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Original Full Article

Subclinical Muscle Injuries in Dogs Infected with *Leishmania (Leishmania) infantum chagasi*

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Abstract

Although canine visceral leishmaniasis (CVL) has been extensively studied, muscular damage due to *Leishmania (Leishmania) infantum chagasi* infection remains to be fully established. The aim of this study was to describe the electromyographic and histological changes, as well as search for the presence of amastigote forms of *Leishmania spp*, CD3+ T-lymphocytes, macrophages and IgG in skeletal muscles of dogs with visceral leishmaniasis (VL). Four muscles (triceps brachial, extensor carpi radialis, biceps femoris and gastrocnemius) from a total of 17 naturally infected and six healthy dogs were used in this study. Electromyographic alterations such as fibrillation potentials, positive sharp waves and complex repetitive discharges were observed in, at least, three muscles from all infected dogs. Myocyte necrosis and degeneration were the most frequent muscular injury seen, followed by inflammatory reaction, fibrosis and variation in muscle fibers size. Immunohistochemistry in muscle samples revealed amastigote forms in 4/17 (23.53%), IgG in 12/17 (70.58%), CD3+ T-lymphocytes in 16/17 (94.12%) and macrophages in 17/17 (100%) dogs. Statistically positive correlation was observed between: inflammatory infiltrate (p=0.0305) and CD3+ immunoreaction (p=0.0307) in relation to the number of amastigote forms; inflammatory infiltrate (p=0.0101) and macrophage immunoreaction (p=0.0127) in relation to the amount of CD3+; and inflammatory infiltrate (p=0.0044) and degeneration / necrosis (p<0.0001) in relation to the presence of macrophages. Our results suggest that different mechanisms contribute to the development of myocytotoxicity, including celular and humoral immune responses and direct muscular injury by the parasite. Nevertheless, the catabolic nature of the disease can probably interact with other factors, but cannot be incriminated as the only responsible for myositis.
Key Words: histopathology, immunohistochemistry, muscle, visceral leishmaniasis.

Introduction

Visceral leishmaniasis is a zoonotic disease caused by a protozoan of the genus *Leishmania*, being the species *Leishmania (Leishmania) infantum chagasi* the agent described in the Americas (33). Approximately 500,000 human cases and 59,000 deaths are recorded every year all over the world (13). Canine leishmaniasis (CL) is a multisystemic disease and dogs may present variable clinical signs. Most of them present progressive weight loss, anemia, pyrexia, generalized lymphadenomegaly, hepatosplenomegaly, cutaneous, renal and ocular lesions (11, 17, 20, 25, 35). Some animals have decreased physical activity associated with episodic weakness, paresis, lameness, exercise intolerance,

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myalgia, arthralgia and muscular atrophy caused by polyarthritis, polymiositis or osteomyelitis (2, 8, 23, 32, 34).

There are some case reports of myositis in dogs with leishmaniasis, this disease being included as one of the causes of inflammatory myopathies in domestic carnivores (6, 23, 26, 34). Although histological and immunohistochemical alterations involving smooth and skeletal muscles of dogs with VL have been described, including masticatory (27), appendicular (21, 27), intra, periocular and extra-ocular (25), cardiac (4, 22), bronchopulmonary (4) and intestinal muscles (29), the pathogenesis of muscular lesions has not been totally explained (23, 28).

Muscle atrophy in dogs with VL is often attributed to the catabolic nature of the disease (10, 19). However, some studies have demonstrated that the histological changes observed in infected animals, such as degeneration and necrosis of myofibers, inflammatory infiltration, fibrosis and variation in size of muscle fibers are due to an inflammatory response against the parasite and not exclusively by a catabolic process (26, 34).

