Diffuse Intralobular Liver Fibrosis in Dogs Naturally Infected with *Leishmania* (*Leishmania*) *chagasi*  

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**Abstract.** The aim of this study was to evaluate the diffuse intralobular fibrosis in dogs naturally infected with *Leishmania* (*Leishmania*) *chagasi*. One hundred five infected animals in positive serologic tests for *Leishmania* were divided into two clinical groups: 69 symptomatic animals and 36 asymptomatic. Special staining with Gomori, Heidenhain, Silver, and Picrosirius Red was applied to characterize fibroplasia. The tissue parasite load was measured by immunohistochemistry and associated histomorphometric analyses. Intralobular fibrosis was observed in all dogs, and more collagen deposition was confirmed in the infected animals than in the controls by these histomorphometric studies. There were significant differences among the distinct clinical groups. In fact, symptomatic dogs showed an increased collagen deposition in the liver compared with asymptomatic ones. A peculiar diffuse intralobular fibrosis, where the collagen fibers encircled small groups of hepatocyte(s), was observed in two cases (1.9%).

**INTRODUCTION**

Canine visceral leishmaniasis (CVL) is caused by *Leishmania infantum* (*syn. L. chagasi in America*) and is transmitted by phlebotomine sand fly bites (*Lutzomyia longipalpis* species in America).1,2 Protozoa of the genus *Leishmania* are dimorphic obligate intracellular parasites that reside within mononuclear phagocytes in the mammalian host. Classic histopathologic lesions have been described mainly in organs rich in cells of the mononuclear-phagocytic system such as liver, spleen, lymph nodes, bone marrow, gastrointestinal tract, and skin. In general, an intense chronic inflammatory reaction consisting of infiltration by mononuclear cells (macrophages, plasma cells, and lymphocytes) is observed in the liver and spleen, skin, bone marrow, gastrointestinal tract, and kidneys.3,12-13 In many parts of the world, with some geographical variation, liver disease and various associated histopathologic changes have been described in CVL4-16 and human visceral disease.17-23 These changes include hypertrophy and hyperplasia of Kupffer cells, portal and intralobular granulomas, bilirubin deposition, and diffuse intralobular fibrosis. Fibrotic tissue is formed in response to inflammation or direct toxic insult to the liver.24-26 The liver seems to attract many parasites, which may either inhabit the organ, pass through it during normal development, or be carried there and destroyed. The Kupffer cells are the target cells for *Leishmania* (*Leishmania donovani* complex), causing visceral leishmaniasis or kala-azar.25 The protozoan, *Leishmania*, has been implicated in hepatic fibrosis in chronic cases of kala-azar in India,21,23,27-28 some cases in the Mediterranean,29 and one case in China.30 Fibrosis has been reported in CVL in organs such as lung (chronic interstitial pneumonitis)31,32 and liver (diffuse hepatic intralobular fibrosis).5 Hepatic fibrosis is characterized by increased deposition and altered extracellular matrix composition in the portal tracts, around central veins, or in perisinusoidal spaces (intralobular fibrosis).24 The aim of this study was to verify the incidence of diffuse intralobular liver fibrosis in dogs naturally infected with *Leishmania chagasi*. Animals with defined parasitologic and clinical status were obtained from an endemic area of Brazil, the municipality of Belo Horizonte, MG (Southern Brazil).

**MATERIALS AND METHODS**

**Animals.** One hundred five mongrel dogs of unknown age naturally infected with *L. chagasi* were identified during an epidemiologic survey of CVL carried out by the municipality of Belo Horizonte, MG (Southern Brazil). Enzyme-linked immunosorbent assays (ELISA; optical density > 100 > 1:400 dilutions) were positive for all infected animals. We also analyzed serum samples using a commercial kit containing an immunochromatographic strip that uses recombinant leishmanial antigen k39 and a dominant amastigote antigen of *L. chagasi* (*rK39*), which is highly sensitive and specific for *Leishmania donovani* complex infection, as previously described.33-35 Sera from all infected dogs were also positive for this test. Another 17 dogs with serologic examinations negative for *Leishmania* were obtained as control animals.

**Groups and clinical aspects of infected dogs.** All infected dogs were clinically classified and divided into three groups as follows: Group 1, symptomatic dogs—69 animals that exhibited the classic signs of the disease such as lymphadenopathy, cutaneous alterations (alopecia, dry exfoliative dermatitis, or ulcers), onychogryphosis, keratoconjunctivitis, weight loss or cachexia, and anemia; Group 2, asymptomatic dogs—36 apparently healthy animals with no signs of the visceral disease; Group 3, control dogs—17 uninfected animals with serologic and parasitologic examinations negative for *Leishmania*. 

