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# Presence of amastigotes in the central nervous system of hamsters infected with *Leishmania* sp.

Presença de amastigotas em sistema nervoso central de hamster infectado com *Leishmania* sp.

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## Abstract

Visceral leishmaniasis (VL) is a severe chronic disease caused by *Leishmania* (*Leishmania*) *infantum chagasi*. Better knowledge on the effects caused by this disease can help develop adequate clinical management and treatment. Parasitological and immunohistochemical studies were performed golden hamsters *Mesocricetus auratus* infected with bone marrow from individuals with VL in the State of Mato Grosso do Sul, central-west Brazil. The effects of parasitism in the spleen, liver, kidneys, lungs, heart and brain of the animals were examined. Eighteen hamsters were inoculated intraperitoneally, and six healthy animals were used as negative controls. The animals were kept in the animal house and checked for clinical signs. Specimens of each organ were examined for the presence of amastigotes. Immunohistochemical technique was performed in all brain specimens and organs negative on the direct examination of parasites. Direct examination of amastigotes was positive in the spleen and liver of all infected animals; 33.3% showed the parasite in the kidneys and lungs, and 16.7% in the heart. Parasitic forms were seen in 83.3% (15/18) of the brain examined. Immunohistochemistry confirmed the results of the direct examination, except in two specimens of lung tissue and in the brain specimens. Other studies are needed to further clarify the effect of the parasite in the central nervous system.

**Keywords:** Visceral leishmaniasis, immunohistochemistry, brain, hamster.

## Resumo

A leishmaniose visceral (LV) é uma doença crônica grave, causada pelo parasito *Leishmania* (*Leishmania*) *infantum chagasi*. Esclarecer as alterações provocadas pela doença é fundamental para que se adotem condutas clínicas e de tratamento adequadas. Com o objetivo de analisar a infecção experimental em hamsters da linhagem golden, *Mesocricetus auratus*, infectados com tecido de medula óssea de pacientes com LV no Estado de Mato Grosso do Sul, foram realizados estudos parasitológicos e de imunomarcação. Foi verificada a distribuição do parasitismo no baço, fígado, rim, pulmão, coração e encéfalo desses animais. Foram utilizados 18 hamsters experimentalmente inoculados via intra-peritoneal, e seis animais sadios como controles negativos. Os animais foram mantidos em biotério de experimentação e observados, em busca de alterações clínicas. Com fragmentos de cada órgão, procedeu-se a confecção de lâminas por aposição para pesquisa de amastigotas. Nos órgãos com resultado negativo na pesquisa direta do parasito, e em todas as amostras de encéfalo, foi realizada a técnica de imunohistoquímica. A pesquisa direta de amastigotas foi positiva no baço e fígado de todos os animais infectados; 33,3% apresentaram o parasito em rim e pulmão, e 16,7% no coração. Quando realizada

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a pesquisa em encéfalo, formas parasitárias foram observadas em 83,3% (15/18) dos animais. A imunomarcação confirmou os resultados da pesquisa direta, exceto em duas amostras de tecido pulmonar e nas amostras de encéfalo. Mais estudos são necessários, para esclarecer o real papel do parasito no sistema nervoso central.

**Palavras-chave:** Leishmaniose visceral, imunohistoquímica, encéfalo, hamster.

## Introduction

Visceral leishmaniasis (VL) is a severe chronic disease of public health concern. VL is endemic in Brazil occurring in 21 states (PENNA, 2008). In the Central-west State of Mato Grosso do Sul, there were 1618 confirmed and reported cases in the period from 2001 to 2008 (MATO GROSSO DO SUL, 2009).

VL is a systemic disease that infects phagocytic monocytic cells (BRASIL, 2006), and the most affected organs are the spleen, liver (PRAKASH et al., 2006), lymphnodes and bone marrow (MELO, 2004).

The best experimental model to study VL is the golden hamster *Mesocricetus auratus* because the pathogenesis and clinical manifestations in this animal are similar to those seen in human disease with splenomegaly, pancytopenia, hypergammaglobulinemia and suppression of T-cell proliferation (OLIVEIRA et al., 2004; GOTO; LINDOSO, 2004).

