Does Leishmaniasis disease alter the parenchyma and protein expression in salivary glands?

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Abstract

Leishmaniasis is considered a serious public health problem in several regions in Brazil and worldwide. This research aimed to perform a histopathological and proteomic study of parotid, submandibular and sublingual glands of BALB/c mice infected by *Leishmania (L) infantum chagasi* using histological, immunohistochemical and epifluorescence techniques. Twelve isogenic BALB/c male mice, around six- to eight-weeks old, were separated into two groups: the animals of the control group were injected with 0.15 ml of NaCl, while those in the experimental group were inoculated with 5×10^6 amastigote forms of *Leishmania (L) infantum chagasi* by the ip route. After 50 days, animals were euthanized and major salivary glands were collected to perform histological, immunohistochemical and epifluorescence techniques using anti-Caspase-2, anti-Ki-67 and anti- β -catenin antibodies, respectively. The histological and morphometric evaluation showed clusters of mononuclear inflammatory cells and a higher area and perimeter of the parotid gland. However, none of the salivary glands had morphophysiological impairment. There was no immunoreactivity to the anti-caspase-2 antibody and Ki67 expression in acinar and ductal cells in both groups. According to the immunofluorescence staining, the β -catenin antibodies did not show nuclear expression, suggesting no uncontrolled proliferation. The data obtained in this study showed population and morphological stability of major salivary glands after 50 days post-infection by *Leishmania (L) infantum chagasi*.

Keywords: Leishmaniasis, salivary glands, immunohistochemistry, antibodies, caspase, beta catenin

Experimental Biology and Medicine 2016; 241: 359–366. DOI: 10.1177/1535370215614658

Introduction

Leishmaniasis is endemic and a major public health problem worldwide. In the Americas, it is caused by protozoan *Leishmania* spp.¹ The genus *Leishmania* includes protozoa that cause various clinical syndromes in humans, ranging from the visceral form of cutaneous Leishmaniasis. The Visceral Leishmaniasis or Kala-Azar is a chronic course of the disease and current estimates are that 0.5 million new cases occur each year worldwide.^{2–7}

Protozoa are transmitted between mammalian hosts during the blood meal of flebotomíneos vectors, whose shape infecting species of *Leishmania* spp. In the new world, they are carried out by the sandfly *Lutzomyia*

subgenus, which inject saliva with promastigotes in susceptible hosts.^{8,9}

The infection can be controlled by the host immune response or evolve quickly for its clinic form, depending on the infecting inoculum and the immune compromised individual.^{10,11}

Many animal models such as mice of the BALB/c strain represent the course of natural infection of visceral leishmaniasis, presenting clinical signs such as ascites, hepatosplenomegaly and progressive cachexia, consistent with aspects described in infected human patients.^{12–14}

The knowledge related to the disease will, in most cases, be limited to the understanding of organ damage, both

individually and systemic. These are based on histopathological and immunohistochemical patterns that identify changes in the expression of regulatory proteins, cell division and death, tissue injury and loss of parenchymal function in organs.¹⁵

This disease is still responsible for a high degree of morbidity and mortality in Mammalian hosts, including humans. It is characterized by the infiltration of amastigotes in different organs such as the liver and lungs,^{16–20} spleen and kidneys,^{17,21} and there are also reports of infection in the oral cavity,²² reaffirming the specificity and ability of *Leishmania* to cause different reactions in each region, resulting in the loss of parenchymal function in addition to local inflammation and cell death.

The salivary glands produce saliva, which is an important component of oral and systemic health maintenance, aiding with digestion, and speech, maintaining the integrity of the teeth and having antibacterial, antifungal and antiviral activity.²³ Mammals possess three pairs of major salivary glands: the parotid, submandibular and sublingual, all of which have a rich vascular plexus and nervous structure, surrounding the secretory and ductal components and relating directly to blood infiltrates.^{24–26}

However, although there are studies linking protozoan infections and the consequent impairment of the salivary glands, such as those associated with *Trypanosoma cruzi*, which causes Chagas disease,^{27,28} research correlating *Leishmania* spp. infection and salivary glands in mammals are lacking in the literature, despite the predilection of the parasites in the salivary glands. Therefore, with the epidemiological importance of the disease in question and the need to know the relationship between visceral leishmaniasis and the salivary glands, the objective of this research was to conduct a histopathological and proteomic study of the parotid, submandibular and sublingual glands in BALB/c mice experimentally infected with *Leishmania (L) chagasi infantum*.

