BJVP
Brazilian Journal of Veterinary Pathology

Original Full Paper

Choroid plexus involvement in dogs with spontaneous visceral leishmaniasis: a histopathological investigation

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Submitted April 27th 2009, Accepted July 8th 2009

Abstract

Visceral leishmaniasis is associated with chronic inflammatory stimulation, resulting in different clinical manifestations, ranging from unapparent infection to a systemic disease. In dogs, there are descriptions of neurological involvement with inflammatory infiltrates and specific antibodies in cerebrospinal fluid. To investigate the involvement of the choroid plexus and the blood-CSF barrier during the infection, we describe the morphological alterations in the choroid plexi of dogs naturally infected by *Leishmania* (*Leishmania*) *chagasi*. A total of 44 mixed-breed adult dogs were selected from the Veterinary Hospital of UNESP-Araçatuba and from the Zoonosis Control Center in Araçatuba, São Paulo State, Brazil. This area is endemic for canine visceral leishmaniasis. Routine ELISA tests and cytological examination of a tissue smear of the popliteous lymph node were proceeded to diagnose *Leishmania* infection. During the necroscopic examination, the brain was collected and samples containing the choroid plexi were stored in 10% buffered formalin and subjected to histological procedures, following staining with haematoxylin–eosin, Congo red and Masson's trichromic. The dogs were classified in three experimental groups: symptomatic dogs (n=11), oligosymptomatic dogs (n=21) and uninfected dogs (n=12). The choroid plexi of symptomatic and oligosymptomatic dogs presented a chronic inflammatory reaction with lymphoplasmacytic infiltrate in higher intensity than the control dogs (P=0.0044). These findings give additional support to confirm that the choroid plexus is an important mediator between periphery and the brain, and that the choroid plexus acts as pathway to initiate an inflammatory process within the nervous tissue in visceral leishmaniasis.

Key Words: Blood-cerebrospinal fluid barrier, central nervous system, choroid plexus, inflammation, *Leishmania chagasi*

Introduction

In dogs, an important and systemic disease, characterized by chronic and immunomediated stimuli is visceral leishmaniasis (VL), caused by the parasitic protozoan *Leishmania* (*Leishmania*) *chagasi* (syn. *L. infantum*, 19). There are three distinct clinical manifestations of VL in dogs: asymptomatic; oligosymptomatic form, characterized by nonspecific symptoms such as fever, diarrhea, discrete anemia; and symptomatic form, featured by alopecia, onycogryphosis, progressive weight loss, hepatomegaly, splenomegaly, hypergammaglobulinemia, generalized lymphadenopathy, cutaneous lesions, hemorrhagic diathesis and, disorders in locomotor, renal, ophthalmologic, and nervous systems (3, 6, 8, 9, 16, 21, 22, 23, 33).

The symptoms of VL are related with the immune response involving T-lymphocytes (17, 28). The response effectuated by $CD4^+$ Th1 cells and mediated by IL-2, IFN-γ and, TNF-α prevail in asymptomatic dogs, apparently resistant to the disease. However, the mechanisms of $CD4^+$ Th2 cells response in symptomatic dogs are still unclear, and cytokines such as IL-4, IL-10 and, TGF-β could be

related with the progressive character of the disease (1, 28).

There are few reports regarding neurological symptoms in dogs with VL. Feitosa *et al*., (7) and Ikeda *et al*., (12) reported signs of generalized central involvement, with occurrence of seizures, visual disorders, cranial nerves alterations, vestibular and cerebellar signs, motor incoordination, tetraparesy and tetraplegy, myoclonies and, vocalization. The most frequent histopathological findings in the central nervous system (CNS) of these dogs, including animals without neurological symptoms, were described as leptomeningitis, choroiditis, satellitosis, neuronophagia, gliosis, perivascular lymphoplasmacytic infiltrate, vascular congestion and presence of hemorrhages.

Classically considered as an immunologically privileged site, we currently know that the CNS is target of immunosurveillance (11, 25), even though it contains particularities capable to modulate the inflammatory process.

