Histol Histopathol (2010) 25: 877-887

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Histopathological and parasitological investigations of ear healthy skin of dogs naturally and experimentally infected with *Leishmania (Leishmania) chagasi*

Maria Marta Figueiredo[1], Eliane Perlatto Moura[1], Miriam Maria Costa[2], Vitor Marcio Ribeiro[3], Marilene Suzan Michalick[4], Washington Luiz Tafuri[5] and Wagner Luiz Tafuri[1]

[1]Department of General Pathology, [2]Department of Biochemistry, [4]Department of Parasitology, Federal University of Minas Gerais, [3]Veterinary Preventive Medicine, Catholic Pontificia University of Minas Gerais, Belo Horizonte, Brazil and Institute of Biological Sciences, [5]Department of Anatomic Pathology, School of Medicine Institute, Belo Horizonte, Brazil

Summary. Although 90% of clinical cases of American visceral leishmaniasis (AVL) occur in the northeastern region of Brazil, the incidence of cases in recent years has increased in southeastern states such as Minas Gerais (MG), where the disease has been reported in several cities, including Belo Horizonte, the state capital. Some studies have shown a strong correlation between the incidence of AVL and canine visceral leishmaniasis (CVL) in Belo Horizonte. A study of 108 dogs with parasite *Leishmania chagasi* detected by immunohistochemistry in healthy ear skin was obtained from two distinct geographical areas: 55 from a metropolitan area of the municipality (Santa Luzia, MG) and 53 dogs from a central area of Belo Horizonte. In parallel, a group of 10 beagles were experimentally infected with *L. chagasi*. Considering the clinical aspects of all naturally infected dogs, symptomatic dogs were more frequent than asymptomatic ones, especially animals from the metropolitan area compared with the central area (79.6% and 20.3%, respectively). A chronic exudate was observed in the ear of 51 out of 55 dogs naturally infected from the metropolitan area (92.7%) and 45 out of 53 dogs naturally infected from the central area (84.9%). Importantly, asymptomatic dogs from the central area harbor more parasites in the skin than the asymptomatic ones from the metropolitan area. In addition, a profound difference was noted in the intensity of the inflammatory reaction and parasite load in the skin of experimental infected dogs.

Offprint requests to: Wagner Luiz Tafuri, Universidade Federal de Minas Gerais, Instituto de Ciências Biológicas – Depto de Patologia Geral – Avenida Antônio Carlos, 6627, CEP 31.270-901, Campus Pampulha - Belo Horizonte – MG/Brasil. e-mail wagner@icb.ufmg.br

Key words: Canine Visceral Leishmaniasis, Ear Skin, Histopathology, Immunohistochemistry

Introduction

Human Visceral Leishmaniasis (HVL), commonly known as "kala-azar", from the Hindu vernacular ("kal" = death; "kala" = dark skin and "azar" = disease) (Ross, 1899; Brahmachari, 1928), is a systemic disease caused by intracellular protozoan of genus *Leishmania sp*. Over half a million new cases occur worldwide each year, of which 10% die because the disease is fatal if left untreated (Liew and O'Donnell, 1993). HVL remains a serious public health problem in the world. Dogs are the most important reservoir of the disease, indeed, the importance of dogs as a reservoir of HVL has been acknowledged for a long time because canine outbreaks have often preceded human cases in the Mediterranean basin, Central Asia, China and South America (Nicolle, 1910; Yakimoff, 1913; Young and Hertig, 1926; Chagas, 1937; Deane and Deane, 1954; Desjeux, 2004).

In Brazil, HVL is known as American Visceral Leishmaniasis (AVL), it is more than a chronic infection with high lethality, being recognized as a zoonotic disease. Although 90% of clinical cases of AVL occur in the northeastern region of the country, in recent years the incidence of cases has increased in southeastern states such as Minas Gerais, where it has been reported in several cities, including Belo Horizonte, the state capital (Luz et al., 2001). Ecological studies using spatial analysis show an association between the incidence of AVL and seropositivity in dogs between 1994 and 1997 in Belo Horizonte (Oliveira et al., 2001; Margonari et al., 2006). Both AVL and canine visceral leishmaniasis