Materials and methods

Ethical and legal aspects

All procedures related to the use of animals were performed according to the standards proposed in the “Guide for the Care and Use of Laboratory Animals” (Institute of Laboratory Animals Resources, National Academy of Science, Washington, DC, 2008), by directive 2010/63/EU (the European parliament and of the council of 22 September 2010), the ethical principles established by the Animal Experimentation of the National Council of Animal Experimentation Control – CONCEA and national legislation as animal-vivisection in force (Law 11,794, of 08.10.2008 and Law 9605 of 02/12/98). The project was approved by the Ethics Committee on Animal Experimentation (CEEA) of the Federal University of Piauí (UFPI), protocol number 095/13.

Animals

To carry out this study, we used 12 inbred mice of the strain BALB/c males, aged six to eight weeks, weighing between

15 and 20 g, and obtained from the vivarium of the Centre for Agricultural Sciences (CCA) UFPI. During the experimental period, the animals received water and balanced food *ad libitum* and were kept in cages with bedding shavings covered with tulle to prevent cross-infection by flies or other insects.

All animals were previously wormed by oral administration (gavage) of Albendazole at a concentration of 0.05 ml/kg (1000 ml oral ricobendazole, 6.0 g of albendazole sulphoxide in 100 ml of vehicle). After 15 days, the procedure was repeated to ensure that all cycles of worms were attained. One week after the last worming, animals were used for the experiment.

Experimental design

The animals were randomly divided into two groups, a control group and an experimental group, each containing six animals. The treatments were as follows: group I (control)—each mouse was injected intraperitoneally with 0.15 ml of 0.9% saline solution (Adv, São Paulo, Brazil) and euthanized after 50 days. Group II (experimental)—each mouse was inoculated intraperitoneally with 5×10^6 purified *Leishmania (L) chagasi infantum* amastigotes (MHOM/BR/72/46 strain) in RPMI 1640 medium (Gibco BRL, Gaithersburg, MD) and euthanized after 50 days.

Identification and dissection of salivary glands

Mice were pre-treated with tramadol hydrochloride (4 mg/kg) and placed in a 10-min lag period. The following were anesthetized with 10 mg/kg Xylazine (Bayer, Istanbul, Turkey) and 60 mg/kg of ketamine (Parker Davis, Istanbul, Turkey) and euthanized with an overdose of sodium thiopental intraperitoneally. The major salivary glands (parotid, submandibular and sublingual) were removed by dissection and immediately immersed in buffered paraformaldehyde solution 0.1 M, pH 7, 40×, where they remained for a period of 48 h. Subsequently, samples were subjected to routine histological processing for haematoxylin and eosin (H&E) and analyzed under a light microscope.

They were also taken from the liver and spleen of the experimental group mice for histological processing and staining (H&E) in order to identify changes due to the infection by *Leishmania (L) chagasi infantum* to prove the efficiency of the experimental model used.

Immunohistochemistry

For immunohistochemistry, the caspase primary antibody mouse anti-2L protein (C-20) (Santa Cruz Biotechnology) was diluted in bovine serum albumin (BSA) at a ratio of 1:150, and the monoclonal primary antibody was mouse anti-protein Ki-67 (Clone MIB-1) Spring®.

After fixation of the salivary gland fragments, they underwent dehydration in ascending alcohol, diaphanization in xylene and were embedded in paraffin. The blocks were submitted to microtomy to a thickness of 4 µm and placed on silanized histological slides. The sections were de-paraffinized in an oven at 60°C for 1 h. Then, the

blades passed through three baths of xylene for 5 min each were hydrated with three baths in absolute alcohol and washed with alcohol in decreasing concentrations (90%, 80% and 70%) for 2 min each and washed in water for 2 min.

