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Apoptosis, inflammatory response and parasite load in skin of *Leishmania (Leishmania) chagasi* naturally infected dogs: A histomorphometric analysis

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ABSTRACT

The skin has an important role in infection by *Leishmania chagasi*. Apoptosis modulates the inflammatory response acting distinctively either on the progression or regression of the lesions. The parasites interact with multiple regulatory systems inducing apoptosis in host cells, during cell invasion, stabilization and multiplication of pathogens. In this context, the aim of this study was to evaluate cell death within the inflammatory infiltrates, and to correlate these results with parasite load and clinical features of dogs naturally infected with *L. chagasi*. Fragments of skin pinnas (8 symptomatic + 8 asymptomatic + 6 negative controls) were used to characterize and measure the inflammatory response, parasite load and apoptosis. Diagnosis of canine leishmaniasis was confirmed by the detection of *anti-Leishmania* antibodies by IFA and ELISA in serum, direct visualization of the parasite and culture in spleen, liver, pinna, bone marrow and lymph nodes, and PCR (pinna). Histomorphometry was performed with images obtained from 20 representative histological fields in a light microscope. Ultra-thin sections were mounted over a 300 mesh grids, contrasted with 2% uranyl acetate and lead citrate and examined under a Transmission Electronic Microscopy. Amastigotes were only found in the skin of symptomatic animals (31.94 ± 18.81). The number of foci and cellularity of the inflammatory infiltrates in symptomatic dogs were higher than in other groups and in asymptomatics were higher than in controls ($p < 0.05$; Tukey). The average area, perimeter and extreme diameters of the inflammatory infiltrates obtained in symptomatic dogs were higher than in controls ($p < 0.05$; Tukey). The apoptotic index was higher in symptomatic than in other groups and there was no difference between asymptomatics and controls ($p < 0.05$; Tukey). Ultrastructurally, apoptotic cells were shrunken, with condensed nuclear chromatin and cytoplasm. Condensed nuclei were frequently fragmented. Internucleosomal DNA fragmentation occurred only in symptomatic cases. Amastigotes were observed within neutrophils and macrophages. Apoptosis is directly related to parasite load, intensity of inflammatory response and clinical manifestations in *L. chagasi* naturally infected dogs.

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1. Introduction

Visceral leishmaniasis (VL) is an endemic zoonosis caused, in Brazil, by the *Leishmania chagasi*, similar to *Leishmania infantum* (Mauricio et al., 2000). It is transmitted by the phlebotomine sand fly *Lutzomyia longipalpis* (Deane and Deane, 1955; Lanzaro and Warburg, 1995). Dogs are the main domestic and peridomestic reservoir host for human visceral leishmaniasis in endemic foci of zoonotic leishmaniasis (Gramiccia and Gradoni, 2005; Baneth et al., 2008).

The diagnosis of VL in endemic areas is not always an easy task. Several current methods present low sensitivity and/or specificity rates (Reed, 1996). Even though it is not widely used, the PCR technique is a valuable tool, eliminating false negative results, especially in scientific research. In these cases, the PCR technique is indispensable for showing the absence of contact between animal and parasite (Solano-Gallego et al., 2001).

Apoptosis is a mechanism of regulated elimination of cells (Kerr, 1993) which takes part in the evolution of lesions triggered by several microorganisms, including *Leishmania* sp. (Moreira et al., 1996; Das et al., 2001; Lee et al., 2002). It participates actively in modulation of the inflammatory response (Weinrauch and Zychlinsky, 1999; Carrero et al., 2004). The resolution of inflammation is characterized by large numbers of cells in apoptosis within the inflammatory sites (Fadok et al., 1998; Huynh et al., 2002; Maderna and Godson, 2003; Eda et al., 2004).

