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RESEARCH ARTICLE

HARPAGOPHYTUM PROCUMBENS MODULATES EOSINOPHILIC RESPONSE DURING INFECTION BYTOXOCARA CANIS

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ABSTRACT

Humans are infected with *Toxocara. canis* by accidental ingestion of embryonated eggs (larvae) from the environment or contaminated food and water. The mechanisms of molecular and immunological development of the symptomatic syndromes of VLMS are largely unclear. Moreover, the presence of antigen-secreting (TES) eggs in tissue initiates a Th2-type immunological response that is marked by eosinophilia and high levels of IgE. *Harpagophytum procumbens* (Hp) has been used as treatment for a variety of inflammatory illnesses. The present study examined the role of Hp in the recruitment of eosinophils as well as the immune response in a murine model of VLMS. Our results demonstrated that Hp led to persistent lower eosinophilia during *Toxocara canis* infection. The Decrease on the levels of IL-5, IL-4 and IgE was observed after the administration of Hp, suggesting strongly that Hp has therapeutic benefits regarding the eosinophilic inflammation triggered by *Toxocara canis.*

INTRODUCTION

Helminthic infections are potent inducers of Th2- type responses in both humans and experimental models. Th2-type responses are characterized by eosinophilia, high titles of circularizing IgE, enhanced Th2 cytokine profile, and reduced Th1 cytokine profile (Silveira*et al*, 2002; Lawrene 2003). *Toxocara canis* is an intestinal parasite of dogs, and is the etiologic agent of Visceral Larva Migrans Syndrome (VLMS) in humans (Beaver, *et al*1952). Humans are infected by ingesting embryonated eggs which develop into larvae in the gastrointestinal tract and then penetrate through its wall invading multiple organs (ex. lung, liver, kidney, brain, and eye) (Beaver, *et al*1956; Hoffmeister*et al*, 2007).

The presence of antigen-secreting (TES) eggs in the tissues initiates Th2-type immunological response which is marked by both eosinophilia and high levels of IgE (Despommier, 2003; Maizels*et al* 2000). Eosinophilia found in patients with VLMS is part of the host immune defences against the parasite leading to an inflammatory tissue reaction (Beaver, *et al* 1956; Tonelli, 2005). The study of Th1 and Th2 subsets of T helper cells has facilitated an understanding of the host immune response tohelminthic infections. The Th1 immune response releases IL-2 and IL-12 cytokines and IFN-γ that are involved in the delayed type hypersensitivity reaction and in the activation of macrophages (Else andFinkelman, 1998;Ferreira*et al*, 2011).
Th2 type immune response, involves IL-4, IL-5 and IL-13 cytokines release which, among other functions, induces IgE production by B cells and activation of eosinophils, basophils and mast cells (Medeiros*et al*, 2010) which then act as key components in defence mechanisms against helminthic infections, because it modulates the host immune system and have a protective role against the parasite (Machado*et al*, 2004).The *Toxocara canis* larvae in humans have a certain ability to survive in its host for several months, causing stimulation on the T helper 2 (Th2) response and hence the production of IgE and eosinophilia for a long period of time(Medeiros*et al*, 2006). Important disorders such as allergic diseases, asthma and parasitic infections can provide remarkable accumulation of eosinophils(Tunon de Lara and Magnan, 2000).

Natural products have long been used in medicine as alternative treatment for various diseases, including inflammatory processes of various origins. *Harpagophytum procumbens* (Hp) has been used as treatment for a variety of illnesses, including, indigestion, diabetes mellitus, hypertension, fevers,skin cancer, infectious diseases (including tuberculosis), allergies, osteoarthritis, fibrosis and rheumatism, and it is particularly effective in small joint diseases (Van Wyk and Gericke, 2000).*In vitro* studies have evaluated the ability of Hp to inhibit the lipid mediators generated by arachidonic acid metabolism (prostagladin and eicosanoid) (Grant *et al*, 2007).