**Parasitological diagnosis of *Leishmania* infection.** All dogs were anesthetized with 2.5% (1.0 mL/kg) intravenous thiopental. The experimental protocol using dogs was approved by CETEA-UFMG (Brazilian Animal Experimental College, number 106/2004). Touch aspirates of bone marrow were obtained for parasitologic diagnosis of infected and control animals. The smears were air-dried and stained with 10% Giemsa. *Leishmania* amastigotes were detected in all in-
ected animals by light microscopy using oil immersion (×1,000 magnification). Control animals were parasitologically negative.

**Necropsy and histopathology.** The animals were anesthetized with a dose of 0.5 mL/kg thiopental intravenously (2.5%) and killed with T-61 (0.3 mL/kg). During necropsy, the livers were weighed, and tissue touch preparations (smears) were obtained from small samples as described for the bone marrow aspirates. *Leishmania* amastigotes were observed in slides stained with 10% Giemsa. Amastigote forms of *Leishmania* were observed in all smears of infected animal livers.

Other liver fragments were collected for histopathology. These samples were fixed in 10% neutral-buffered formalin and were dehydrated, cleared, embedded in paraffin, cut into 4- to 5-μm-thick sections, and stained with hematoxylin and eosin (HE). For collagen studies, all liver fragments were stained with Gomori trichrome, Hedeihen blue, Gomori ammoniacal silver, and Picrosirius red. In Gomori trichrome, fibrillar collagen appears green and in Hedeihen, it appears blue. In silver staining, the fibrillar collagen becomes black, and in Picrosirius, some fibers (probably type I) stain red or yellow, whereas reticular collagen (probably type III) appears green under polarized light. For Picrosirius staining, fibrillar collagen was estimated at ×110 resolution using polarized light.

**Histomorphometric analysis.** Liver sections stained with Gomori trichrome, Hedeihen blue, and Gomori ammoniacal silver were analyzed morphometrically to characterize the intralobular collagen deposition, excluding perivascular collagen. This analysis was carried out using an Axioslab light microscope (Zeiss) with ×440 resolution. The images were transferred to a computer video screen using software and relayed to a computer-assisted image analysis system (Kontron Elektronik/Carl Zeiss, Oberkochen, Germany). Using a digital pad, the total area occupied by stained collagen fibers was measured from real images and segmented to generate binary images. The results are expressed in square micrometers.

**Immunohistochemical method for labeling amastigotes of *Leishmania* and morphometric analysis.** The streptavidin–biotin immunohistochemical method for detecting *Leishmania* in formalin-fixed paraffin-embedded canine tissues described by Tafuri and others was used. 39 *Leishmania* amastigotes were readily observed within macrophages in fragments of the livers from all naturally infected dogs.

For histomorphometric study, 40 randomly chosen images from histologic slides of liver tissue fragments were used to assess the number of immunolabeled amastigotes in a Kontron Elektronick/Carl Zeiss image analyzer (KS300 software) as described above, using an Axioslab light microscope (Zeiss) with a ×440 resolution.

**Statistical analysis.** All collagen staining results were compared between asymptomatic and symptomatic dogs by a one-way analysis of variance (ANOVA) test. \( P < 0.05 \) was considered significant.

### RESULTS

**Clinical aspects of the animals.** The most frequent clinical signs were lymphadenopathy (67%), chronic dry desquamation (61%) mainly restricted to the ears and limbs, followed by onychogryphosis (57%), weight loss (41%), alopecia (32%), anemia (27%), and skin ulcerations (19%).

**Anatomic pathology.** Macroscopically, the livers of the naturally infected animals were generally enlarged. However, hepatomegaly was not necessarily found in symptomatic dogs; it varied markedly in association with a dark red coloration indicating congestion. The relative liver weight (liver weight/body weight) was higher in symptomatic dogs (5.12%) than asymptomatic dogs (4.16%) or controls (4.08%; \( P < 0.05 \)).

We observed idiopathic macroscopic lesions in one symptomatic dog. In this case, the liver was pale with a slightly nodular surface, suggesting a prominent reticular pattern on the surface and the cut section. The liver was of firm consistency.