(CVL) are caused by *Leishmania chagasi (*syn = *L. infantum)* (Mauricio et al., 2001), which is transmitted by the bite of an infected phlebotomine, *Lutzomyia (Lutzomyia) longipalpis*. Brazil has experienced a sharp increase in the number of cases of CVL and in consequence HVL since 1999. In this country, which has historically experienced rural epidemics in ten-year cycles, the disease is also appearing in an urban form in addition to the rural form. There has been a large migration of the population from rural areas to the peripheral suburbs of large cities, creating densely populated settlements with minimal infrastructure and sanitation. Primitive houses, inevitably with dogs, chicken houses and other animal shelters, provides an excellent habitat for *L. longipalpis* females that have repetitive feeding habits and quickly invade such habitations (Lainson and Rangel, 2005; Tesh, 1995; Moreno et al., 2005). Thus, considering that infected phlebotomines are able to adapt easily to a new habitat, they will encounter a vast number of non-immune hosts who are often malnourished (Deusjeux, 2004; Bern et al., 2006), or they may already exist in these impoverished urban areas (Tesh, 1995; Arias et al., 1996).

Earlier reports of CVL described various macroscopical skin lesions (desquamation, alopecia, pustular dermatitis, ulcerative dermatoses and nodular disease) the type depending on the immune response provoked (Adler and Theodor, 1931; Torres, 1941; Ferrer et al., 1988; Ciaramella et al., 1997; Lima et al., 2004; Melo et al., 2008). The skin is seen as an important source for parasites in healthy and sick *Leishmania*-infected dogs, hence the important role of dogs in VL transmission is supported by the high parasite loads found in the skin of infected animals (Deane and Deane, 1962; Abranches et al., 1991). Histopathological changes in the skin of *Leishmania*-infected dogs consist of variable degrees of focal or diffuse inflammatory infiltrate in the dermis, and variable numbers of plasma cells, macrophages (parasitized or not by amastigotes of *L. chagasi*), lymphocytes and isolated neutrophils (Torres, 1941; Tafuri et al., 2001; dos-Santos et al., 2004; Solano-Gallego et al., 2004; Xavier et al., 2006). Histological changes in CVL are probably triggered by the type of host immune response. Many authors have reported differences between the development of a natural and experimental infection, which could be explained by the inoculum not including sandfly saliva (Santos-Gomes et al., 2000).

CVL was firstly reported in the suburbian area of the municipality of Belo Horizonte in 1989 (Michalick et al., 1993). Since these studies a significant increase of the visceral disease in dogs and in human beings has been reported. In this work we have made thorough histological (morphometry of the chronic inflammatory reaction) and parasitological (immunohistochemistry) analyses of ear skin of dogs from Belo Horizonte (Margonari et al., 2006). A parallel group of experimentally infected beagle dogs with *L. chagasi* was also investigated. The main objective was to compare the differences between the groups, and examine the correlations between the distinct clinical status, histological aspects and the parasite-load in the ear healthy skin.

Materials and methods

Animals

Group I (Control dogs)

The first group included 25 uninfected mongrel dogs unselected for gender and age (but mature), but which were serologically (indirect immunofluorescence - IFI and enzyme-linked immunosorbent assay - ELISA) and parasitologically negative exams for Leishmania (immunohistochemistry) (Tafuri et al., 2004). These animals were divided into two subgroups: (1) 20 dogs from a metropolitan area of municipality of Belo Horizonte (Santa Luzia, state of Minas Gerais, Brazil), obtained from the Zoonoses Center of Santa Luzia; and (2) 5 healthy and well-fed mongrel dogs maintained in the Kennels of Instituto de Ciências Biológicas (ICB), Universidade Federal de Minas Gerais (UFMG) from the non-endemic area (Barbacena, MG). These dogs were given water *ad libitum* and were kept under constant scrutiny for health problems by veterinarians. During the experiments, all invasive procedures were done by ethically approved procedures for animal experimentation and biosafety.

Group II (Naturally infected dogs)

This consisted of 108 mongrel dogs naturally infected with *L. chagasi* obtained from two distinct Zoonoses Centers: (1) 55 were from a suburban area of metropolitan area of Belo Horizonte (Municipality of Santa Luzia, state of Minas Gerais, Brazil); (2) 53 were from a central area of municipality of Belo Horizonte, state of Minas Gerais, Brazil, obtained from Veterinary Particular Clinical Santo Agostinho.