Vamvakidis et al. performing electromyography in the masticatory and cranial tibial muscles of 24 dogs with VL observed myopathy in all of them. Performing histological analysis, the authors identified myofibre atrophy with varying degrees of necrosis, mononuclear inflammatory infiltrate and neutrophilic vasculitis in 16 dogs with, as well as in six animals without muscle atrophy. *Leishmania* amastigotes were found in sixteen (67%) dogs. Thus, the authors suggested that the muscular lesions in VL are caused both by the presence of the parasite and as a consequence of an immune-mediated response (34). Moreover, Paciello et al., evaluating skeletal muscles of 15 dogs with VL with signs of muscle atrophy and weight loss, detected *Leishmania* spp., CD3 +, CD4 + and CD8 + lymphocytes, suggesting that amastigotes in the muscles may act as a causing factor triggering an inflammatory response and that muscle damage might be related to immune-mediated mechanisms associated with leishmania infection (26).

Based on the hypothesis that dogs with VL have subclinical muscle damage and that muscular lesions can occur by the presence of the parasite in the muscles or by an immune response, the aim of this study was to describe the electromyographic and histological changes, as well as search for the presence of *Leishmania* amastigotes, CD3+ T-lymphocytes, macrophages and IgG in four muscles (triceps brachial, extensor carpi radialis, biceps femoris and gastrocnemius) of 17 *Leishmania (Leishmania) infantum chagasi* naturally infected dogs, in order to better understand the pathophysiology of the disease in the muscular system.

Material and methods
Dogs

A total of 17 mixed-breed adult dogs, of both sexes, aged between two and five years, naturally affected by visceral leishmaniasis, were selected from the Veterinary Teaching Hospital of São Paulo State University in Araçatuba, Northwestern São Paulo state, Brazil, an endemic area for CVL (canine visceral leishmaniasis). The study included only dogs with positive parasitological and serological diagnosis. Six healthy dogs from the same area, serologically and parasitologically negative for *Leishmania (Leishmania) infantum chagasi*, were used as a control group. No dog in the present study had anti-*Toxoplasma gondii* (9) or anti-*Neospora caninum* antibodies (14). The present study was approved by the Ethics Committee in Animal Experimentation and Animal Welfare (Protocol number 000842/05).

Parasitological and serological diagnosis

Diagnosis of CVL was based on the enzyme linked immune sorbent assay (ELISA) and confirmed by direct observation of *Leishmania* amastigote forms in lymph nodes smears obtained by needle aspiration biopsy and stained with a quick Romanovsky-type stain (Panótico Rápido®, Laborclin®, Pinhais, Brazil) (21).

Experimental design

All dogs were subjected to a complete physical examination, and an electromiographic examination of the triceps brachial, extensor carpi radialis, biceps femoris and gastrocnemius muscles was performed under general anesthesia. These muscles were chosen because of their size and ease of electrodes placement. Muscle biopsy specimens were collected immediately after EMG from each contralateral muscle to prevent histological changes due to the insertion of the needle during electromyography. In compliance to a federal law and under the owners' signed consent, after electromyography *Leishmania (Leishmania) infantum chagasi* infected dogs were euthanatized.

Electromyography (EMG)

Needle electromyography was performed using a portable EMG (Viking Quest -Nicolet Compass Meridian - Nicolet Biomedical Inc. - USA) with monopolar recording electrodes. EMG was performed on one side of the body with dogs lying in lateral recumbency.

Muscle biopsy

Muscle samples were slightly stretched on small corks and fixed in 10 per cent neutral buffered formalin for 24 hours, stored for 24 hours in alcohol 70 per cent, embedded in paraffin and subjected to the routine histological procedure. Five micrometer thick transverse and longitudinal sections were stained with hematoxylin-eosin (HE) and modified Gomori trichrome and also subjected to immunohistochemical procedures. Degree of muscle injury such as inflammatory infiltrate and myocyte necrosis and degeneration were semi quantitatively classified from 0 to 3+, as follows: 0 for absent, (1+) for mild alterations (small foci lesions), (2+) for moderate (larger, multifocal or focally extensive lesions) and (3+) for severe (affecting mostly evaluated areas, often with

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coalescence among them), and subjectively compared to healthy dogs. Muscle fibers size was also subjectively compared to samples from healthy dogs.