Other studies demonstrated that Hp extracts were able to suppress the biosynthesis of cysteinyl-leukotrienes (Cys-LT; LTC_4, LTD_4 and LTE_4) and TXB_2. (Tippler*et al* 1997; Loew, 2001). However,no reports on its parasite related anti-

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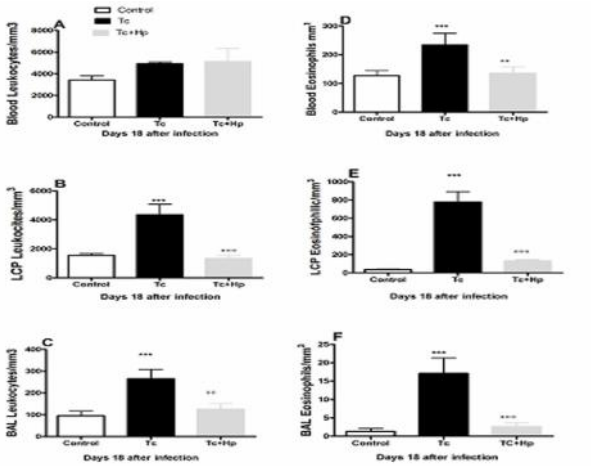
inflammatory capability -have been yet described.The anti-inflammatory effectand the potential for modulation and recruitment of eosinophils that cause systemic eosinophiliaof an ethanolicextractof*H. Procumbens*will be evaluatedduring VLMS, our experimental model.



Graph 1 Counts on the total number of leukocytes in the blood (A), peritoneal cavity fluid (B) bronchoalveolar fluid (C) and eosinophils in the blood (D), peritoneal cavity (E) and bronchoalveolar fluid (F). Data represent mean+/-SEM (n=7 animals) two independent experiments. *** P<0,001 represents significant difference between the results obtained from the groups Tc compared with control group, °°°P< 0.001 and °°P< 0.01 represents significant difference between the results obtained from the Hp+Tc groups compared with Tc group using the test TWO – ANOVA.

MATERIALS AND METHODS

Animals

Female Balb/c mice, weighting between 15 and 18 grams, 4 weeks old, from the animal facilities from the Faculty of Pharmaceutical Sciences in Ribeirão Preto (FCFRP-USP), pathogen free (Specific Pathogen Free - SPF) were used. This project was approved and conducted in accordance with the guidelines established by the Federal University of São Carlos (UFSCar) Animal Care Committee (Protocol n° 046/2009). Animals were divided into 3 groups: control, infected and untreated (Tc) and infected and treated with 100 mg/Kg/animal of *H. Procumbens* ethanolic extract of (Tc + Hp). Infected animals received 500 animal eggs of *T. canis* by gavage. Either kept treated or untreated with *H. procumbens.*

Experimental infection

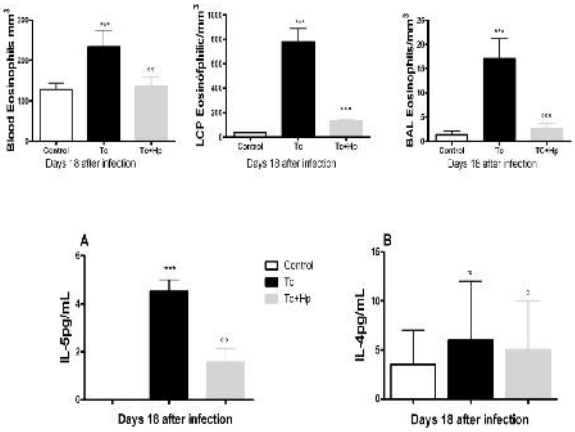
Eggs were obtained from female parasites recovered from young dogs by administration of mineral oil with piperazine hydrochloride. Female *T. canis* were recovered and dissected to extract their wombs, which were the dissolve in Petri dishes containing 2% formalin. Material was the filtrated on gauze to obtain eggs and stored in a formalin solution in a Petri dish, left at room temperature to reach the infective stage (L3). Animals were infected with eggs containing larvae (L3) of *T. canis* (gavage/500 eggs/0.5 ml saline/animal) (Sugane and Oshima, 1982). Animals were euthanized on day 18 post parasite infection.

Preparation of H. Procumbens ethanolic extract

Crude ethanol extract of *Harpagophytum procumbens* (Hp) was prepared at Natural Products Laboratory at the Chemistry Department of the Federal University of São Carlos (DQ-UFSCar), with the guidance of Prof. Dr. Cezar Paulo Vieira. Hp secondary roots maceration was performed using ethanol and water. After that, aliquots of the extract were kept refrigerated at - 20 ° C until use.