All dogs were positive for *Leishmania* by indirect immunofluorescence (IFI) (title > 40) and enzyme-linked immunosorbent assay (ELISA with optical density > 100 > 1:400 dilutions). Previous works with dogs obtained from the municipality of Belo Horizonte were positive for *Leishmania chagasi*, as shown by a polymerase chain reaction (PCR) protocol. Indeed, liver tissue from a naturally infected dog showed a conserved region of kinetoplastidae (kinetoplast mini-circle DNA or kDNA) and hybridization with kDNA probes verified the presence of *L. chagasi*. kDNA, present at ~1000 copies per cell, has been used as a target for the selective amplification of parasite DNA (Tafuri et al., 2001). Immunlabeled amastigotes forms of *Leishmania*, which were present in all skin samples of these animals, were detected in accordance with Moura et al. (2008)

proceedings.

Group III (Experimentally infected dogs)

A group of ten 3-9-month-old laboratory-bred beagles (5 males and 5 females) were inoculated intravenously (saphenous vein) with $5x10^7$ promastigotes/mL resuspended in PBS. The infection was confirmed by seroconversion (IFI and ELISA), immunohistochemistry and polymerase chain reaction (PCR) tests of ears skin tissues (parasitological tissue confirmation) was evident after 90 days post-infection. In ELISA, cut-off values were determined from average optical densities plus two standard deviations, obtained from negative sera. Only healthy and well-fed animals under constant scrutiny for health problems by a veterinarian, presenting normal hematological and biochemical parameters and that had received their routine vaccinations against leptospirosis, distemper, adenovirosis-2, hepatitis, parainfluenza and parvovirosis were included in the study. Dogs were also treated with anti-helminthes drugs, and maintained according to the International Guiding Principles for Biomedical Research Involving Animals. The experimental design and all the conditions of animal maintenance and handling were approved by the Brazilian Public Animal Health and Agriculture authorities (MAPA21028. 007698/2003-15) (Fernandes et al., 2008).

Ethical approval

All animal studies were performed under the guidance and approval of the institute's animal welfare committee under the supervision of a certified veterinarian. The experimental protocol using dogs was approved by CETEA (Comitê de Ética em Experimentação Animal – UFMG), number 190/2006.

Preparing parasites for experimental infection

Promastigotes of *Leishmania (Leishmania) chagasi* strain (MCAN/BR/2000/BH400) were maintained in hamsters and cultivated *in vitro* with α-MEM medium (Gibco BRL) with 10% fetal bovine serum (Sigma) supplemented with 200U of penicillin/ml (Sigma), 100 μg of streptomycin/ml (Sigma) at pH 7.4 and maintained at 23°C in a FANEM® incubator (model 347). The *Leishmania* promastigotes in stationary phase were washed once with phosphate buffered saline (PBS), centrifuged at 200g for 10 min/18°C, and resuspended to $5x10^7$ parasites/mL in PBS.

Clinical aspects of the infected dogs

All infected dogs were clinically classified as follows: (1) symptomatic dogs: animals exhibiting the classical signs of the disease - cutaneous alterations (alopecia, dry exfoliative dermatitis or ulcers, onychogryphosis, keratoconjunctivitis), cachexia, and anemia, lymphadenopathy and weight loss, and (2) asymptomatic dogs - apparently healthy animals (Lima et al., 2007).

Histopathology

Healthy ear skin samples, without scars or other pathologies, were obtained in different protocols because samples could be obtained from different conditions. Except for the dogs obtained from the Veterinary Clinics (53 dogs), all the others (90 dogs = 25 control dogs, 55 infected dogs from the metropolitan area and 10 experimentally infected dogs) were anesthetized with a dose of 0.5ml/kg Thiopental® iv 2.5% and put down with T61® (0.3 ml/kg). Samples of ear skin were collected and fixed in 10% neutral buffered formalin. In addition, ear samples from Veterinarian Clinical Dogs were received by our laboratory as ear biopsies that had been taken by professional veterinarians, and fixed immediately in 10% neutral buffered formalin. These samples are part of our own departmental material (Departamento de Patologia Geral, ICB/UFMG), because of its co-operative with many Veterinarian Clinics in Belo Horizonte for Canine Visceral Leishmaniasis diagnosis (extension of project number 5830/28, University CENEX/UFMG).