Slides underwent an antigen retrieval process in 10 mM citrate buffer, pH 6, heated by microwave in a water bath for 10 min. Afterwards, the slides were cooled in two baths of distilled water and rinsed in PBS. Subsequently, the blocking of endogenous peroxidase was achieved by immersing the slides in 3% hydrogen peroxide solution and rinsing in PBS for 3 min. The cuts followed to step inhibition of non-specific binding and is subject to blocking protein (Kit Reveal-Biotin-free Polyvalent-Spring) for 10 min in a dark humid chamber and then incubated with anti-caspase 2 primary antibodies and ki67 in a moist chamber at about 4°C for 35 min.

After this period, the slides were subjected to two rinsing baths in PBS buffer/Tween for 5 min each. Afterwards, we used the biotinylated secondary antibody (LSAB + System, HRP Universal Code: K0690, Dako) for 30 min, followed by two immersion baths in PBS washing buffer. Then, the streptavidin-biotin-peroxidase complex was applied for 30 min in a humid chamber at room temperature, followed by another two immersion baths in PBS wash buffer for 5 min.

To reveal the samples, diaminobenzidine chromogen substrate (Liquid DAB + substrate, Chromogen System Bulletin: K3468, Dako) was used. The slides were rinsed with distilled water and then counterstained with Harris haematoxylin for 45 s, followed by four baths with distilled water. Dehydration was performed with alcohol baths from 70 to 100% for 5 min each, and two baths of xylene for 5 min each. From this stage, the slides were mounted with Entellan to fix the cover slip and then examined by light microscopy.

Microscopic analysis

The photomicrographs record of preparations was performed by light microscopy using an Olympus light microscope (Olympus Tokyo, Japan) with the Nikon Eclipse E200 digital photographic system.

Immunofluorescence

The slides were incubated with anti- β -catenin antibody (Invitrogen, Life Technologies Brazil) diluted in BSA to 1:100 and kept in a moist chamber at room temperature for 30 min. With these intervals, the blades were subjected to three 5 min baths in PBS solution. Subsequently, under light, the secondary antibody used was Alexa Fluor 488 (green) (goat anti-mouse, Invitrogen), diluted in 1% BSA/PBS (1:250) for 1 h at room temperature. The slides were mounted in glycerol and distilled water (2:1) and then observed in an epifluorescence microscope (Leica Microsystems MD 2000); the objective was increased by 10 $\times$ and 40 $\times$ and photographed using a digital photomicrography system (Leica) and evaluated on the Leica Image software program Manager.

Morphometric analysis

Morphometry, which in this study consisted of the quantitative analysis of acinar dimensions, was performed using imaging software (Image ProPlus Software). Perimeter values were obtained (μ M), and area (μ m²) was photomicroscopically acquired by the analysis of images.

Statistical analysis

Data were tabulated and analyzed using SPSS version 18.0 (SPSS Inc., Chicago, IL). To analyze the groups, ANOVA with Tukey's post-test was applied.

Results

The model used in this study was effective. BALB/c mice infected during the experimental period showed clinical signs of ascites, wasting, loss of hair and apathy. Also, amastigotes were found to imprint in the spleen and lymph nodes; fragments processed in the liver and spleen of these mice revealed histopathological changes. In the spleen, disruption of the splenic architecture was revealed, along with lymphoid depletion with loss of germinal centers, and a large number of megakaryocytes diffusely distributed throughout the parenchyma. The liver tissue showed moderate passive congestion, which also affected three areas of the liver, with hyperplasia of Kupffer cells; some of these cells were still parasitized with amastigotes and the presence of soft, diffuse lymphocytic infiltrate in addition to the focal array of immature granuloma forms of amastigotes (Figure 1).