Treatment

Animal treatment was administrated using the mean weight of the animals on the group. Aliquots of the extract were kept refrigerated at - 20 ° C, daily treatment was prepared with the diluted extract. Treatment with *H. Procumbens* extract at a 100 mg/Kg/animal dose began after infection and was maintained throughout 18 days. The administration was performed orally by gavage daily. After this period the animals were euthanized and the cells obtained from the peritoneal cavity lavage (LCP), bronchoalveolar fluid (BAL) and whole blood were analysed.



Graph 2 Top panels Eosinophils in the blood, peritoneal cavity fluid (LPC) and bronchoalveolar (BAL).Bottom panels, Evaluation of IL-5 (A) and IL-4 (B) on day 18 post parasite infection in the plasma. Data represent mean±SEM (n=7 animals) of two independent experiments. *** P <0.001. * P<0,05represents significant difference between the results obtained from the Tcgroupscompared with control group and °°P< 0.0,1 ° P < represents significant difference between the results obtained from the Hp+Tc groups compared with Tc group using the test TWO – ANOVA.

Collection of lavage of the peritoneal cavity (LPC), bronchoalveolar fuid(BAL) and Plasma

Blood, LPC and BAL smears were prepared and stained with dye-Panótico (Laborclin). On each slide 100 cells were counted using light microscopy with a final magnification of x 1000. The total number of cells /mm³ in the different compartments was determined using Turk solution to lyse the erythrocytes (Stibbe et al. 1 985) at 1:20 dilution and subsequent counting in a Neubauer chamber, and stained with dye-Panótico (Laborclin). After blood coagulation, plasma was collected and stored at -20°C.

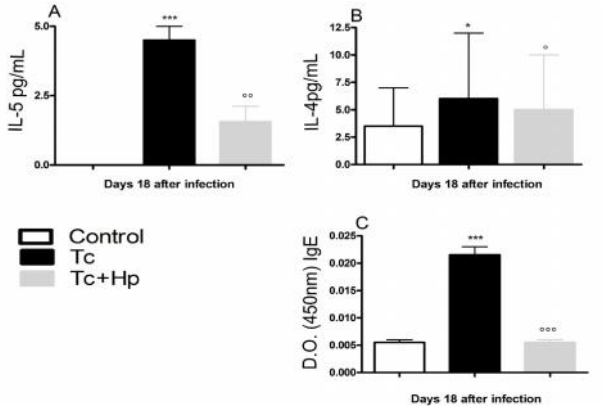
Cytokines analysis in the plasma

ELISA were performed according to the manufacturer instructions (BD) using purified mAb as capture Abs and biotinylatedmAb as developing Abs, followed by incubation with streptavidin-alkaline phosphatase and substrate. Plates were read in a 96-well spectrophotometer (Microquant-Sellex, Inc 450nm) and data was analysed using software by comparison against a standard curve generated using recombinant cytokine at known concentration.

Analysis of anti- T.canis IgE – ELISA

Plasma samples were collected on day 18 post parasite infection and stored at -20°C until its use for IgE analysis. For detection of this immunoglobulin, IgE Kit (BD) was used - plates with 96 wells coated with 200ng of total protein antigen

of *T. canis* (diluted in carbonate buffer and applied 100µl/well) incubated at 4°C overnight.



Graph 3 Top panels evaluation of IL-5 (A) and IL-4 (B) in the plasma. Bottom panels, IgElevels of anti-*T canis* present in serum on day 18 post parasite infection. Data represent mean±SEM (n=7 animals) of two independent experiments. *** P <0.001 represents significant difference between the results obtained from the Tcgroupscompared with control group and °°°P< 0.001 represents significant difference between the results obtained from the Hp+Tc groups compared with Tc group using the test TWO – ANOVA.

Blocking was then performed using 1% BSA in PBS for 60 min at 37°C. 50 ul of each diluted sample (plasma dilution 1:2) in carbonate buffer was added to each well. Subsequently, the plates were washed and 100 mL of biotin-conjugated secondary Ab (4 µl Ac + 10 ml of carbonate buffer) was added. After 1h, the plates were washed and then added 100 ul of enzyme streptavidin (1:200 dilution). The plates were washed and 100 ml of a substrate 1:1 mixture of H_2O_2tetramethylbenzidine (TMB) (BD-OpTEIA) was added. The reaction was stopped by the addition of 50 µL/well of H_2SO_4. Absorbance was read at a wavelength of 450 nm in an ELISA – READER (Microquant – Sellex Inc.).

