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The effects of increased heme oxygenase-1 on the lymphoproliferative response in dogs with visceral leishmaniasis

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ABSTRACT

Canine visceral leishmaniasis (CVL) is known to affect the cellular immunity of infected dogs, through impairing lymphoproliferation and microbicidal mechanisms. This study examined heme oxygenase-1 (HO-1) and its metabolites, oxidative stress and IL-10 levels in CVL and investigated correlations between these parameters. Additionally, the effects of HO-1 inhibition on the lymphoproliferative response and cytokine production in lymph node cells (LNCs) from infected dogs were evaluated. Forty-four dogs, 24 controls and 20 dogs with CVL were selected. Plasma and splenic levels of HO-1, haptoglobin, soluble CD163 receptor, ferritin and IL-10 were determined using capture ELISA. The HO-1 levels and relative gene expression in peripheral blood and bone marrow mononuclear cells were also determined. LNCs proliferation was evaluated with an HO-1 activator and with an HO-1 inhibitor, in the presence of the *Leishmania infantum* soluble antigen (SAgL), using flow cytometry. HO-1, IL-2, IFN-gamma and IL-10 were also determined in these cultures using capture ELISA. Infected dogs presented oxidative stress and increased HO-1 levels and relative gene expression, with correlation between oxidative stress and HO-1. The substances from heme metabolism and IL-10 were also elevated in the plasma and spleens of infected dogs. IL-10 and HO-1 levels were positively correlated with one another. Inhibition of HO-1 increased LNCs proliferation and decreased IL-10 and IL-2 production in the presence of SAgL. The increased HO-1 metabolism observed in CVL is probably associated with oxidative stress and increased IL-10, which could be one of the mechanisms responsible for inhibition of the lymphoproliferative response in sick dogs.

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMMC, bone marrow mononuclear cells; CBC, complete blood count; CFSE, carboxyfluorescein diacetate succinimidyl ester; CK, creatine kinase; CO, carbon monoxide; ConA, concanavalin-A; CoPP, cobaltic protoporphyrin IX chloride; CPDA-1, citrate-phosphate-dextrose-adenine-1; CVL, canine visceral leishmaniasis; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; GGT, gamma-glutamyl transferase; HO-1, heme oxygenase-1; IL, interleukin; LDH, lactate dehydrogenase; LNCs, lymph node cells; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffer solution; PCV, packed cell volume; PHA-M, phytohemagglutinin-M; qPCR, real-time polymerase chain reaction; qRT-PCR, real-time reverse transcription-polymerase chain reaction; RBC, red blood cells; ROS, reactive oxygen species; RT, reverse transcription; SAgL, soluble antigen of *Leishmania infantum*; sCD163, soluble CD163 receptor; SnMsP, Sn(IV) mesoporphyrin IX dichloride; TAC, total antioxidant capacity; TBARS, thiobarbituric acid reactive substances; TOC, total oxidant capacity; TPP, total plasma protein; UPC, urinary protein/creatinine ratio; VL, visceral leishmaniasis; WBC, white blood cells.

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1. Introduction

Visceral leishmaniasis (VL) is a parasitic zoonosis that is widely distributed throughout the world and is among the six most important tropical diseases according to the World Health Organization (WHO, 2015). Over recent years, increases in the incidence of VL have been reported in Brazil (Alvar et al., 2012). The increased incidence of human cases appears to be associated with increases in canine infections (Coura-Vital et al., 2011; Nunes et al., 2010) because dogs are the primary source of this disease in urban areas (Gramiccia and Gradoni, 2005).

This dog-susceptible disease is characterized by skin lesions, generalized lymphadenopathy, progressive weight loss with muscle atrophy and weakness, exercise intolerance, decreased appetite, lethargy, splenomegaly, polyuria and polydipsia, eye lesions, epistaxis, onychogryphosis, vomiting and diarrhea (Solano-Gallego et al., 2009).

It has been accepted that susceptibility is associated with an exacerbated humoral immune response (Th2) and a reduced cellular immune response (Th1) (Alvar et al., 2004; Baneth et al., 2008). Increased production of IL-10, a major Th2 cytokine, is associated with progression of the canine infection (Boggiatto et al., 2010), while higher expression of IFN-gamma, a Th1 cytokine, is correlated with a lower blood parasite load (Solano-Gallego et al., 2016).

Recently, it has been suggested that oxidative stress in infected dogs presenting clinical signs of canine visceral leishmaniasis (CVL) is associated with increased production of reactive oxygen species (ROS), which may be a critical mechanism of the pathogenesis of this disease (Almeida et al., 2013b; Bildik et al., 2004; Heidarpour et al., 2012). This increased ROS production has been demonstrated in human macrophages (Luz et al., 2012) and canine neutrophils (Almeida et al., 2013a,b).