Apoptosis modulates distinctively the progression (Das et al., 1999) or regression (Conceição-Silva et al., 1998; Huang et al., 1998) of the lesions caused by *Leishmania* sp. The parasites interact with multiple regulatory systems inducing apoptosis in host cells, during the cell invasion, stabilization and multiplication of pathogens (Carmen and Sinai, 2007). Furthermore, apoptosis also occur in other cellular elements and even on the own parasite (Lindoso et al., 2004), as a form of population control or due to nutritional restrictions (Welburn and Maudlin, 1997; Knight, 2002).

When the infected phlebotomine bites the vertebrate host, both apoptotic and viable forms of promastigotes are inoculated into the skin. Being located in the phlebotomine' superior part of the digestive tract, the apoptotic promastigotes are the first cells to be inoculated (Wanderley et al., 2009). Apoptotic promastigotes dysfunction the leishmanicidal activity of the host cells, increasing the parasite's virulence (Van Zandbergen et al., 2006) and contributing to the survival of viable parasites (Wanderley et al., 2009). Infection causes tissue irritation, recruiting neutrophils to that location, which recognize and phagocytize both apoptotic and viable promastigotes. The infection of these cells increases the levels of macrophage inflammatory protein-1β which recruits macrophages and performs phagocytosis of apoptotic polymorphonuclear (PMN) neutrophils containing several viable forms of *L. chagasi* (Van Zandbergen et al., 2004).

In this context, the aim of this study was to evaluate the cell death in inflammatory infiltrates, its cellularity and number of foci, and to correlate these results with parasite load and clinical features of dogs naturally infected with *L. chagasi*. To our knowledge, this is the first morphometrical approach of inflammation and the first report of occurrence of apoptosis in inflammatory cells *in vivo* involving natural infection with *L. (L.) chagasi*.

2. Materials and methods

2.1. Animals and diagnosis of visceral leishmaniasis

A total of 16 positive and six negative-tested dogs previously examined for VL were used. Macroscopic skin lesions due to secondary infections in the pinna region were considered as criteria of exclusion. To confirm *L. chagasi* infection, blood samples were taken to detect *anti-Leishmania* antibodies by IFA and ELISA and needle aspiration of the popliteal lymph node and bone marrow was performed in each dog, to direct visualization of the parasite and culture. Once confirmed the infection, they were euthanatized and submitted to necropsy for sample collection. Before fixation of the samples (spleen, liver, skin and lymph nodes), imprints of the cut surface on cleaned slides were taken to direct visualization of the parasite and confirm visceralization of the infection. Myelograms and imprints of popliteal lymph nodes, spleen, liver and skin were stained with Giemsa, for parasitological visualization (Mikel, 1994). Aspirates from spleen, liver, bone marrow and lymph node were also cultured for promastigotes in NNN-phase Schneider's liquid medium. Polymerase Chain Reaction was performed to detect parasites only in pinna skin extracted DNA, using a target sequence of *Leishmania donovani* complex.

Anti-*Leishmania* antibodies were detected in all infected animals, the titers ranging from 1:40 through 1:640. All infected animals (symptomatic and asymptomatic) were positive in PCR and at least two of the three parasitological tests (Giemsa, culture and immunohistochemistry) in different organs. Animals regarded as non-infected controls had negative results in all tests, including PCR.

Efforts were made to avoid all unnecessary distress to the animals. Housing, anesthesia and all procedures concurred with the guidelines established by our local Institutional Animal Care and Use Committee that also reviewed and approved this work (*CETEA, Universidade Federal de Minas Gerais*, protocol n° 198/2007, approved on 03/27/08).

2.2. Experimental design

Eight VL-positive dogs (by serological and parasitological analysis) were used in this experiment, with the exception of the control group. Animals were divided into three groups: (a) Eight VL-positive animals with clinical signs of the disease; (b) eight positive animals, with no clinical signs; and (c) six VL-negative control animals. Standards used to group the animals followed the Pozio et al. (1981) classification.

2.3. Collecting and processing post-mortem samples:

The animals were tranquilized with Acepromazine 1%, anesthetized with Sodium thiopental 2.5%. After this procedure, the animals were euthanatized with an overdose of sodium thiopental 7.5% (75 mg/kg) for further pos-mortem