The salivary glands of BALB/c mice in the control group showed homogeneity between the serous acinar cells of the parotid gland, with pyramidal aspect morphology, willing nucleus in the basal region and finely granular cytoplasm (Figure 2(a)). The submandibular and sublingual glands present as mixed tubulointerstitial alveolar glands with the presence of granular convoluted tubules in the submandibular gland (Figure 2(b)), whereas the sublingual mucous gland acinar cells were fusiform, with mucus in the core periphery and the cytoplasm, and the presence of striated appearance ducts with cells (Figure 2(c)).

The histological findings of the three major salivary glands of animals infected by *Leishmania (L.) infantum chagasi* at 50 days showed that the cytoplasm of serous acinar cells of the parotid gland lost its homogeneity and had a slightly granular appearance (Figure 2(d)), with the presence of multifocal clusters of mononuclear inflammatory cells (arrow). There was an increase in granulation of the granular convoluted tubules cells of submandibular glands compared to the control group (Figure 2(e)), while acinar cells of both mucosal, submandibular and sublingual areas showed preserved morphology at 50 days post-infection (Figure 2(f)).

Table 1 shows the average perimeter and area of acini of the salivary glands; a significant difference can be observed between the means and the perimeter of the area around the parotid gland. The same was not observed in other glands.

There was no expression of anti-caspase-2 protein in any of the three glands studied in both the control group and the experimental group (Figure 3). Immunohistochemical

Figure 1 Photomicrograph of the fragments. (a) Liver and (b) spleen from BALB/c mice infected with 5×10^6 amastigotes of *Leishmania (L.) infantum chagasi* 50 days post-infection. The liver is sinusoidal congestion with hepatocytes having proliferation nucleus pyknosis and Kupffer cells and granuloma (arrowhead) with amastigotes (*). In the spleen megakaryocytes are observed diffusely distributed throughout the parenchyma (arrows). Liver (HE400 $\times$, bar—40 μ m) (100 $\times$, bar—5 μ m*). Spleen (HE400 $\times$, bar—40 μ m). (A color version of this figure is available in the online journal.)

Figure 2 Photomicrograph fragments from the parotid, submandibular and sublingual BALB/c uninfected (a, b and c) and experimentally infected (50 days) with *Leishmania (L.) infantum chagasi* (d, e and f). Presence of inflammatory infiltration (arrow) (d). There is a subtle loss of homogeneous and slightly granular cytoplasmic characteristic of acinar cells (HE 400 $\times$, bar—40 μ m). (A color version of this figure is available in the online journal.)

analysis of the anti-Ki67 antibody of the salivary glands cuts showed no nuclear staining in the acinar and ductal cells in both the control and experimental groups (Figure 4).

Immunostaining for the expression of β -catenin was based on nuclear localization, and the cytoplasmic and membrane surface. Thus, there was immunostaining on the surface of the mucous membrane of sublingual gland acinar cells, both in tissue samples from the control group and the experimental group. Still, the striated ducts of the sublingual gland in both groups showed strong cytoplasmic labeling. However, no labeling was observed in tissue

samples of the parotid and submandibular gland, in both the control and experimental groups 50 days post-infection (Figure 5).

Discussion

The murine model for the experimental study of visceral leishmaniasis is well established and mimics numerous immunological and pathogenic aspects of canine and human natural infection.^{12,17,29} As expected, it was also effective for the study of morphological characteristics of

Table 1 Mean and standard deviation, perimeter and area of acini of the salivary glands of BALB/c mice

	Parotid M ($\pm$ DP)	Submandibular M ($\pm$ DP)	Sublingual M ($\pm$ DP)
Perimeter (μ m)			
Control	10.92 ($\pm$ 4.06)	5.75 ($\pm$ 1.504)	11.71 ($\pm$ 2.98)
Trial	14.36 ($\pm$ 2.14)	5.11 ($\pm$ 1.620)	10.55 ($\pm$ 4.26)
<i>p</i>	0.059	0.614	0.559
Area (μ m ²)			
Control	168.66 ($\pm$ 45.20)	137.0 ($\pm$ 137.07)	356.76 ($\pm$ 52.72)
Trial	302.45 ($\pm$ 95.59)	379.05 ($\pm$ 109.2)	398.94 ($\pm$ 173.8)
<i>p</i>	0.031	0.579	0.501