Macroscopically, histologic examination after HE staining showed a general chronic inflammatory reaction involving the entire architecture of the liver including the capsule, portal tracts, and central veins or perisinusoidal spaces. The portal space showed no fibrosis, but there was mild infiltration of lymphocytes, plasma cells, and macrophages with or without amastigotes (Figure 1A and B). Kupffer cells showed hyperplasia and hypertrophy. Additionally, intralobular granulomas comprising of plasma cells, epithelioid cells, and lymphocytes were found in almost all the infected animals (Figure 1C and D). The search for *Leishmania* parasites by HE and specifically by immunohistochemistry showed amastigotes mainly in macrophage granulomas (Figure 1E) and Kupffer cells (Figure 1F). Hepatocellular necrosis foci were found, but they were not intensely or diffusely distributed throughout the liver parenchyma.

None of the hepatic lesions were specific to asymptomatic or symptomatic animals. All infected animals showed the same qualitative histologic picture. The general histopathologic changes in the livers are summarized in Table 1.

Intralobular collagen deposition (intralobular fibrosis) was also observed in all infected animals, but the intensity was highly variable. Even thought it could be observed by HE, Gomori trichrome, and Heidenhain blue staining, it was most evident after Gomori ammoniacal silver staining (Figure 1A–D).

Two cases of symptomatic animals merited attention. First, under HE analysis, we observed a peculiar and diffuse intralobular fibrosis compatible with the description of Rogers23 (so-called “Rogers' cirrhosis”) in Indian Kala-azar. Here, the fibrosis had developed to link the portal areas and central veins. Gomori trichrome and Heidenhain blue showed intense proliferation of intralobular collagen deposition, but this histologic picture was again most evident after Gomori ammoniacal silver staining, as previously mentioned. In fact, silver staining showed conspicuous collagen (reticular fibers) thickening in the space of Disse. In some areas, hepatic cells were isolated from the sinusoidal blood by fibropoiesis (Figure 2E and F). However, there was no subversion of the architecture of the organ with intersection of the classic lobules to produce pseudolobules. Swelling of liver cells was associated with changes in fat content, but no intense necrosis was observed. There was also hypertrophy and hyperplasia of the Kupffer cells, which were commonly parasitized. Portal inflammatory cellular exudate and intralobular granulomas were also frequently found as described above. Regenerating
FIGURE 1. Liver sections of dogs naturally infected with \textit{L. (L.) chagasi}. (A,B) Asymptomatic dog. (A) Portal space with a cellular infiltrated of plasma cells (arrows), lymphocytes and macrophages. Note hepatocytes swelling (hydropic degeneration) (arrowheads) HE (Bars = 16 μm). (B) Same field showing immunolabeled amastigotes forms of \textit{Leishmania} in macrophages (large arrows). Note an intense swelling of the hepatocytes (“balloon cells”) (arrowheads). Streptavidin-peroxidase method (Bars = 16 μm). BD = Bile duct; PV = portal vein; LV = lymphatic vessel; S = sinusoids. (C) Symptomatic dog. Observe three intralobular granulomas formation comprising macrophages (epithelioid cells) (large arrows), plasma cells and lymphocytes (small arrows). Hepatocytes with hydropic degeneration is also present (arrowheads) HE (Bars = 16 μm). (D,E) Asymptomatic dog. (D) Observe an intralobular granuloma formation comprising epithelioid cells with an elongated or oval nucleus and a pink cytoplasm with indistinct cell boundaries (appearing to merge into one another) (large arrows), plasma cells (arrowhead) and lymphocytes (small arrow), HE (Bars = 16 μm). Fatty change (FC) of the hepatocytes (hepatic steatosis) is easily observed, HE (Bars = 16 μm). (E) Same field showing immunolabeled amastigotes into granulomas macrophages (arrows), Streptavidin-peroxidase method (Bars = 16 μm). (F) Symptomatic dog. Note in the center of the figure Kupffer cells intensely parasitized (large arrows). On the right Kupffer cells with less intracellular immunolabeled amastigotes (small arrows) (Bars = 16 μm). This figure appears in color at www.ajtmh.org.
liver cells plates were two cell layers thick, and acinar-like regenerative processes were present. Hepatocellular necrosis foci could be observed.

Picrosirius analysis showed that a large proportion of the total collagen was generally fibrillar collagen type I (yellowed color). Fibrillar collagen with a reticular disposition (probably type III in green color) was less prominent.

Morphometric analysis of hepatic intralobular collagen deposition (Gomori trichrome, Heidenhain blue, Gomori amniacal silver-staining, and Picrosirius red methods). The focus of this study was to evaluate intralobular fibrosis. This was apparent with all staining methods, but collagen fibers were best visualized by silver staining. The morphometric results obtained by all methods showed that infected animals had more hepatic intralobular collagenopoiesis than controls. There were significant differences among the distinct clinical groups: symptomatic dogs showed more collagen deposition in the livers than asymptomatic ones (Table 2).