Immunohistochemistry (IHC)

Deparaffinized slides were hydrated and incubated in 2% hydrogen peroxide 10 vv in 0.01 M PBS, pH 6.0, to block endogenous peroxidase activity, followed by incubation with 6% nonfat dry milk (Molico®- Nestlé – São Paulo, Brasil) to block nonspecific immunoglobulin absorption to tissues. Antibodies against *Leishmania* spp. (produced in mouse by the Laboratory of Pathology of Infectious Diseases, Medical School, São Paulo University), CD3+ lymphocytes (Polyclonal Rabbit Anti-Human CD3 –Dako, CA, USA), IgG (Goat anti-dog IgG – Bethyl Laboratories Inc. – Montgomery, TX, USA) and macrophages (Mouse Anti-Human Macrophages – MCA874G - AbD Serotec – Morphosys Co. – Munich, Germany), in 1:1000, 1:120, 1:2400 and 1: 600 dilutions, respectively, were used as primary antibody. Slides were incubated for 18 hours at 4ºC in a humid chamber. After washing in PBS, the slides were incubated with biotinylated secondary antibody (LSAB kit - K0690 - Dako CA, USA), washed in PBS again, and then incubated with the streptavidin-peroxidase complex for 45 minutes at 37ºC. The reaction was developed with a diaminobenzidine (Liquid DAB + Substrate Chromogen System – K3468 – Dako CA, USA) solution and hydrogen peroxide 10 vv. Finally, slides were dehydrated, cleared, counter-stained with Harris Hematoxylin, and mounted with coverslips. The immunoreactivity was analyzed, semi-quantitatively, as the average number of immunostainings identified in five optical fields (at 400 magnification) and classified as: (–) absent, (1+) mild (1-50), (2+) moderate (50-200) and (3+) intense (> 200).

Statistical Analysis

Correlation between the intensity of histologic lesions and immunostaining for *Leishmania* spp., CD3+ lymphocytes, IgG and macrophages was evaluated by Spearman test. Differences regarding lesion intensity among the four muscles were evaluated by Friedman test. Statistical analysis was performed by a commercially available software program (InStat®, GraphPad Software Inc., La Jolla, CA, USA). A value of p < 0.05 was considered statistically significant.

Results

Dogs

All 17 infected dogs were presented with clinical signs of systemic leishmaniasis, such as skin lesions, lymphadenopathy, hepatosplenomegaly, weight loss, onychogryphosis and ocular lesions (Table 1). Only one dogs had clinical signs of neuromuscular disorder, characterized by paraparesis. This dog was alert, with normal cranial nerves responses and spinal reflexes, negative serology for toxoplasmosis and neosporosis and was also routinely vaccinated against canine distemper.

Electromyography

Fibrillation potentials, positive sharp waves and complex repetitive discharges were detected at least in three muscles from all dogs with VL. EMG was normal in all dogs from control group.

Histology

The most frequent muscular injury were myocyte degeneration and necrosis, followed by variable degrees of inflammatory response (Fig. 1A), mostly composed of lymphocytes and plasma cells, with few macrophages in some of the areas. Usually, it was diffusely located around muscle fibers throughout the perimysium and endomysium (Fig. 1A e 1B). There were also noted either perimysial or endomysial fibrosis and fatty infiltration, identified as increased number of adipocytes within the endomysium (Fig. 1C) and variation in muscle fibers size (Fig. 1D). *Leishmania* amastigotes were identified in HE stained sections of some dogs. Pathological changes in muscle from all affected dogs were similar and are presented in table 1. Control healthy dogs were histologically normal.