An important epithelial barrier in the CNS is the blood-cerebrospinal fluid (CSF) barrier, constituted by the choroid plexus (CP) epithelium. Herein, the blood-brain-barrier (BBB) lacks, and the blood-CSF barrier avoids the direct contact between blood and CSF, nevertheless the access of blood molecules and cells to choroidal stroma occurs (32, 36). Recently, the role of the CP as a mediator between the CNS and the periphery has been focus of researches. CP is more responsive to a systemic immune activation than the encephalon, showing up-regulation of pro-inflammatory cytokines gene expression such as IL-1β and TNF-α (18) and adhesion molecules on vascular endothelium (13, 26). Due to these characteristics, CP could be an important pathway for leukocytes and pathogens migration from blood to nervous tissue. Bacterias, parasites and virus such as *Neisseria meningitidis*, *Trypanossoma brucei*, Sendai virus, Measles virus, AIDS (HIV-1) and leukemia (HTLV-1) viruses present tropism for the CP (14, 34).

In view of the probable disruption of the encephalic barriers due to chronic immune stimulation in dogs with VL, we investigated the compromising of the CP and the blood-CSF barrier during the infection and we describe the morphological alterations in the CP of dogs naturally infected by *L. (L.) chagasi*.

Material and Methods

Animals

A total of 44 mixed-breed adult dogs were selected from the Veterinary Hospital of UNESP-Araçatuba and the Zoonosis Control Center in Araçatuba, São Paulo State, Brazil, which is an endemic area for canine VL. Euthanasia was requested by owners in compliance with the State Law instructions. Blood samples were collected before the dogs were euthanized with an overdose of pentobarbital (Nembutal®) and potassium chloride. Necropsies were performed immediately after euthanasia, and macroscopic lesions were recorded. None of these dogs presented neurological signs.

Routine ELISA tests and cytological examination of a tissue smear of the popliteous lymph node were performed to diagnose *Leishmania* infection. Immunofluorescence tests were also conducted to exclude other infections that could result in CNS inflammation, such as toxoplasmosis and neosporosis.

In this study, 11 dogs were enrolled into the group of symptomatic infected dogs (SD), 21 animals were enrolled into the group of oligosymptomatic infected dogs (OD), and 12 animals were classified as uninfected dogs (UD) and enrolled as control group.

Histopathology

The brain was sagitally sectioned after removal and the right hemisphere was fixed in 10% neutral buffered formalin. Tissue samples containing lateral and fourth ventricular choroid plexi were paraffin-embedded, sectioned (5 μm) and, stained with Haematoxylin and Eosin (HE), Congo red and, Masson's trichromic using routine protocols.

The presence and distribution of the inflammatory infiltrate were scored semiquantitatively in HE-stained tissue on a four-point scale of 0-3. A score of 0 represented absence of inflammatory cells; a score of 1, discrete, characterized focal cellular groups; 2, moderate, represented diffuse or multifocal inflammatory infiltrates; and, a score of 3, intense, described high inflammatory cellularity, resulting in evident morphological alteration of the choroid plexus' connective tissue, characterizing a choroiditis. Scoring was attributed without knowledge of the experimental groups.

Statistical analysis

Significant differences among groups were determined by Kruskal-Wallis test followed by Dunn's multiple comparison test. Chi-square test was used to check the association between the intensity of inflammation in symptomatic and oligosymptomatic dogs. A value of $P<0.05$ was considered statistically significant. Data are expressed as median values (minimum-maximum). Statistical analyses were performed using Prism software (GraphPad, California, USA).

Ethical issue

This study was approved by the institutional Ethics and Animal Welfare Committee (CEEA – Comissão de Ética e Experimentação Animal, UNESP, process number 05/06).

Results

Remarkable lesions were observed in choroid plexi of symptomatic and oligosymptomatic infected dogs but not in the uninfected dogs. We detected the presence of infiltration of inflammatory cells with lymphoplasmacytic morphology around blood vessels and also diffusely in choroidal stroma (Figure 1C, 1E). Both SD and OD groups presented the median score of 1 (0-3) while UD group exhibited the median score of 0 (0-2). Inflammatory infiltrate was more intense in symptomatic and oligosymptomatic dogs (Figure 2), when compared with control group (Kruskal-Wallis test: P=0.0044). SD and OD exhibited no difference regarding the intensity of inflammation. Furthermore, there was no significant association between SD and OD regarding the intensity of the inflammation in the CP (Chi-square test: P=0.6853).