Statistical analysis

The results were expressed as mean±SEM. The results obtained in different experiments were analysed using ANOVA. Statistical analysis was performed using the PRISM-(TWO-ANOVA) (San Diego, California, USA). The level of significance was 5%.

RESULTS

Eosinophils accumulation after T. canis infection and treatment with H. procumbens

Graph 1 Eosinophils accumulation after *T. canis* infection in blood, LPC and LBA

During the course of *T. canis* infection, an intense inflammatory reaction characterized by an increase in the total number of leukocytes and eosinophils in the blood, LPC and BAL was observed in the infected groups when related to the control group (Figure 1). Our data also demonstrated that *H. procumbens* administration was able to decrease total numbers of eosinophils on Tc+Hp groups when compared to the infected untreated group both for blood, LCP and BAL compartments. (Figure 1D, 1E and 1F).

Cytokines analysis in the plasma

Graph*2* Plasma levels of IL-5 and IL-4 cytokines

Analysis of the serum obtained 18 days after infection revealed a significant increase in plasma IL-5 in infected animals (Tc) when compared to the control group. Additional analysis revealed that IL-5 concentration significantly decreased in the *H. Procumbens*treated group(Tc+Hp) when compared to the infected untreated animals (Figure 2).

Analysis of anti- T.canis IgE – ELISA

Graph*3* Antibody production IgE

The infected (Tc) group showed a significant decrease in the levels of IgE when compared to the group control in the 18th day post infection. Levels of serum IgE showed a significant decrease in treated (Tc+Hp) mice with *H. procumbens* compared to the group only infected (Tc) the 18th day post infection (Figure 3).

DICUSSION

Parasitic infections represent a serious problem for human health (Menezes *et al*, 2008), mainly caused by helminthic infections, which are characterized by long-term infections and can lead to a specific state of morbidity or even death (Maizels and Yasdanbakhsh, 2003). Immune responses during nematode infections include mainly a Th2-response pattern which is characterized by the production of cytokines such as IL-4, IL-5, IL-13 and also regulatory cytokines such as IL-10, resulting in the differentiation of B lymphocytes into serum cells secreting high levels of antibodies (Lawrene,2003; Maizels and Yasdanbakhsh, 2003; Pinelli*et al*, 2007].

There are a wide variety of disorders associated with eosinophilia (Mendes*et al*, 2000). However, in our country this increase is related mainly with intestinal helminthic infections, asthma and various allergic processes (Ponte*et al* 2007). Eosinophilia has been implicated by several authors as one of the most outstanding characteristic of the VLMS in naturally infected human or experimental models (Beaver, *et al*1952; Sugane andOshima, 1994; Meeusen andBalic, 2000). VLMS eosinophilia peaks on day 18 post parasite infection and has been correlated with the production of IL-5 by Th2 CD4+ lymphocytes in the lungs as well as with elevated production of IgE (Faccioli*et al*,1996; Anibal*et al*, 2007).There is no proven effective treatment for VLMS, although anthelmintic-drugs such as diethylcarbamazine, ivermectin, thiabendazole, mebendazole, and albendazolehave been used, these being sometimes associated with an antihistamine (Cunha, 2005).

Plant-derived compounds are sources of anti-inflammatory agents. *Harpagophytum procumbens* has been used as an analgesic, as a medication for fevers and allergies and by San bushmen in Africa to stimulate gastric enzymes and digestion (Chrubasik*et al*, 2000*)*, and it has been used traditionally for centuries by indigenous people for treating a range of conditions (Wegener andLupke, 2003). Other studies demonstrated the anti-inflammatory effect of *Harpagophytum procumbens* in various disorders such as rheumatic diseases, diabetes, atherosclerosis, malaria, indigestion, and fever among others (Lanhers*et al*, 1992; Andersen*et al*, 2004*;* Clarkson*et al*, 2006). Here, we have investigated the effect of *H. procumbens* administration in mice infected with *T. canis*.

Similar to previous data (Faccioli*et al*. 1996, Anibal *et al*. 2007, D. Carlos *et al*, 2011) our findings demonstrated an increased numbers of eosinophils in blood (1D), LCP (1E) and BAL (1F) in infected animals when compared to the control group. Eosinophilia have long beenrecognized as a

characteristic feature of helminthic infections (Ponte*et al*, 2007).