Immunhoistochemistry

Immunohistochemistry revealed amastigote forms in 4/17 (23.53%), IgG in 12/17 (70.58%), CD3+ T-lymphocytes in 16/17 (94.12%) and macrophages in 17/17 (100%) dogs (Table 1). Immunostainings for *Leishmania* were found inside the cytoplasm of macrophages (Fig. 2A) in areas with intense inflammatory infiltrate in the endomysial region. The observed CD3+ lymphocytes had multifocal and diffuse distribution, variable intensity, especially in areas with inflammatory infiltrate (Fig. 2B) and myofibril degeneration and necrosis, in the endomysial region. All muscles in which the parasite was detected showed reactivity for CD3+, macrophages and IgG. Statistically positive correlation was observed between: inflammatory infiltrate (p=0.0305) and CD3+ immunoreaction (p=0.0307) in relation to the number of amastigotes; inflammatory infiltrate (p=0.0101) and macrophage immunoreaction (p=0.0127) in relation to the amount of CD3+; and inflammatory infiltrate (p=0.0044) and degeneration / necrosis (p<0.0001) in relation to the presence of macrophages. There was no correlation between: macrophages (p=0.0811) and IgG (p=0.1063) in relation to the presence of parasite amastigotes. Macrophages were detected in the perimysial and perivascular regions of all animals (Fig. 2C), while immunohistochemical staining for IgG was observed among the inflammatory cells and perivascular areas, mainly in the endomysial region (Fig. 2D). Although there was no statistical difference (p=0.064) among the four evaluated muscles for the assessed immunostainings, biceps femoris was most affected, showing the highest reactions intensity.

Discussion

A wide variety of clinical signs were observed among the 17 dogs; however, although most

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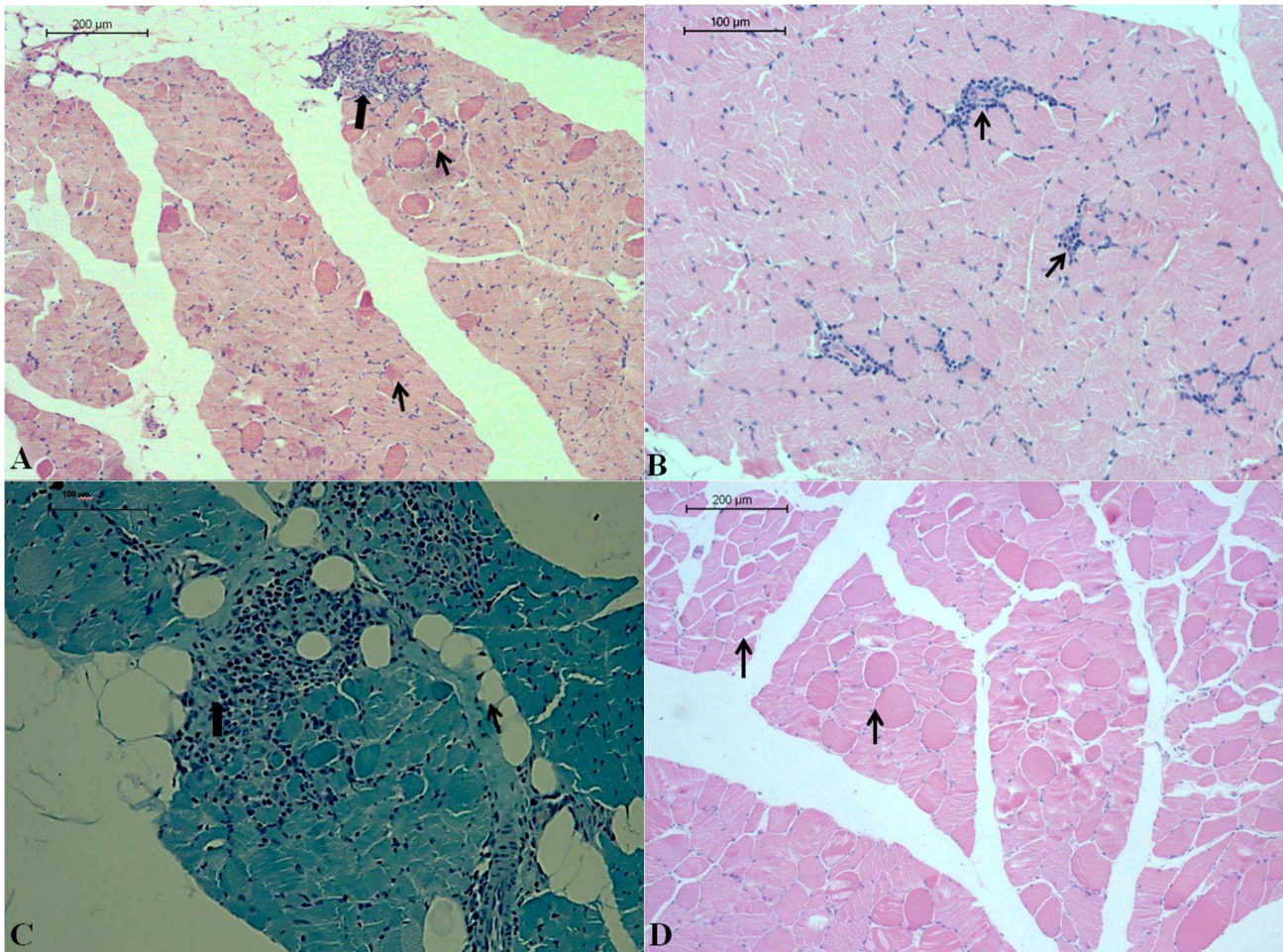*(Leishmania) infantum chagasi*. Braz J Vet Pathol, 2012, 5(3), 108-115