M: mean; $\pm$ DP = $\pm$ standard deviation.Test-t, *p* < 0.05. **Figure 3** Photomicrograph of fragments of the parotid glands, submandibular and sublingual BALB/c mice uninfected (control) and experimentally infected (50 days) with *Leishmania (L.) infantum chagasi*. Immunohistochemical analysis using anti-caspase-2 antibody, which is not observed marking ductal and acinar cells (400 $\times$ magnification, bar—40 μ m). (A color version of this figure is available in the online journal.)

the salivary glands of infected animals and is reflected in a model that can be used in other studies.

The major salivary glands of mice have morphological and physiological characteristics similar to those of humans, except for the presence of granular convoluted ducts located between the interim and striated ducts of the submandibular gland.³⁰ It was observed that the parotid gland in the experimental group showed multifocal clusters of mononuclear inflammatory cells, but with maintenance of normal histological structure and the submandibular and sublingual glands showed no significant change 50 days after infection.

Histologically, the salivary glands are composed of different cell types: acinar cells, ductal cells and myoepithelial cells. The acinar cells are innervated by both sympathetic and parasympathetic branches of the autonomic nervous system (ANS) and are responsible for stimulating salivary secretion.^{31–33} Furthermore, stimulation of the parasympathetic SNA promotes increased α -amylase synthesis and

restores its stock serosa acinar cells, a characteristic that means that the enzyme is currently used as a biomarker for several organic systemic changes.^{31,33} By morphometric analysis, an increase in perimeter and serous acini areas was revealed at late stages of infection. It is believed that chronic inflammation may cause this parasympathetic stimulation and consequent increase in glandular size, since these animals are able to monitor the total parasite load in the final post-infection stages by maintaining a chronic low-grade infection.

Still, histologic and morphometric analysis revealed population stability of major salivary glands in infection. It is known that the tissue population control is orchestrated by apoptosis mechanisms, and the loss of this control is closely related to the emergence of cancer. Indeed, apoptosis is associated with the elimination of unwanted cells and is mediated by the family of cysteine proteases called caspases.^{34–36}

Figure 4 Photomicrograph of parenchymal cuts salivary glands to verify the Ki67 expression in mice uninfected and experimentally infected with *Leishmania (L.) infantum chagasi* after 50 days. Note that no nuclear immunostaining with Ki67 in acinar cells in the control group and the experimental group glands. 400 $\times$ magnification. (A color version of this figure is available in the online journal.)

Figure 5 Photomicrograph of fragments of the parotid glands, submandibular and sublingual BALB/c mice uninfected (control) and experimentally infected (50 days) with *Leishmania (L.) infantum chagasi*. Notes a strong cytoplasmic immunostaining for β -catenin in striated ductal cells of the sublingual gland and similarly in both the control group and in post-infection 50 days group (increase of 400 $\times$, bar—40 μ m). (A color version of this figure is available in the online journal.)

Immunohistochemical analysis showed the expression of anti-caspase-2 antibody in both control and experimental groups, which indicates the absence of apoptotic phenomena in the salivary glands, even in animals subjected to a period of 50 days of infection; therefore, it was possible to measure how the three salivary glands are able to maintain homeostasis in the population, even in the presence of mild mononuclear inflammatory infiltrate, primarily in the case of the parotid gland.

Ki-67 monoclonal antibody reacts with the core antigen in all cell cycle phases except G0. The antigenic expression is negative in G0 and early G1 phases of the cell cycle and positive for the remainder of the cycle, reaching peak levels in G2 and M phases³⁷; this is considered a reliable labeling protein in cell proliferation.