We also detected deposits of hyaline substance around blood vessels, associated or not to inflammatory infiltrate (Figure 1B) in 12 dogs, four (36.4%) from SD, five (23.8%) from OD, and three (25.0%) from UD group. Congo red and Masson's trichromic stains were used to identify whether the hyaline deposits were constituted by amyloid substance or collagen fibers, respectively. None of the choroid plexi exhibited congophilic fibers (Figure 1D), indicating no choroid plexus amyloidosis. Analysis of Masson's trichromic-stained tissue revealed deposition of diffuse collagen fibers in the choroidal stroma and around blood vessels (Figure 1F) in all of the 12 dogs.

Furthermore, amastigotes forms of *Leishmania*, inside cells or free in choroidal stroma, were not found in HE-stained tissue at magnification of 100x.

Discussion

We detected a significative morphological difference comparing the choroid plexus of normal and of *Leishmania* infected dogs, even if we had not found the parasites. The high intensity of the inflammatory infiltrate in the CP of the infected groups may possibly indicate that the CP acts as a pathway for inflammatory cells gaining access to the CNS during *Leishmania* infection, suggestive of blood-CSF barrier disruption. On the other hand, there was no significant difference between SD and OD groups regarding the intensity of the inflammation in the CP. This could be expected once it is not representative of the lesions in the nervous tissues.

Due to absence of the BBB, CP cells are more exposed to the stimulation of peripherical cytokines and in these areas also exists higher concentration of cytokine-responsive cells (27). Therefore, under VL infection, certain leukocyte population and inflammatory mediators migrate into the ventricles through this route. In a recent work, Melo *et al.*, (20) describe the presence of great number of $CD3^+$ T lymphocytes in the brain of *L. chagasi* infected dogs and, according to Petito & Adkins (26), the CP acts as a source of T cells, which can reach adjacent brain areas or the CSF.

Moreover, there are also evidences of disruption of the blood-CSF barrier's selective filtration in leishmaniasis, caused by the peripherical inflammatory processes. Lima *et al.*, (15) and Melo *et al.*, (20) report the expressive increase of anti-*Leishmania* antibodies titers in the CSF of dogs with VL, presenting also, strong correlation with the serum anti-*Leishmania* antibodies titers. The presence of lymphocytes and plasma cells in the CP could trigger a local inflammatory process, changing the blood-CSF barrier permeability.

In this study the parasite was not detected in the CP of the dogs. These animals did not receive any treatment and they were euthanized prematurely, as soon as *Leishmania* infection was diagnosed. The reports that confirm the presence of the parasite within the CNS of naturally infected dogs comes from the Mediterranean basin, mainly from Spain (22, 37), region where the treatment to VL is recommended (4, 5, 10, 24), promoting the chronification of the disease, with more intense parasite dissemination, perhaps via macrophages as described for others infectious agents (14, 34).

We cannot explain the morphological changes in CP by means of a direct response to the presence of parasite in the tissue. However, the presence of *Leishmania* circulating antigens was already detected in the CSF and in the choroidal stroma (9). The presence of the recent described pan-antigens in VL (29, 30) would activate a peripherical inflammatory response, independent on the presence of the parasite in the nervous milieu. Activated leucocytes should accumulate in the CP before immigrate to the CSF and periventricular encephalic areas.

Even though the animals exhibited different patterns of clinical signs, we did not find difference between the morphological alterations in the CP of symptomatic and oligosymptomatic dogs, suggesting that the inflammatory response in the CNS is associated with VL, although not related with the worsening of the clinical signs, in agreement with Melo *et al*., (20) who did not find variation in symptomatic and asymptomatic dogs, regarding both the intensity of the inflammation and the inflammatory cells population.

The amyloid substance deposition could be a secondary alteration to a chronic inflammatory stimulus, such as VL, and, in the CP is related with Alzheimer's disease and neoplasias (2, 31, 35). Garcia-Alonso *et al*., (9), detected amyloid deposition in the CP of three *L. infantum* naturally infected dogs, which presented cutaneous and visceral lesions. In this study, we did not detect amyloidosis in the CP of any dog, maybe because the animals had developed only the initial stage of the infection.