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Figure 1. Histological sections of skeletal muscles from dogs naturally affected by visceral leishmaniasis. (A) Inflammatory infiltrate (wide arrow) and myocyte necrosis and degeneration (thin arrows) in the endomysium. HE. Bar: 200µm; (B) Endomysial multifocal lymphoplasmocitic inflammatory infiltrate (arrows). HE Bar: 100µm; (C) Endomysial fibrosis associated to inflammatory response (wide arrow) and fatty infiltration (thin arrow) Bar: 100µm. Modified Gomori trichrome; (D) variation in muscle fibers size (arrows). HE Bar: 200µm.

dogs had progressive weight loss and poor body condition, only one dog (5,9%) with visceral VL exhibited signs of neuromuscular disease, characterized by paraparesis and muscle atrophy. Since this dog had no other alteration on neurologic examination except paraparesis, and other diseases such as toxoplasmosis, neosporosis and canine distemper were ruled out as possible causes, we believe that paraparesis was due to intense myoatrophy. Although asymptomatic, all dogs presented electromyographic changes in, at least, three of the four tested muscles. The electromyographic abnormalities, characterized by abnormal spontaneous activity in the form of fibrillation potentials, positive sharp waves and complex repetitive discharges, evidenced the occurrence of myopathy (15, 30). Such findings were also verified in a previous study, particularly in dogs with muscle atrophy (34). This result suggests that the polymyositis in CVL may be subclinical (34). The histological assessment of the muscles confirmed the occurrence of inflammation, degeneration and necrosis in all dogs, suggesting a chronic evolution of the myopathy,

similar to the data described by Paciello et al. (26) and Vamvakidis et al. (34), who identified changes in 100% (15/15) and 91.67% (22/24) of the dogs with VL, respectively. Of the four evaluated muscles, biceps femoris showed the highest intensity of lesions and immunostainings. This finding cannot be compared to previous studies since, in those, only one skeletal appendicular muscle (26) or one appendicular and one masticatory muscle (34) were assessed.

Only 4/17(23.53%) dogs presented immunoreactivity to *Leishmania* amastigotes, differing from a previous study in which the parasite was observed in the biceps femoris of 13/15 (86.7%) VL dogs presenting muscle weakness and atrophy (26). The only dog with paraparesis, associated to electromiographic and histological changes of the four assessed muscles, did not present amastigotes in the muscles. Thus, we cannot state that the parasite was the only responsible for the development of inflammatory myopathy in the evaluated animals. Absence of *Leishmania* amastigotes in naturally affected dogs with severe muscle damage were already

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reported by other authors, who considered direct muscular injury from the parasite and immune-mediated myositis a