In the present study, the nuclear staining for Ki-67 in the control and experimental groups was not observed. Herrera-Esparza et al.³⁸ evaluated proliferation and apoptosis in the

salivary glands of patients with Sjögren's syndrome; also no Ki67 labeling was detected in acinar cells, only lymphoid cell infiltration of the gland. Costa et al.³⁹ performed a study using Ki-67 (MIB-1) on renal injuries of dogs naturally infected with LV and observed markings indicative of cell proliferation only in inflammatory cells located in adjacent areas of the glomeruli; this was absent in glomerular cells.

β -catenin functions as a component of adherents junctions in simple epithelia and is an essential element for the connection of various cadherins to the cytoplasm, such as E-cadherin, which is involved in the differentiation of acinar cells during morphogenesis.^{40,41} With respect to anti- β -catenin antibody, a slight tag of this protein was observed at the plasma membrane surface in acinar cells; similarly, mucosal cells showed no difference in immunostaining in the nucleus between the experimental group and the control group, thereby suggesting the maintenance of tissue homeostasis, even in the 50-day post-infection period.

The greater intensity of immunostaining for anti- β -catenin was observed in the ducts of the sublingual glands, in both control and experimental samples. This may be associated with the type of secretion produced by these glands and transported actively by the duct system. The mucous secretion is rich in mucins and synthesized in the sublingual; this is believed to have an important role in protecting the oral epithelial surfaces, as well as the host defense system of the oral cavity.⁴²

It is known that Wnt/ β -catenin signaling plays an important role in the renewal of hematopoietic stem cells, which have a constant capacity to renew and give rise to all lineages of the blood system.⁴³ This significant immunostaining provides evidence to suggest that the results of this study are consistent, since there was higher expression of the protein in blood cells found in the vessels supplying the salivary glands.

The salivary glands of BALB/c mice, when inoculated with 5×10^6 U *Leishmania infantum chagasi* and evaluated after infection (50 days) using regulatory proteins of the cell cycle caspase-2, Ki-67 and β -catenin, showed no significant changes in parenchyma and ducts. Future research should be conducted in an attempt to identify the expression of other proteins in addition to the mechanisms that produce these characteristics to the salivary glands.

Authors' contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; AMCJ: Effective scientific, intellectual contribution to the study, creator of the design; FAAC; WOD; LCM; ABSS: Technical procedures and acquisition of data; MMASC; IMO: Manuscript writing; BBS; MSR; CMCL; SMSM: Macroscopic and histopathological examinations; LFADM; MDML: Interpretation of data and critical review. Approved the version to be published.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

FUNDING

The author(s) received no financial support for the research, authorship, and/or publication of this article.