The presence of morphological alterations in the CP gives additional support to the idea that *Leishmania*

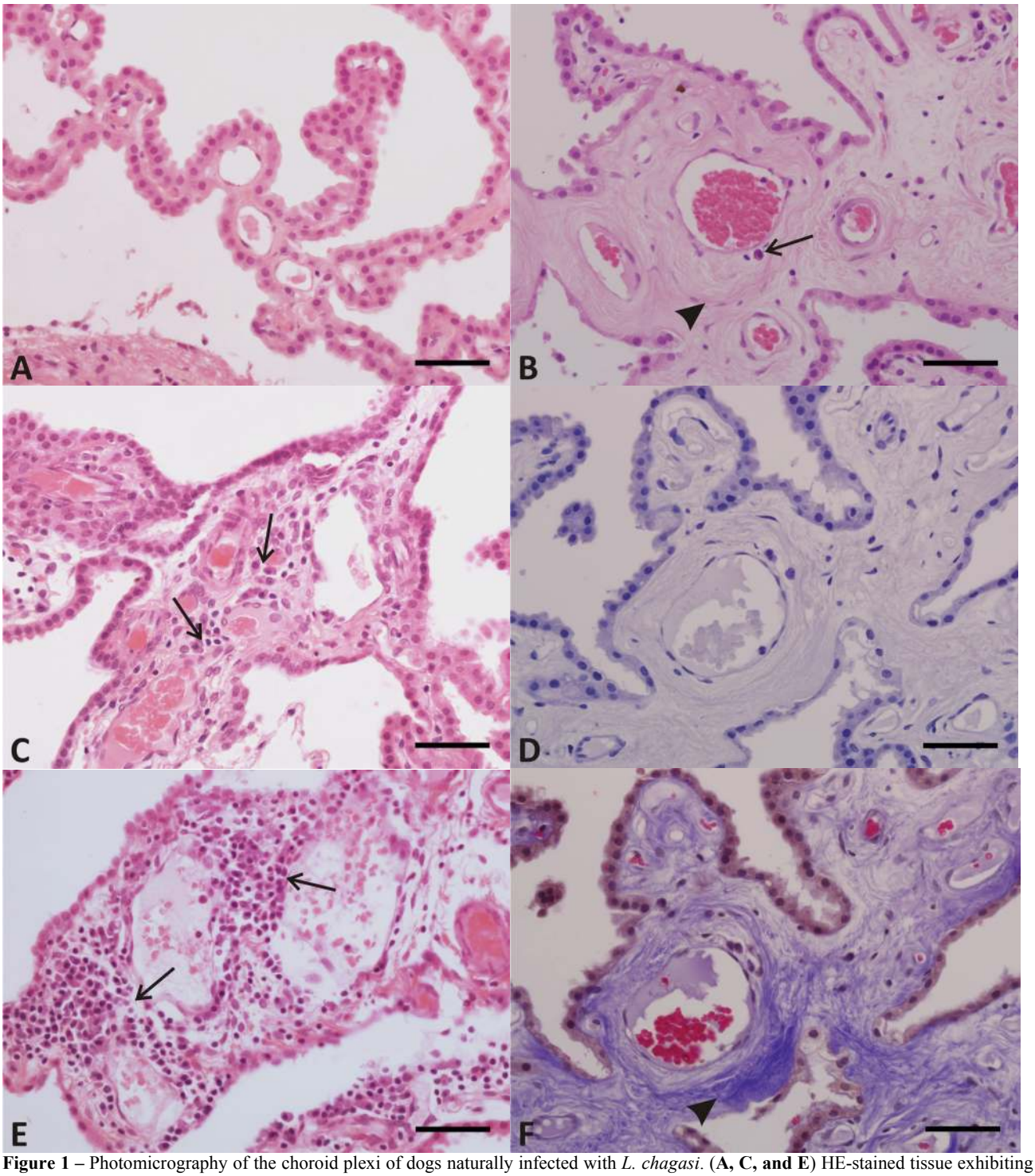

Figure 1 – Photomicrography of the choroid plexi of dogs naturally infected with *L. chagasi*. (**A, C, and E**) HE-stained tissue exhibiting different degrees of the inflammatory infiltrate in the CP. (**A**) Normal feature of the CP of a dog from control group. Observe contiguous epithelial cells covering capillaries which are sustained by a small amount of loose connective tissue. (**C**) Diffuse cellular groups (*arrows*), classified as moderate, presenting morphologic diversity. (**E**) High inflammatory cellularity resulting in evident morphological alteration of the CP's connective tissue, with multifocal distribution (*arrows*), classified as intense. (**B, D, and F**) Histochemical analysis of the perivascular hyaline deposits in the same areas of the CP. (**B**) HE-stained tissue showing the perivascular deposition of hyaline substance (*arrowhead*) and the presence of few mononuclear cells (*arrow*) in the CP. Observe, further, the connective tissue