The mechanisms associated with CVL susceptibility are not well-understood. Information regarding CVL disease pathogenicity is fundamental for future treatments for dogs. Involvement of heme oxygenase-1 (HO-1) in human VL susceptibility has been reported (Das et al., 2013; Luz et al., 2012). HO-1 is responsible for degrading the heme in hemoglobin into iron, carbon monoxide and biliverdin, which is rapidly converted into bilirubin (Tenhunen et al., 1969). Under physiological conditions, hemoglobin from erythrocyte destruction binds with haptoglobin and this complex is then internalized by macrophages via the CD163 receptor. Inside the macrophage, heme is metabolized by HO-1 and iron stimulates ferritin production (Kristiansen et al., 2001).

Human patients with clinical VL have been found to present increased HO-1 levels, which then decreased after disease treatment. This clinical finding demonstrates the involvement of HO-1 in VL susceptibility (Das et al., 2013; Luz et al., 2012). Furthermore, both the lipophosphoglycan and promastigote parasite forms of *Leishmania* spp. are responsible for increasing HO-1 expression in macrophages, resulting in the persistence of the infection in humans (Luz et al., 2012). Because heme is necessary for complete NADPH oxidase activation, the underlying mechanism causing persistence of this macrophage infection is possibly associated with heme degradation caused by HO-1, which prevents complete activation of NADPH oxidase. Thus, ROS production by infected macrophages is impaired, and this allows the parasite to survive inside the host cell (Pham et al., 2005).

HO-1 also presents immunoregulatory functions, mainly those associated with lymphocytes, by inhibiting activation and proliferation of T lymphocytes and stimulating apoptosis in these cells (Pae et al., 2004). To date, the involvement of HO-1 in the lymphoproliferative response of dogs with CVL has not been studied. However, it is known that infected dogs with clinical signs of CVL exhibit low lymphocyte proliferative responses (Pinelli et al., 1994). The mechanisms involved in this impaired cell response may be related to increased T lymphocyte apoptosis (Chiku et al., 2016; Lima et al., 2012; Perosso et al., 2014). Therefore, gaining an understanding of the mechanisms that improve the lymphoproliferative response in sick dogs is critical for development of treatments and elimination of this parasite in dogs.

For the present study, it was assumed that HO-1 production is activated under stress conditions, that oxidative stress occurs in CVL and that anemia is a common finding in relation to this condition. Hence, this study aimed to evaluate HO-1 levels and relative gene expression, along with HO-1 metabolites, in infected dogs and to determine the correlations between HO-1, oxidative stress markers, IL-10 levels and parasite load. Additionally, we evaluated the regulatory role of HO-1 relating to the antigen-specific proliferative response of lymph node cells from infected dogs and determined the effects of HO-1 activation and inhibition on the lymphoproliferative response and on IL-10, IL-2 and IFN-gamma production.

2. Material and methods

2.1. Ethics approval

This study was approved by the Ethics Committee for Animal Experimental Research (Comitê de Ética em Pesquisa Experimental Animal, COBEA) and the Ethics Committee for Animal Use (Comitê de Ética no Uso Animal, CEUA) of the School of Veterinary Medicine (Faculdade de Medicina Veterinária, FMVA) at the Araçatuba Campus of São Paulo State University (Universidade Estadual Paulista "Júlio de Mesquita Filho", Unesp), under procedural number FOA-00532-2013. The participation of dogs in the control group was authorized by their keepers, who provided free and informed consent.

2.2. Dog selection

The control group consisted of 24 dogs of various breeds (10 males and 14 females; average age of 4 ± 2 years), which were recruited in the city of Araçatuba. The keepers of these dogs authorized their participation in the experiment. All the dogs were clinically healthy with normal physical examinations. These dogs presented normal hematological, biochemical and urinary profiles, and negative indirect ELISA reactions (Lima et al., 2003) for the *L. infantum* antigen obtained within the last three months before the experiment. There was no *Leishmania* DNA amplification (Perosso et al., 2014) in the spleen or bone marrow samples taken at the time of the experiment.

The infected group consisted of 20 dogs (10 males and 10 females; average age of 5 ± 2 years) of varying breeds that were naturally infected with *Leishmania* spp. These dogs were acquired from the Zoonosis Control Center of Araçatuba. General clinical examinations, complete blood counts, plasma biochemistry analyses and urinalyses were performed on these dogs to stage the disease in accordance with the LeishVet Consensus (Solano-Gallego et al., 2009). All the animals were reactive to total crude antigen of *L. infantum* through indirect ELISA (Lima et al., 2003) and presented positive *Leishmania* DNA amplification (Perosso et al., 2014) in spleen and bone marrow samples.

2.3. Sample collection and laboratory analysis

Prior to blood collection, the dogs were kept fasting for 8–12 h. Twenty-one milliliters of blood were extracted via jugular venipuncture; 20 mL was placed in heparinized tubes (BD Vacutainer®, Franklin Lakes, NJ, USA) and 1 mL of ethylenediaminetetraacetic acid (EDTA)-treated blood was used to determine the complete blood count (CBC).