Parasitism burden. As depicted in Figure 1E and F, Leishmania parasites were readily observed in Kupffer cells and macrophage granulomas, and the tissue parasitism load was evaluated. Our results showed a positive correlation between parasite load and collagenopoiesis. The difference was statistically significant ($r^2 = 0.0451; P = 0.0303$, linear Pearson correlation). The parasite tissue load was higher in symptomatic dogs ($20.1 \pm 5.7$) than asymptomatic ($14.7 \pm 3.7$) dogs ($P = 0.0310$, unpaired $t$ test). Although our results showed only a weak correlation between parasite load and collagenopoiesis ($r^2 = 0.0131$), it was statistically significant ($P = 0.0303$, linear Pearson correlation).

DISCUSSION

Diffuse intralobular fibrosis or a systemic form of fibrosis in the sinusoids has been reported in human visceral leishmaniasis. Rogers described a peculiar diffuse hepatic intralobular fibrogenesis in a chronic case of kala-azar. A Hindu male, ~30 years of age, died with all the classic symptoms of liver cirrhosis. The most striking histopathologic picture of the liver was described by Rogers, the universal distribution of the cirrhotic process throughout the liver lobules, so that the hyperplastic connective tissue widely separates each column of epithelial liver cells. A careful study of a number of sections showed that there is extremely little alteration in the general arrangement of the liver lobules, which retain to a great extent their shape and size, although the intralobular veins are somewhat less prominent than usual. Visentini, in southern Italy, described a case of human visceral leishmaniasis with hepatic fibrosis similar to Rogers’ description. However, as cited by Bogliolo, this author did not know the previous work of Rogers. Thus, this peculiar intralobular fibrosis was subsequently only cited by Laveran, but Nattan-Larrier, in a study of hepatic specimens from Indian adults, gave a detailed histologic description. This author, who was familiar with the earlier work of Rogers, called the hepatic lesion “Rogers’ cirrhosis” but described more details, showing that the collagen fibers isolated hepatocytes as single cells. He called the condition cirrhoses dites monocelulares. It was subsequently characterized as “monocellular cirrhosis of Nattan-Larrier.”

In American visceral leishmaniasis, a diffuse intralobular fibrosis such as that described by Rogers and Goswami in Indian Kala-azar (25–60%, respectively) is rarely found. In Brazil, Bogliolo gave a detailed description of an intense and diffuse hepatic intralobular fibrosis in a 19-year-old male patient diagnosed with visceral leishmaniasis. He described the formation of reticular collagen fibers on the sinusoidal walls and around the hepatocytes, where they induced atrophy of the hepatocytes. Since these studies, intralobular fibrosis as depicted by Rogers has been shown by histopathologic studies in six isolated Brazilian cases of human visceral leishmaniasis. In this study, we found two morphologic cases (1.9%) of intralobular fibrosis of the kind described by Rogers in Indian kala-azar.

Duarte and Corbett, in a study of 47 patients with visceral leishmaniasis, found a high incidence of intralobular fibrosis, with 74.4% of cases showing at least scattered small foci of perisinusoidal fibrosis. In this study of 105 infected animals, we observed intralobular fibrillar collagen deposition in all cases, and this was confirmed by the histomorphometric data (Table 2).

According to Goswami and Duarte and Corbett, this diffuse intralobular fibrosis appears mainly in adults with long-standing disease. Children often have a rapidly progressing disease, allowing no time for the fibrotic changes to occur. In parallel studies with beagles experimentally infected with L. chagasi, our group did not find intralobular fibrosis 1–2 years after experimental infection (data not shown).

The “Rogers’ cirrhosis” as described is not nodular but presents a marked diffuse fibrosis, isolating small groups of liver cells with regenerative changes. In two cases described herein, we observed no subversion of liver architecture with intersection of the classic lobules to produce pseudolobules. This means that “Rogers’ cirrhosis” does not represent real cirrhosis according to the 1997 definition of cirrhosis by a working group sponsored by the World Health Organization. Fibrosis is usually part of the picture of chronic progressive hepatic disease, and spreading changes in the sinusoids may further compromise the liver cells. This very delicate and widely fenestrated structure is loosely associated with the hepatocyte cords and separated from them by the Space of Disse, an arrangement that brings plasma into direct contact with the hepatic cells. As chronic fibrosis progresses, reticulin and subsequently collagen is deposited in the Space of Disse, and the hepatic cells become isolated from the si-

**Table 1**

Histopathologic features of liver (hematoxylin–eosin staining) of naturally infected dogs with *Leishmania infantum* from Belo Horizonte, MG, Brazil