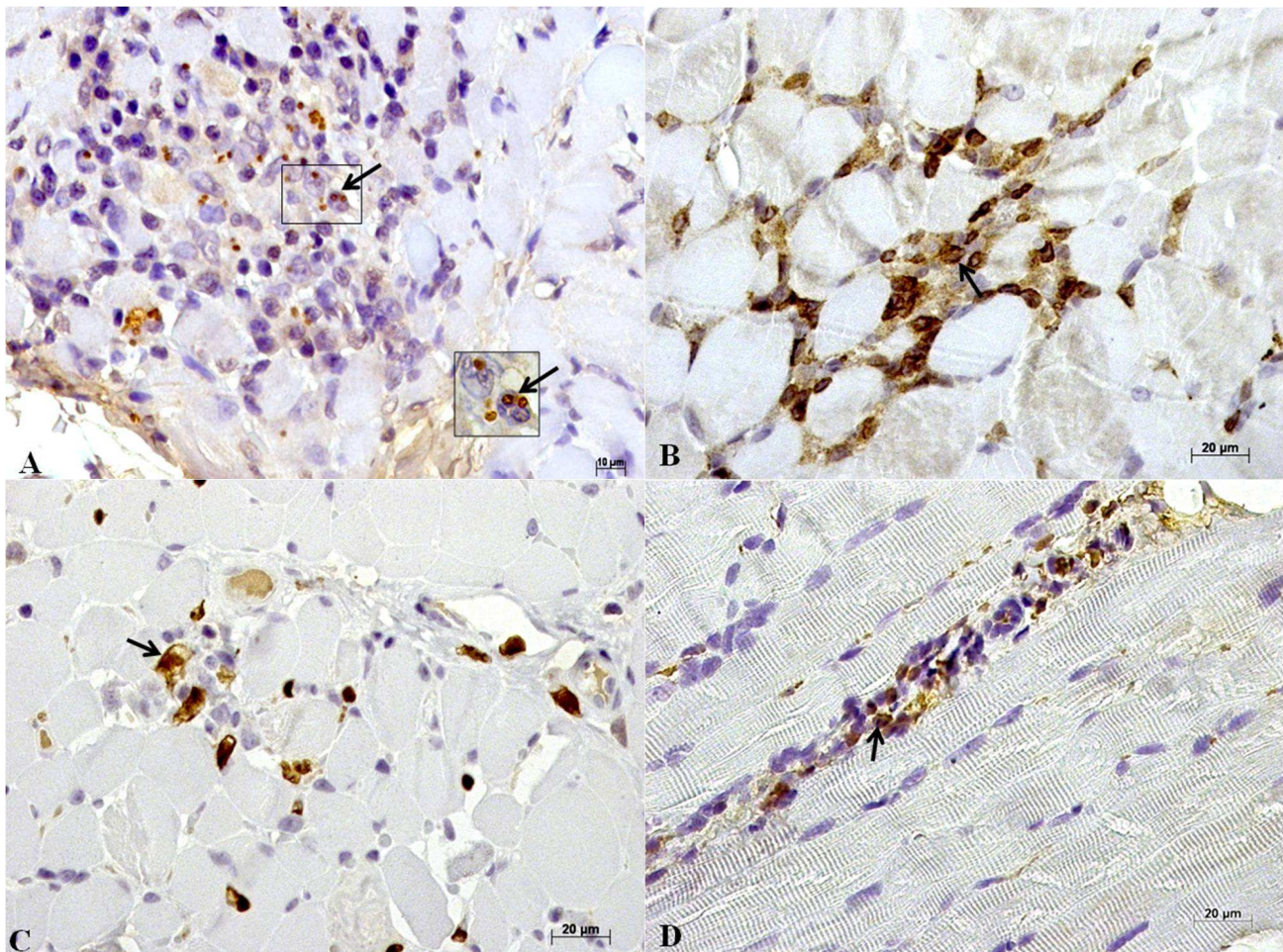possible causes involved in polymiositis induced by

Figure 2. Immunohistochemical staining of skeletal muscles from dogs naturally affected by visceral leishmaniasis. (A) *Leishmania* amastigotes in macrophages cytoplasm (arrows) wide picture and inset. Bar: 10µm; (B) CD3$^+$ T-lymphocytes in the inflammatory focus (arrow) Bar: 20µm ; (C) Macrophages in the inflammatory focus (arrow) Bar: 20µm; (D) Immunoglobulin G in the inflammatory focus (arrow) Bar: 20µm. Streptavidin-biotin peroxidase complex method.

leishmaniasis (34).