A complete necropsy was carried out on dogs that had been euthanized. Samples of liver, spleen, cervical lymph nodes, bone marrow, kidneys, lungs and skin were collected. They were dehydrated, cleared, embedded in paraffin, cut into 4 μm thick sections and stained with hematoxylin and eosin (H&E) for histological and parasitological studies.

Histological studies: semi-quantitative and quantitative (morphometrical analysis) of the inflammatory reaction of canine skin tissues

Under light microscopical analysis, chronic inflammatory reaction in the dermis was quantified. Twenty images were randomly chosen and used to count the mononuclear nucleated cells in dermis. For the semi-quantitative analysis the inflammatory mononuclear cells in the dermis were counted by ocular analysis following the score: (1) discrete: 1-9 cells per field/20 fields; (2) moderate: 10-30 cells per field/20 fields; (3) intense: > 30 cells per field/20 fields. For the quantitative analysis the images were analyzed by software, viewed on a computer video screen and sent to a computer-assisted image analysis system (Kontron Elektronic/Carl Zeiss, Germany) (Caliari, 1997; Gonçalves et al., 2003; Lima et al., 2007).

Immunohistochemical (IHC) studies: semi-quantitative and quantitative (morphometrical analysis) of the parasite tissue burden of canine skin tissues

Deparaffinized slides were hydrated and incubated with 4% hydrogen peroxide (30 v/v) in 0.01 M PBS, pH

7.2, followed by incubation with normal goat serum (diluted 1:50). Heterologous immune serum from dogs naturally infected with *L. chagasi* (diluted 1:100 in 0.01 M PBS) was used as the primary antibody "cross-reactive as described by Tafuri et al. (2004)". The slides were incubated for 18–22 h at 4°C in a humid chamber, washed in PBS, incubated with biotinylated goat anti-mouse and anti-rabbit (Link-DAKO, LSAB2 kit, California, USA), washed once with PBS, and incubated with streptoavidin-peroxidase complex (Link-DAKO, LSAB2 kit, California, USA) for 20 min at room temperature. The reaction was developed with 0.024% diaminobenzidine (DAB: Sigma, Saint Louis, USA) and 0.16% hydrogen peroxide (40 v/v). The slides were counterstained with Harris's hematoxylin, dehydrated, cleared and mounted with coverslips. For the semi-quantitative analysis the parasite load in the dermis was counted by ocular analysis following the score: (1) discrete: 1-9 amastigotes per field/20 field; (2) moderate: 10-30 amastigotes per field/20 fields; (3) intense: > 30 amastigotes per field/20 fields. Quantitative study was carried out for optical microscopy and morphometrical analysis. Twenty images were randomly chosen and used to count immunolabeled amastigotes. The images were analysed as described in the previous section obtained by software, viewed on a computer video screen and relayed to a computer-assisted image analysis system (Kontron Elektronic/Carl Zeiss, Germany), as previously discussed (Lima et al., 2007).

Statistical analysis

Descriptive analyses were given for results on histological and parasite load. The program Prism 4.0 was used for analyses. The Kruskal Wallis test was carried out on differences between H&E and IHC. The Spearman test was used to detect any correlation between parasite load and inflammatory response. $P<0.05$ was considered significant.

Results

Clinical aspects

Of all the naturally infected dogs from the metropolitan and central area of the municipality of Belo Horizonte, we found 86 symptomatic (79.6%) and 22 asymptomatic dogs (20.3%). The principal clinical signs were lymphadenopathy followed by skin lesions. Exfoliative dermatitis was found mainly in the face, around the eyes and in the pinna. Only 3 experimentally infected dogs (30%) developed clinical signs of the disease. Lymphadenopathy was the most obvious clinical alteration and only one of the symptomatic dogs had skin lesions.

Histopathology

Chronic dermatitis was seen histologically in 5 control dogs (20%), 51 dogs (92.7%) naturally infected from the metropolitan area of the municipality of Belo Horizonte (41 symptomatic and 10 asymptomatic), 45 dogs (84.9%) naturally infected from the central area of municipality of Belo Horizonte (38 symptomatic and 7 asymptomatic) and 4 dogs from the experimentally infected group (2 symptomatic and 2 asymptomatic) (Fig. 1).