In view of this disease severity and increasing rates of parasitosis, and also taking into consideration the study of the disease in an experimental model that permits the accomplishment of infeasible research in humans, and the fact that the knowledge about the disease is essential for appropriate prophylactic and treatment procedures, this study aimed to assess the effects of VL parasitism in different organs of hamsters *Mesocricetus auratus* experimentally infected with tissue from bone marrow of patients with VL.

## Material and Methods

Twenty-four 30-day-old *Mesocricetus auratus* hamsters, Golden strain, both males and females, were used in the study. The animals were provided by the animal house of the Universidade Federal de Mato Grosso do Sul (UFMS). The study was approved by the CEUA/UFMS Research Ethics Committee (protocol no. 125/2006). Bone marrow specimens from 18 patients attended at health care centers institution in the capital city of Campo Grande were used. The material was sent to the UFMS Laboratory of Parasitology for VL diagnosis. Five isolates of the parasite were characterized as *Leishmania* (*Leishmania*) *infantum chagasi* at the UFMS Laboratory of Molecular Biology by polymerase chain reaction (PCR standard) test with RV1/RV2 initiators (LACHAUD et al., 2002; FERROGLIO et al., 2006; SILVA et al., 2008). The remaining specimens are still in process of identification.

Eighteen animals were inoculated (0.5 mL) intraperitoneally with a bone marrow specimen from each patient. Six healthy hamsters were used as negative controls. The animals were kept in the animal house of the Parasitology Laboratory with animal feed and water ad libitum, and examined for clinical alterations consistent with VL on a weekly basis. Four months post-inoculation, or ith the development of clinical signs such as weight loss, hepatosplenomegaly and ascites, the animals were anesthetized intraperitoneally with 0.70 $mg.kg^{-1}$ of 10% sodium pentobarbital solution and euthanized by sectioning their abdominal aorta. Specimens were collected from the brain, heart, lungs, kidneys, liver and spleen. Slides were made after Giemsa staining and examined with the aid of a light microscope at 1.000× magnification for the presence of amastigotes. Specimens of negative organs by impression technique (15 hearts, 12 kidneys, 12 lungs) and all brain specimens were stored in 10% formaldehyde and embedded in paraffin for immunohistochemistry.

From the material embedded in paraffin 3 µm thick sections were obtained, deposited on silanized slides without paraffin and hydrated in PBS buffer. Antigens were retrieved using a commercial solution (Target Retrieval Solution) and 3% hydrogen peroxide for blocking endogenous peroxidase. The primary antibodies were sera from dogs naturally infected with VL diluted at 1/100. Biotinylated secondary antibody (LSAB + Sys HRP), streptavidin/biotin complex and diaminobenzidine liquid chromogen (DAB) 60 mg% were also used. The slides were counterstained with hematoxylin, and mounted in Enttelan and examined under light microscopy (1.000×). It was considered a positive reaction when there was a brown marking consistent with morphological structures of amastigotes. A spleen section of an animal parasitologically positive was used as a positive control. Sections of the spleen, heart, kidneys, lungs and brain of a healthy animal were used as negative controls.

## Results

Of the 18 animals infected, 13 (72.2%) had splenomegaly, 10 (55.5%) had apparent weight loss and hepatomegaly and ascites were seen in 6 (33,3%) animals. The average time for symptom onset was 3.5 months.

The direct examination of amastigotes was positive in the spleen and liver of all infected animals, 33.3% had parasites in the kidneys and lungs, and 16.7% in the heart. When brain specimens were examined, amastigotes were seen in 83.3% of the animals examined (Figure 1).

Clinical alterations and parasites were not seen in negative controls on direct examination (Figure 2).

Immunohistochemistry markings with an intense background staining were seen in 12 kidney specimens, and also in negative controls, making the analysis of this organ infeasible. In other organs there was no reaction in negative controls.

Immunomarking did not reveal the presence of parasitic forms in the 15 negative heart specimens in the direct examination of parasites, or in the 18 brain specimens. In the lungs, two specimens were positive by immunohistochemistry.