thickening. (**D**) Congo red-stained tissue evidencing absence of congophilic fibers. (**F**) Masson's trichromic-stained tissue showing that the hyaline substance was constituted by collagen fibers (*arrowhead*). Scale bar = 50 µm.

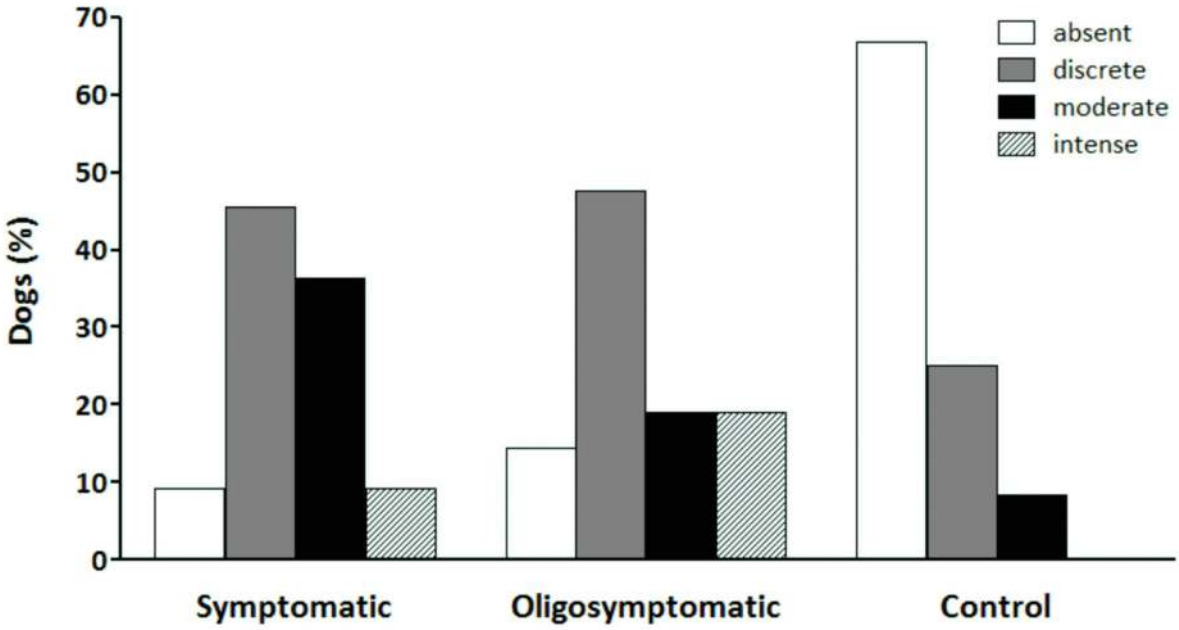

Figure 2 – Percentage of dogs presenting morphological alterations in the choroid plexus, classified according the intensity of the inflammatory infiltrate and the experimental groups.

infection promotes changes in the blood-CSF barrier, rendering favorable the increase of CNS permeability to molecules, such as antibodies, inflammatory cells and mediators, allowing the activation and perpetuation of the inflammatory process within the nervous environment.

Acknowledgements

This work was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, Process number 05/60132). G.D. Melo was financed by FAPESP scientific initiation scholarship (Process number 06/56724-3). We wish to thank M.Y.I. Campos and F.A. Ardisson for brilliant histotechnical support and, T.F. Antello for the help with the English version.

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