Chronic inflammatory reaction was characterized by a cellular exudate mainly composed by mononuclear cells (plasma cells, macrophages and lymphocytes). In general, the cellular inflammatory exudate was diffuse in the upper dermis and around vessels, glands and pylus follicles in the deep dermis. A presence of many macrophage epithelioid cells was noticed within the mononuclear cell exudate, parasitized or not, but we did not find granuloma formation in any of the cases (Fig. 2a-f).

Plasmocytosis were observed in 62 animals (57.4%), of naturally infected dogs (Fig. 2f). However, this histological picture was more frequently seen in dogs from the metropolitan area (76.3%) than from the central area of Belo Horizonte (37.7%). In experimental dogs, plasmocytosis was less obvious than in the naturally infected dogs.

With regard to the infected dogs with chronic dermatitis (100 dogs), these lesions were more frequent in symptomatic dogs (81 dogs) than in asymptomatic ones (19 dogs). The semi-quantitative study analysis revealed that the chronic inflammatory reaction ranged in intensity from discrete to intense, and an intense inflammatory process was more frequently observed in skin sections obtained from dogs from the municipality of Santa Luzia (metropolitan area), followed by Santo Agostinho (central area), and finally by the experimental dogs. This data was confirmed by the quantitative morphometrical analysis and a statistical difference

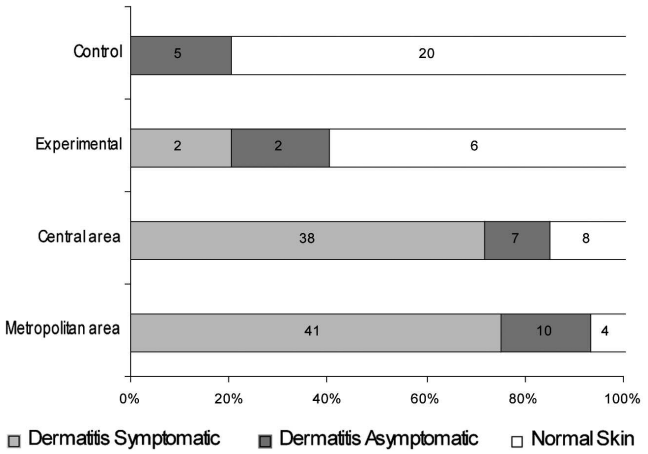

Fig. 1. Frequency of chronic dermatitis of all groups of dogs with different clinical status: Control group (n=25); Experimental group (n=10); Central area group (n=53) and Metropolitan area group (n=55).