Amastigotes were always found inside mononuclear cells, similarly to previous reports (25, 26, 34). According to Paciello et al. (26), the parasite is not dispersed in the muscle fiber since real-time PCR could not identify *Leishmania* DNA in the sarcoplasm of myocytes. *Leishmania* specimens were present especially in areas of inflammatory infiltrate with a direct correlation between the inflammatory process intensity and the number of parasites, as previously reported (26). Although the parasite was not identified in all muscles with inflammatory infiltrate, the positive correlation between the number of parasites and the inflammatory intensity suggests that the presence of the parasite can aggravate the inflammatory myopathy. Although all dogs were serologically negative for toxoplasmosis and neosporosis, we cannot rule out the possibility of involvement of some other disease in the occurrence of muscle damage.

In the present study, there was also a correlation between the intensity of the inflammatory process and the number of parasites in relation to the amount of CD3+ lymphocytes in areas of inflammatory infiltrate. Nevertheless, CD3+ was also verified in not parasitized muscles. A previous study performed on Syrian hamsters, an experimental animal model of VL, demonstrated that hamsters infected with *L. infantum* develop also an inflammatory myopathy, with mononuclear inflammatory cells composed of CD3+, CD4+ and CD8+ cells, even in muscles without immunoreactivity to *Leishmania* amastigotes (27). CD3+ molecules are present in all subtypes of T lymphocytes, including T helper, T suppressor and Tγδ lymphocytes, and it is known that VL symptomatic dogs develop a significant reduction in the number of circulating CD3+ lymphocytes, probably as a consequence of the recruiting of these cells to the parasitized organs (3, 7, 24).

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Table 1. Clinical signs, histopathology (H&E) and immunohistochemistry (IHQ) for *Leishmania* spp., CD3+ T-lymphocytes, macrophages and IgG in muscles of 17 *Leishmania (Leishmania) infantum chagasi* naturally infected dogs.