The larvae of *T. canis* have an allergenic fraction responsible for the stimulation of eosinophils, which explains the eosinophilia (Pellou*xat al*, 2004). In our results the *H. procumbens* treatment was able to inhibit eosinophilia in the blood, LCP and BAL. Given these data our next step was to investigate whetherHP treatment was able to reduce interleukin-5 (IL-5) that has been correlated with an increased eosinophilia. From our results, we can say that infected mice showed a significant increase in the plasma IL-5 concentrations when compared with the control group (Graph 2A). Thereby, eosinophilia peaks are correlated with production of IL-5 in this experimental infection. However, the role of IL-5 on eosinophilia has been demonstrated in several parasitic and allergic models. Experiments using anti-IL-5 or mice lacking IL-5 gene show that eosinophilia is IL-5-dependent.(Nakajima*al et*, 1996; Hamelmann*et al*, 2000).

H. procumbens treatment was able to significantly reduce the concentrations of IL-5 in animals receiving treatment when compared with those infected and untreated (Graph 2A). Thus we suggest that the reduction in eosinophilia in the compartments LBA, LCP and blood, observed after HP treatment could be explained because IL-5, a cytokine produced by CD4 + T cells, plays an important role in the inflammatory response observed during the Th2 immune response.In addition to eosinophilic inflammation and a shift in the cytokine pattern toward a Th2 profile, one of the clinical signs seen during VLMS is an increment in the production of antibodies specific for *T. canis* antigens. IL-4 is a cytokine essential for development of a Th2 pattern response (Bacharier and Geha,2000).

We found, however, that treatment with *H. procumbens* generally reduce IL-4 in serum, on day 18 post *T. Canis* infection using Balb/c mice treated with *H. procumbens* (Graph 2B) when compared to infected untreated mice. These findings are consistent with other studies, which report the prevalence of these cytokines in this model and that specify a pattern of Th2-type response as characteristic in VLMS (Buij*set al*, 1997; Altcheh*et al* 2003).

Among others, *Toxocara canis* is one of the most commonly parasites associated with increased levels of IgE, a proposed mechanism to explain the increase in total IgE levels is the secretion by parasites of factors that stimulate the production of IL-4 leads to an increased levels of IgE (Sorensen *et al*, 2006). By analysing the pattern of specific antibodies in serum from animals infected with *T. canis*, an increase of IgE antibody was found when compared with infected animals treated with *H. procumbens*. IgE levels in the infected mice were higher than those in the control group (Graph 3). Ours findings also clearly suggests that the administration of *H. procumbens* inhibit production of IgE on day 18 post parasite infection. IgE immunoglobulin plays a central role in the pathogenesis of immediate hypersensitivity reactions due to its ability to bind specifically to receptors with high (FceRI) and low activity (FceRII-CD23) present on various cells such as mast cells, basophils, B lymphocytes and T and activated eosinophils with the objective of expanding cellular and humoral immune responses in allergic reactions (Delespesse*et al* 1992; Turner and Kinet, 1999; Mutizani *et al*, 2012). The increase in total IgE levels might be explained by the fact that factors that stimulateIL-4 also increases the level of IgE production (Pinelli and Aranzamendi, 2012). This finding is in agreement with others that report *Toxocara canis* as one of the most commonly parasites associated with IgE increased levels (Mendonça *et al*, 2012). Thus, our results corroborates previous studies, which report that IL-4 acts in the regulation of the immune response by acting on the induction of IgE (Mutizani *et al*, 2012;Zavadniak and Rosário, 2005], because in our study concentrations of IL-4 (2B) are reduced in the same period in which it was possible to observe the reduction in serum levels of IgE in animals that were treated with *H. procumbens* (Graph 3).

In conclusion, our findings suggested an early blockage on IL-5, IL-4 and specific IgE productionapparently in the effective on subsequent blockage of eosinophilic inflammation. So, given the above, we suggest that the extract of *H. procumbens* presented an anti-inflammatory effect in the model of VLMS. However, further investigations will be required to contribute to the understanding of the mechanisms by which the extract of *H. procumbens* can interfere with the migration of eosinophils, production of cytokines and antibodies in this model, since this event is characterized by factors of order complex and multifactorial.

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