REFERENCES

- Santos DO, Coutinho CER, Madeira MF, Bottino CG, Vieira RT, Nascimento SB, Bernardino A, Bourguignon SC, Corte-Real S, Pinho RT, Rodrigues CR, Castro HC. Leishmaniasis treatment a challenge that remains: a review. *Parasitol Res* 2008;**103**:1-10
- Silva-Almeida M, Pereira BAS, Guimarães MLR, Alves CR. Proteinases as virulence factors in leishmania spp. *infection in mammals*. *Parasit Vectors* 2012;**5**:1-10
- Melo EC, Fortaleza CMCB. Challenges in the therapy of visceral leishmaniasis in Brazil: a public health perspective. *J Trop Med* 2013;**2013**:1-5
- Verma S, Kumar R, Katara GK, Singh LC, Negi NS, Ramesh V, et al. Quantification of parasite load in clinical samples of leishmaniasis patients: IL-10 level correlates with parasite load in visceral leishmaniasis. *Plos One* 2010;**5**:101-7
- Murray HW, Berman JD, Davies CR, Saravia NG. Advances in leishmaniasis. *Lancet* 2005;**366**:1561-77
- WHO. World Health Organization Disponível em, www.who.int/Leishmaniasis/em (accessed 31 March 2015)
- Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, Cano J, Jannin J, den Boer M, WHO Leishmaniasis control team. Leishmaniasis worldwide and global estimates of its incidence. *Plos One* 2012;**7**:e35671
- McCarthy CB, Santini MS, Pimenta PFP, Diambra LA. First comparative transcriptomic analysis of wild adult male and female *Lutzomyia longipalpis*, vector of visceral leishmaniasis. *Plos One* 2013;**8**:1-20
- Andrade BB, Oliveira CI, Brodskyn CI, Barral A, Barral-Neto M. Role of sand fly saliva in human and experimental leishmaniasis: current insights. *Scand J Immunol* 2007;**66**:122-7
- Murray HW, Hariprasad J, Ficht RE. Models of relapse of experimental visceral leishmaniasis. *J Infect Dis* 1996;**173**:1041-3
- Carrión J, Nieto A, Iborra S, Iniesta V, Soto M, Folgueira C, Abanades DR, Requena JM, Alonso C. Immunohistological features of visceral leishmaniasis in BALB/c mice. *Parasite Immunol* 2006;**28**:173-83
- Loría-Cervera EN, Andrade-Narváez FJ. Animal models for the study of leishmaniasis immunology. *Rev Inst Med Trop Sao Paulo* 2014;**56**:1-11
- Pastorino AC, Jacob CM, Oselka GW, Carneiro-Sampaio MM. Visceral leishmaniasis: clinical and laboratory aspects. *J Pediatr* 2002;**78**:120-7
- Jeronimo SMB, de Queiroz Sousa A, Pearson RD. Leishmaniasis. In: Guerrant RL, Walker DH, Weller PF (eds). *Tropical infectious diseases: principles, pathogens and practice*. Edinburgh, Scotland: Churchill Livingstone Elsevier, 2006, pp. 1095-1113
- Silva FS. Patologia e patogênese da leishmaniose visceral canina. *Rev Trop - Ciênc Agrár e Biol* 2007;**1**:20-31
- Oliveira E, Oshiro ET, Pinto RV, Castro BC, Daniel KB, Guimarães EB, Silva JM, Dorval ME. Presence of amastigotes in the central nervous system of hamsters infected with leishmania sp. *Rev Bras Parasitol Vet* 2011;**20**:97-102
- Awasthi A, Mathur RK, Saha B. Immune response to leishmania infection. *Indian J Med Res* 2004;**119**:238-58
- Diehl AR, Santos RP, Zimmerman R, Luz LP, Weiss T, Jacobson P, Goldani LZ. Microscopy and polymerase chain reaction detection of *Leishmania chagasi* in the pleural and ascitic fluid of a patient with AIDS: case report and review of diagnosis and therapy of visceral leishmaniasis. *Can J Infect Dis Med Microbiol* 2004;**15**:231-4
- Costa NSS, Cerri GG. Interstitial pulmonary alterations in visceral leishmaniose: evaluation with high-resolution computed tomography. *Radiol Bras* 1999;**32**:159-67
- Mana L, Paciello O, Morte RD, Gravino AE. Detection of leishmania parasites in testis of a affected by orchitis: case report. *Parasit Vectors* 2012;**5**:1-4
- Sagnelli C, Martino FD, Coppola N, Crisci A, Sagnelli E. Acute liver failure: a rare clinical presentation of visceral leishmaniasis. *New Microbiol* 2012;**35**:93-5