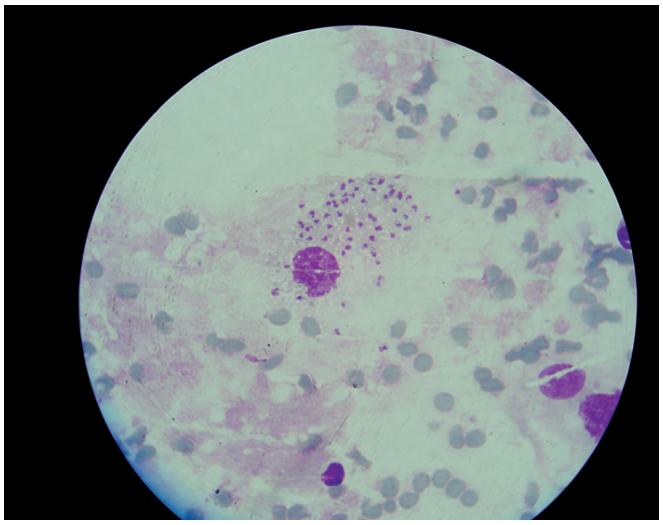

**Figure 1.** Amastigotes in the brain, organ impression, Giemsa staining (1000× magnification).

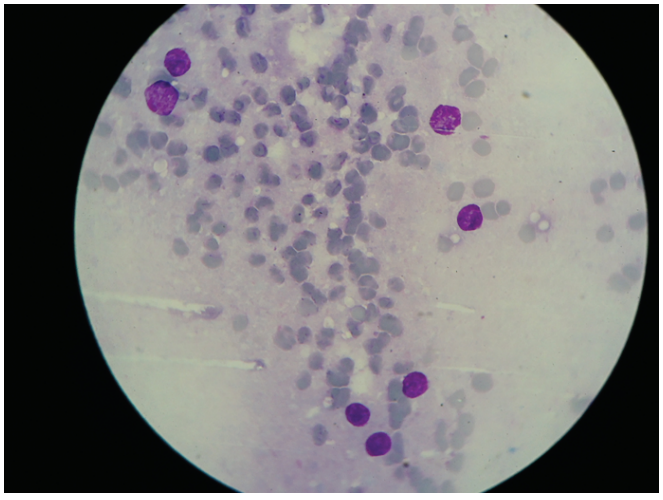

**Figure 2.** Negative control, Giemsa staining (1000× magnification).

## Discussion

The clinical manifestations of weight loss, hepatosplenomegaly and ascites in the inoculated animals are also seen in human and canine cases of VL, in addition to other signs and symptoms that define the broad clinical spectrum of the disease and the confirmation of the parasitic infection (OLIVEIRA et al., 2004; CALDAS et al., 2006).

The frequency and richness of parasites found in the liver and spleen of the animals studied confirm the observations of other authors in experimental infection studies (MELENEY, 1925; GUTIERREZ et al., 1984; MELBY et al., 2001; SOUZA et al., 2001; RIÇA-CAPELA et al., 2003; WYLLIE; FAIRLAMB, 2006), and in canine and human cases of VL undergoing necropsy (MELENEY, 1925; PRAKASH et al., 2006).

A histopathological study in *Cricetulus griseus* hamsters experimentally infected with *Leishmania donovani* has showed that from day 9 post-inoculation parasitized cells in the spleen can be found. In the liver, the presence of Kupffer cells with small numbers of parasites can be seen at day 6 post-inoculation, raising the parasite load in relation to inoculation time (MELENEY, 1925).

The parasitism found in the kidneys (33.3%) can be considered high compared to that reported in the literature, in view of the scarcity of reports about the parasite. Previous studies in humans, dogs and experimental models have not reported the parasite in the tissues here studied and have only described the histological changes induced in the kidneys DUARTE et al., 1983; OLIVEIRA et al., 1985; DUARTE; CORBETT, 1987; POLI, 1991; NIETO et al., 1992; SALGADO FILHO et al., 2003; PRIANTI et al., 2007; ALBUQUERQUE et al., 2008).

Carvalho et al. (2007) reported the absence of parasitic forms of *Leishmania* sp. in hamsters and the finding of antigenic fragments in phagocyte cells in the kidney glomerulus, tubular epithelial cells and interstice, suggesting that the parasite circulates through the kidneys, but it does not remain in the organ.

Background staining in immunohistochemistry in the of kidney slides may be explained by the same factors as described in the literature such as endogenous biotin, hydrophobic interactions, ionic and electrostatic interactions, endogenous peroxidase activity and use of unpurified antibodies (RAMOS-VARA, 2005).

The parasitism in the lungs (33.3%) is less frequently reported in humans and dogs. In humans there are reports of a few infected cells and amastigotes in interstitial tissue, alveolar septa and bronchoalveolar lavage (MELENEY, 1925; ANDRADE, 1959; JOKIPII et al., 1992).