<table>
<thead>
<tr>
<th>Liver histologic analysis</th>
<th>Asymptomatic (N/%)</th>
<th>Symptomatic (N/%)</th>
<th>Infected (N/%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thickened capsule with chronic inflammation of Mo</td>
<td>30/83</td>
<td>42/61</td>
<td>72/69</td>
</tr>
<tr>
<td>Intraportal granulomas</td>
<td>33/92</td>
<td>63/91</td>
<td>63/90</td>
</tr>
<tr>
<td>Congestion</td>
<td>32/89</td>
<td>65/94</td>
<td>97/92</td>
</tr>
<tr>
<td>Portal tract inflammation</td>
<td>36/100</td>
<td>69/100</td>
<td>105/100</td>
</tr>
<tr>
<td>Degenerative lesions (swelling)</td>
<td>36/100</td>
<td>69/100</td>
<td>105/100</td>
</tr>
<tr>
<td>Presence of leishmania</td>
<td>20/56</td>
<td>42/61</td>
<td>62/59</td>
</tr>
<tr>
<td>Total</td>
<td>36</td>
<td>69</td>
<td>105</td>
</tr>
</tbody>
</table>
nusoidal blood. Eventually, the sinusoids begin to resemble capillaries. In the two cases of "Rogers cirrhosis" described herein, we observed areas of enlarged sinusoids with erythrocytes. This could be a result of splenic congestion. Indeed, the spleens of these animals were enlarged, tense, and cyanotic, and on section, they freely exuded blood and the capsule became wrinkled. Cirrhosis may alternatively be defined as nodular regeneration of the liver combined with fibrosis. Thus, "pseudolobule formation," a pathologic condition defined by derangement of the lobular and vascular architecture.

**Figure 2.** Liver sections of dogs naturally infected with *L. (L.) chagasi*. Intralobular Fibrosis. (A–D) Symptomatic dog. (A) Lower magnification showing a diffuse intralobular fibrosis. Note collagen fibers extend through sinusoids from the portal tract (PT), HE (Bars = 62 μm); (B) Higher magnification showing an intense proliferation of collagen and reticulin fibers detected by Gomori ammoniacal silver-staining (Bars = 16 μm). (C,D) Higher magnifications showing intralobular proliferation of collagen fibers (arrows) detected by Gomori’s trichrome and Heidenhain blue (arrows), respectively (Bars = 16 μm). (E,F) Symptomatic dog “Rogers’ Cirrhosis.” (E) Observe dense and coiled fibers extend through sinusoids from the portal tract (white arrows). (PV) Portal vein. Gomori ammoniacal silver-staining (Bars = 16 μm). (F) Note conspicuous collagen thickening in the space of Disse. Hepatic cells had become isolated from the sinusoidal blood by the fibropoiesis (white arrowheads). Gomori ammoniacal silver-staining (Bars = 16 μm). This figure appears in color at www.ajtmh.org.
Morphometrical analysis of collagen (average of collagen deposition in $\mu m^2$) stained by Gomori trichrome, Hedeihen blue, and Gomori ammoniacal silver methods in liver of infected dogs with *Leishmania infantum*.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Gomori trichrome</th>
<th>Hedeihen blue</th>
<th>Gomori ammoniacal silver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>329†</td>
<td>363†</td>
<td>373†</td>
</tr>
<tr>
<td>Infected animals</td>
<td>322†</td>
<td>363†</td>
<td>3955§</td>
</tr>
<tr>
<td>Asymptomatic</td>
<td>287§</td>
<td>364§</td>
<td>3955§</td>
</tr>
</tbody>
</table>

*A* The averages from infected dogs and asymptomatic dogs vs. control were extremely significant under Gomori ammoniacal silver collagen staining analysis (ANOVA test with $P < 0.001$).

† The averages from infected dogs were statistically different vs. controls for all collagen staining (ANOVA test with $P < 0.001$).

§ The averages from symptomatic dogs were statistically different vs. controls for all collagen staining (ANOVA test with $P < 0.001$).

The liver, may be the final common pathway through which almost all chronic liver diseases produce morbidity and mortality.26

In this work, we identified an intralobular fibrosis pattern in dogs naturally infected with *L. chagasi*. Also, we found differences in fibrolipoesis among the clinically defined groups, and we observed a positive correlation between the tissue parasite load and collagen deposition. Moreover, the two cases with “Rogers’ cirrhosis” (two symptomatic dogs) suggest that this intralobular fibrosis pattern may represent chronic progression of long-standing disease.

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