22. Mota LAA, Miranda RR. Manifestações dermatológicas e otorrinolaringológicas na leishmaniose. *Arq Int Otorrinolaringol* 2011;**15**:376–81
23. Choi JS, Park IS, Kim S, Lim JY, Kim YM. Analysis of age-related changes in the functional morphologies of salivary glands in mice. *Arch Oral Biol* 2013;**58**:1635–42
24. Junqueira LC, Carneiro J. *Histologia básica*, 12ed. São Paulo: Editora Guanabara, 2013:488
25. Klatte E. *Atlas de patologia*, 2nd ed. Philadelphia: Elsevier, 2010:570
26. Souza GD, Czeckzo NG, Moreira H, Filho JMR, Mafafaia O, Czeckzo LE, Thiele ES, Aguiar LR. Expressão citofotométrica do fator de proliferação celular ki-67 no bócio colóide e no carcinoma papilífero da tireóide. *Rev Col Bras Cir* 2009;**36**:105–9
27. Moreira A, Napimoga MH, Benatti BB, Silva GA, Rocha-Rodrigues DB, Clemente-Napimoga JT, Vieira GA, Alves JB. Morphological changes and EGF expression in the granular convoluted tubule cells of submandibular glands of *Trypanosoma cruzi* infected rats. *Tissue Cell* 2008;**40**:293–8
28. Aguirre KL, Alves JB, Silva GA, Cardoso JE, Murta SM, Ferreira AJ. Epidermal growth factor receptors, testosterone levels and parotid gland changes in rats infected with *Trypanosoma cruzi*. *Mem Inst Oswaldo Cruz* 2009;**104**:715–9
29. Mukherjee P, Ghosh AK, Ghose AC. Infection pattern and immune response in the spleen and liver of BALB/c mice intracardially infected with leishmania donovani amastigotes. *Immunol Lett* 2003;**86**:131–8
30. Yamagishi R, Wakayama T, Nakata H, Adthapanyawanich K, Kumchanteuk T, Yamamoto M, Iseki S. Expression and Localization of α -amylase in the submandibular and sublingual glands of mice. *Acta Histochem Cytochem* 2014;**47**:95–102
31. Nater UM, Rohleder N. Salivary alpha-amylase as a non-invasive biomarker for the sympathetic nervous system: current state of research. *Psychoneuroendocrinology* 2009;**34**:486–96
32. Actis AB, Lampe PD, Eynarda AR. Cellular basis and clinical implications of biological markers in salivary tissues: their topological distribution in murine submandibular gland. *Oral Oncol* 2002;**38**:441–9
33. Granger DA, Kivlighan KT, El-Sheikh M, Gordis EB, Stroud LR. Salivary alpha-amylase in biobehavioral research: recent developments and applications. *Ann N Y Acad Sci* 2007;**1048**:122–44
34. Bruchhaus I, Roeder T, Rennenberg A, Heussler VT. Protozoan parasites: programmed cell death as a mechanism of parasitism. *Trends Parasitol* 2007;**23**:376–83
35. Kumar S. Caspase function in programmed cell death. *Cell Death Differ* 2007;**14**:32–43
36. Vakifahmetoglu-Norberg H, Norberg E, Perdomo AB, Olsson M, Ciccocanti F, Orrenius S. Caspase-2 promotes cytoskeleton protein degradation during apoptotic cell death. *Cell Death Dis* 2013;**4**:940
37. Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. *J Cell Physiol* 2000;**182**:311–22
38. Herrera-Esparza R, Bollain-y-Goytia J, Ruvalcaba C, Ruvalcaba M, Pacheco-Tovar D, Avalos-Díaz E. Apoptosis and cell proliferation: the paradox of salivary glands in Sjögren's disease. *Acta Reumatol Port* 2008;**33**:299–303
39. Costa FAL, Prianti MG, Silva TC, Silva SMMS, Guerra JL, Goto H. Cells, adhesion molecules and modulation of apoptosis in visceral leishmaniasis glomerulonephritis. *BMC Infect Dis* 2010;**10**:1–12
40. Peifer M, Sweeton D, Casey M, Wieschaus E. Wingless signal and Zeste-white 3 kinase trigger opposing changes in the intracellular distribution of Armadillo. *Development* 1994;**120**:369–80
41. Patel VN, Hoffman MP. Salivary gland development: a template for regeneration. *Semin Cell Dev Biol* 2014;**25–26**:52–60
42. Tabak LA. In defense of the oral cavity structure, biosynthesis and function of salivary mucins. *Annu Rev Physiol* 1995;**57**:547–64
43. Reya T, Ducan AW, Ailles L, Domen J, Scherer DC, Willert K, Hintz L, Nusse R, Weissman IL. A role for Wnt signalling in self-renewal of haematopoietic stem cells. *Nature* 2003;**423**:409–14

(Received August 7, 2015, Accepted October 1, 2015)