Dogs	Clinical signs	H & E	IHQ			
			Leishmania spp	CD3 +	Macrophages	IgG
1	Progressive weight loss, cutaneous lesions	lymphoplasmacytic infiltrate, myocyte necrosis and degeneration	-	2+	1+	-
2	Progressive weight loss, cutaneous lesions, lymphadenomegaly, hepatosplenomegaly	lymphoplasmacytic infiltrate, intense myocyte necrosis and degeneration, intense variation in muscle fibers size	-	1+	2+	-
3	Progressive weight loss, cutaneous lesions, ocular lesions, lymphadenomegaly	lymphoplasmacytic infiltrate, intense myocyte necrosis and degeneration, variation in muscle fibers size	-	2+	1+	1+
4	Progressive weight loss, hepatosplenomegaly	lymphoplasmacytic infiltrate, myocyte necrosis and degeneration, fatty infiltration, intense variation in muscle fibers size	-	2+	1+	-
5	Progressive weight loss, ocular lesions, hepatosplenomegaly	lymphoplasmacytic infiltrate, intense myocyte necrosis and degeneration, *Leishmania* spp (1+), intense variation in muscle fibers size	1+	1+	2+	1+
6	Progressive weight loss, ocular lesions	intense lymphoplasmacytic infiltrate, intense myocyte necrosis and degeneration, intense fibrosis, *Leishmania* spp (2+), intense variation in muscle fibers size	2+	3+	2+	1+
7	Progressive weight loss, cutaneous lesions, lymphadenomegaly	intense lymphoplasmacytic infiltrate, intense myocyte necrosis and degeneration, intense fibrosis	-	2+	2+	1+
8	Progressive weight loss, cutaneous lesions lymphadenomegaly	lymphoplasmacytic infiltrate, myocyte necrosis and degeneration, fibrosis, variation in muscle fibers size	-	2+	1+	1+
9	Lymphadenomegaly, hepatosplenomegaly	lymphoplasmacytic infiltrate, myocyte necrosis and degeneration, fibrosis, variation in muscle fibers size	-	2+	1+	1+
10	Progressive weight loss	lymphoplasmacytic infiltrate, fibrosis	-	2+	1+	-
11	Progressive weight loss, cutaneous lesions, decreased physical activity , lameness	Intense lymphoplasmacytic infiltrate, intense myocyte necrosis and degeneration, fibrosis, *Leishmania* spp (3+)	3+	3+	3+	1+
12	Lymphadenomegaly, hepatosplenomegaly, onycogryphosis	myocyte necrosis and degeneration, fatty infiltration, fibrosis	-	-	1+	1+
13	Hepatosplenomegaly, lymphadenomegaly, fever	intense lymphoplasmacytic infiltrate, intense myocyte necrosis and degeneration, fibrosis, *Leishmania* spp (1+)	2+	1+	2+	1+
14	Progressive weight loss, cutaneous lesions, bronchopneumonia	intense myocyte necrosis and degeneration, fatty infiltration, fibrosis, variation in muscle fibers size	-	1+	1+	-
15	Progressive weight loss, paraparesis, intense muscular atrophy, onycogryphosis	intense lymphoplasmacytic infiltrate, intense myocyte necrosis and degeneration, variation in muscle fibers size	-	2+	1+	2+
16	Progressive weight loss, exfoliative dermatitis, fever	intense myocyte necrosis and degeneration, intense variation in muscle fibers size	-	1+	1+	1+
17	Progressive weight loss, hepatosplenomegaly	lymphoplasmacytic infiltrate, intense myocyte necrosis and degeneration, fatty infiltration, intense variation in muscle fibers size	-	2+	2+	1+

* (-) absent; (1+) mild; (2+) moderate; (3+) severe

According to Vamvakidis et al., canine inflammatory myopathies can be of infectious or immune-mediated origin, and in dogs with polymyositis induced by leishmaniasis both causes may be involved, since IgG was

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identified in all 24 assessed animals, even in those without parasites in their muscles (34). The authors stated that the cross-reactivity of antibodies directed against *Leishmania* with sarcolemmal antigens, or the deposition of immune complexes, may be mechanisms for the initial sarcolemmal injury, which result in the leakage of intracellular proteins from myofibres (34). Similarly, a study conducted on Chagas disease confirmed that T cells infiltrate into the myocardium and play a primordial role in tissue damage; however, the authors could not determine the nature of the target antigens inside the heart. The absence of parasites close to areas of destructive inflammatory infiltrate in infected cardiac tissue suggested that the inflammatory lesion in the heart was of autoimmune nature, involving molecular mimicry between *Trypanosoma cruzi* B13 protein and cardiac antigens. This cross-reaction between myosin and B13 is present among 100% of the patients with chronic chagasic cardiomyopathy and only among 14% of the asymptomatic seropositive individuals (12). Nearly 30% (5/17) of the dogs from the present study had no IgG immunoreaction in their muscles, and a mild immunoreactivity was observed in eleven (65%) dogs, suggesting that, if humoral immunity is also important in the development of muscular lesions, inflammation was not triggered only by circulating immune complexes.

The correlation between the presence of macrophages and the existence of inflammatory infiltrate, CD3+ cells, degeneration and necrosis of muscular tissue can be explained since, besides having phagocytic activity, these cells also secrete pro inflammatory cytokines and tumor necrosis factor (TNF), and additionally repair tissue lesions becoming present in chronic inflammation and in tissues with degeneration and necrosis (16).

Although pathogenesis of muscular lesions in VL has not been fully elucidated, our results suggest a multifactorial mechanism, including celular and humoral immune responses and direct muscular injury by the parasite. Nevertheless, the catabolic nature of the disease can probably interact with other factors, but cannot be incriminated as the only responsible for myositis.

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