In naturally infected dogs, it has been reported the absence of parasites in the lungs or their presence in a small percentage of animals (DUARTE et al., 1986; GONÇALVES et al., 2003, SILVA et al., 2005).

In this study immunohistochemistry and organ impression techniques proved valuable for parasite examination and, when both were combined, they have yielded high positivity of lung tissue (33.3 to 44.4%).

The heart showed the lowest frequency of parasitic forms, which is consistent with other studies that have reported human cases with parasites seen in the lumen of small vessels and myocardium (MELENEY, 1925; RAMOS et al., 1994), and even the absence of amastigotes in this organ (RASO; SIQUEIRA, 1964).

The high percentage of animals with parasites in the CNS is a major finding. Although scarcely reported, it has been seen in other human and animal laboratories, in which amastigotes were seen in few meningeal cells and choroid plexus as well as in the brain (ABREU-SILVA et al., 2003; MELENEY, 1925; PRASAD; SEN, 1995). Silva et al. (2005) have identified *Leishmania* DNA in the cerebellum of dogs naturally infected with VL.

Prasad and Sen (1995) found parasites in the CSF of human patient with relapsing VL after splenectomy and parasitosis treatment and suggested that parasite migration could be explained by splenectomy.

In this study, the negative results in immunohistochemistry may be explained by the stage of disease as, in advanced cases parasites can be found in atypical organs such as the lungs (DUARTE et al., 1989), lumen of heart and CNS vessels (RAMOS et al., 1994). In the same way, in VL/ HIV association almost all organs that have phagocyte cells can be infected and atypical locations may

occur as a consequence of parasite spread and low immunity (RAMOS et al. 1994; LAGUNA, 2003).

Although the negative results of immunomarking indicated absence of tissue amastigotes, parasitic forms were seen in the CNS through direct examination, which suggests that the parasites were not found in the tissues because they were circulating through the organ.

Ikeda et al. (2007) have argued that the absence of parasites in the CNS reported in naturally infected dogs may be due to non treatment of these animals. According to these authors, amastigotes would migrate to the CNS as a means of escaping from drug action in animals under treatment. Contrasting results were presented by Viñuelas et al. (2001) who found parasites within macrophages in the meninges of dogs with VL as well as extracellular amastigotes.

The present study permitted the visualization of circulating parasites which contrasts with Ikeda et al. (2007) assumption on the potential migration of parasites to escape from antileishmanial drug, since the animals studied did not receive any treatment.

Although neurological signs and symptoms have been described in humans and dogs infected with VL parasite, there is no consensus in the literature regarding the pathogenesis of VL in the CNS (CHUNGE et al., 1985; HASHIM et al., 1995; GARCIA-ALONSO et al., 1996; NOLI, 1999; IKEDA et al., 2007). Convulsions, dilated pupils, signs of cranial nerve palsy, facial ptosis, facial paralysis and ataxia are common neurological signs seen in canine VL (FEITOSA et al., 2000; IKEDA et al., 2007; OLIVEIRA et al., 2004; LIMA et al., 2003).

There are also reports in the medical literature of peripheral neuropathy associated with VL (MUSTAFA, 1965; CHUNGE et al., 1985). Hashim et al. (1995) found that patients gradually recover their health after specific treatment. Although the etiology of this picture has not been determined, the authors challenged the hypothesis of Fasanaro et al. (1991) that VL etiology would be a B-complex hypovitaminosis associated with the absorption of vitamin B by the parasite or by the reduction of thiamine and pantothenic acid.

In addition to vascular congestion, coinfected patients have been reported with the presence of microglia cells with vacuolization without visualization of parasites. Amastigotes and particulate antigenic material have been evidenced by immunohistochemistry only inside the cells in lumen CNS vessels (RAMOS et al. 1994; LAGUNA, 2003).

## Conclusion

Infected animals showed weight loss, hepatosplenomegaly and ascites, and leishmania amastigotes were seen in the spleen, liver, lungs, kidneys and heart. The results also allowed us to determine the circulation of parasitic forms in the CNS, serving as an alert to the professionals responsible for the management of VL patient as to neurological manifestation. Further studies are needed to clarify parasite effects in the CNS, contributing to the understanding of neurological pathogenesis of this major public health issue.

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