC groups ($P < .001$); * represents statistical difference between IC and groups that receive curcumin ($P < .05$, $n = 8$ per group).

and cells that release cytokines [25]. In addition, adenosine is an anti-inflammatory molecule [15].

In Figs. 2 and 3, it is possible to observe an increase of the NTPDase activity through degradation of ATP and ADP in *T. evansi* infection (IC group) compared to control group (C group) ($P < .05$). Schenk et al. [26] suggested an important role of NTPDase in controlling the lymphocytes function, including antigen recognition and/or activation of cytotoxic T-lymphocytes. The increase of ATP and ADP hydrolyzes by NTPDase can decrease levels of these nucleotides. ATP and ADP have affinity to P2Y receptors in the surface of lymphocytes, and should stimulate the Th2 immune response, resulting in IL-4 production, activation of eosinophils and mast cells [8,27]. Due to this scenario, there is a reduced immune response and the host is not able to control the infection causing high parasitemia levels.

As a compensatory response to the infection and inflammatory process there was a reduction in ADA activity ($P < .05$) in infected rats (IC group) when compared to not infected control group (C) (Fig. 4). The reduction in ADA activity suggests an increase in the extracellular adenosine levels, as previously described by SILVA et al. [10]. The adenosine reflects what happens in the immune system during acute tissue inflammation [27]. The reduction of ADA activity in lymphocytes increases adenosine concentration and promote interaction of this molecule with its purinergic receptor present in many cells, resulting in an anti-inflammatory response, such as inhibition of Th1 immune response [28]. Consequently, the anti-inflammatory effect mediated by adenosine may be due to its sensibilization of purine type 1 receptors (A1R and/or A2AR) inducing a compensatory effect. Since the *T. evansi* infection activates the immune system, increasing concentration of adenosine could minimize the inflammatory process.

The benefits of curcumin have been investigated, although the mechanism of how it affects the purinergic system are not completely known [29]. Additionally, enzymes of the purinergic system are closely evolved in the modulation of the immune system with anti-inflammatory and proinflammatory effects [12].

In the present study, the effect of curcumin in the NTPDase and ADA activities was investigated in rats infected with *T. evansi*. The results revealed an efficacy of pretreatment with curcumin with both doses tested (PreI20 and PreI60 groups). Animals received curcumin in a period of 30 days prior and 15 days post infection. It was observed a modulation of the immune response via purinergic system that was demonstrated through the reduction of NTPDase activity by degradation of ATP and ADP in lymphocytes of infected rats (Figs. 2 and 3). This, probably maintains higher levels of ATP and ADP, stimulating a more efficient immune response against the parasite, since it induces activation, differentiation and secretion of important mediators in T-lymphocytes such as IL-2 [14]; and cytokines that control the *T. evansi* infection [6,30].

The treatment with curcumin 30 days prior to *T. evansi* infection modulates the proinflammatory cytokines, reducing parasitemia and mortality [16]. In agreement, the present study shows that daily use of curcumin, administered at doses of 20 or 60 mg/kg, as preventive treatment (30 days pre infection) was effective in decreasing parasitemia (Fig. 2). Several evidences reveal that curcumin has an immune modulatory role due to its influence on cytokines, eicosanoids, protolithic enzymes, antioxidants and several molecules involved in cellular inflammation [31].

Related to ADA activity, the pre-treatment with curcumin maintained the enzymatic activity high in both doses (PreI20 and PreI60; $P < .05$), when compared to the untreated infected group (Fig. 4). This enzyme catalyzes the deamination of adenosine in inosine, and closely regulates the extracellular concentration of this nucleoside [32]. Adenosine, which is considered an anti-inflammatory molecule, inhibits lymphocyte activation and diminishes cytokines secretions via A2A receptors. Considering these activities, ADA can increase the deamination of adenosine leading to lower extracellular concentrations of this molecule and this may affect triggering A2A and A2B receptors, and alter inflammation [33]. For this reason, ADA activity in lymphocytes decreases concentrations of extracellular adenosine, which favors an inflammatory response that may control the parasite proliferation.

These data suggest an interaction of curcumin with the purinergic system, leading to a positive effect of curcumin in the control of ADA activation, probably via reduction of adenosine synthesis. Subsequently, curcumin may also regulate the immune response, once herein it prevented increase in proinflammatory cytokines and increased anti-inflammatory cytokine, such as IL-10 [16]. Curcumin reduces and control deleterious effects caused by inflammatory response on hosts. Based on these evidences, we believe that the increase in ADA activity in lymphocytes reduce extracellular adenosine concentrations and this may favors an inflammatory response to control parasite proliferation.

5. Conclusion

The results of the present study highlight that NTPDase and ADA activities were altered in lymphocytes from *T. evansi* infecded rats, suggesting that these alterations play a central role in the inflammatory process of this disease. Oral curcumin pretreatment presents an immunomodulatory effect, preserving NTPDase activity in lymphocytes of *T. evansi* infected rats. We also detected an increase of ADA activity, which favors the immune response against the parasite. This mechanism induces a more efficient inflammatory response favoring parasitemia control.

Conflict of interests

The authors have declared that there is no conflict of interest.

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