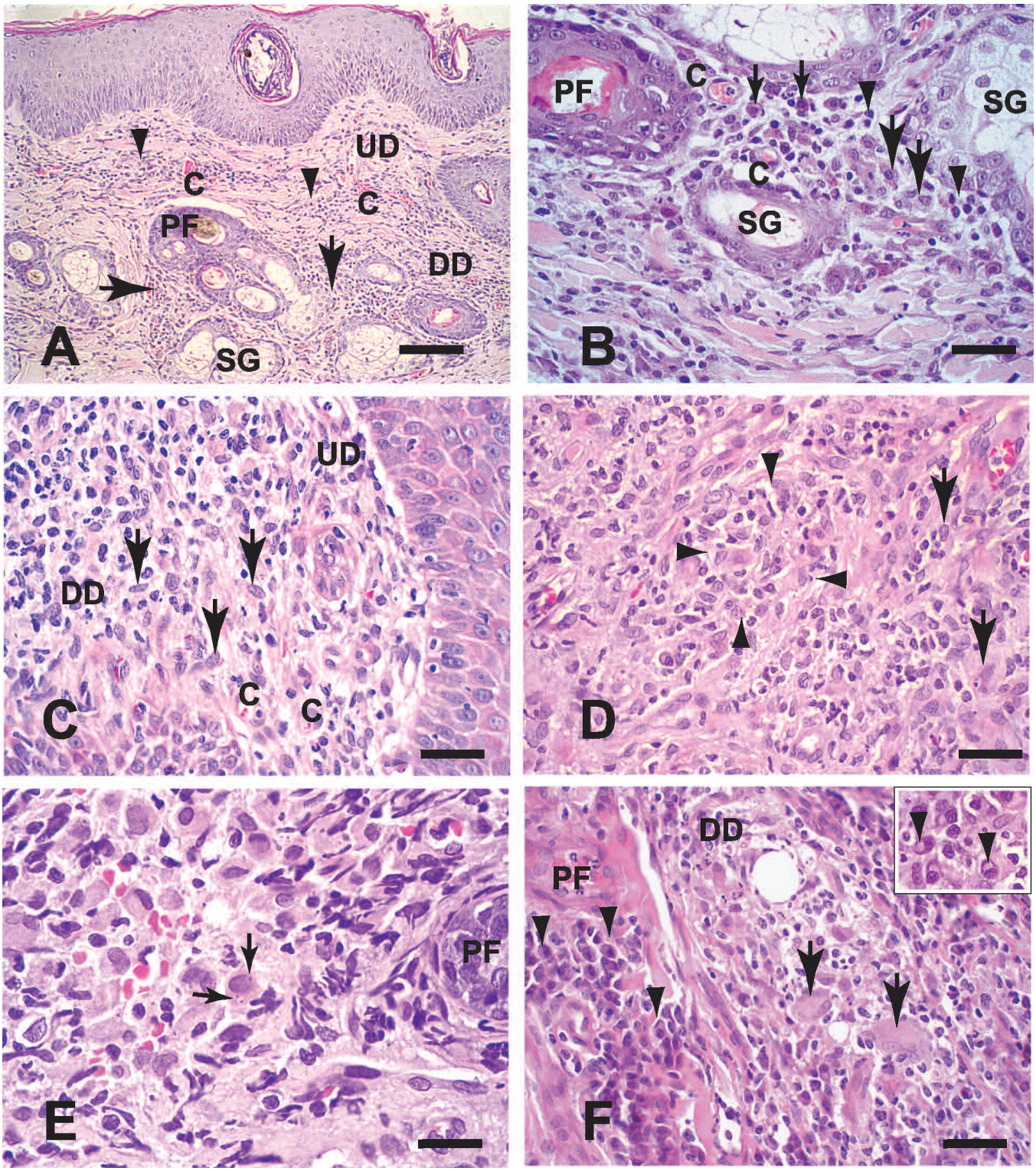

Fig. 2. Ear healthy skin section of dogs infected with *L. chagasi*. **A.** Observe a presence of inflammatory reaction diffuse in the upper dermis (UD) (arrowheads) and focal in the deep dermis (DD) (arrows). **B.** High magnification showing a chronic cellular exudate of macrophages (large arrows); plasma cells (thin arrows); lymphocytes (arrowheads) around the vessels, pyli and glands in the deep dermis. **C.** Note the presence of macrophage epithelioid cells (arrows) in all layers of the dermis. **D.** Epithelioid cells, aggregated macrophages in chronic cellular exudate, where the distribution of these cells was diffuse (arrows) or perhaps slightly aggregated (arrowheads). **E.** High magnification (oil immersion objective) showing amastigotes inside a macrophage epithelioid cell (arrowheads) in the deep dermis. **F.** Observe plasmocytosis (arrowheads) in the left and giant cells (arrows). **Inset.** At the right corner (oil immersion objective) plasma cells with a vacuolated cytoplasm (arrowheads) indicating activity of the Golgi complex. PF: pylus follicles; SG: sebaceous gland; C: capillaries. Hematoxylin-Eosin (H&E). Bars: A, 60 μm; B-D, F, 20 μm; E, inset, 7 μm.

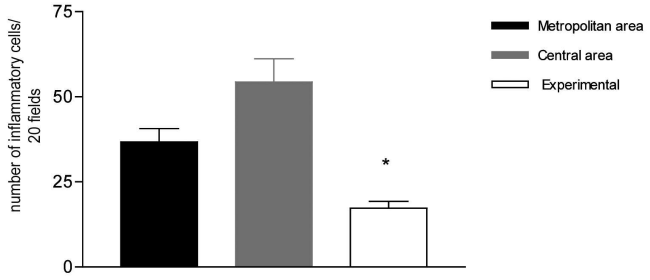

Fig. 3. Number of inflammatory cells (morphometrical analysis) in the dermis of healthy skin ear of infected dogs with *Leishmania (Leishmania) chagasi* in the different groups analyzed: Metropolitan area (n=55); Central area (n=53) and Experimental (n=10). *statistical difference between experimentally and naturally infected dogs (p=0.0144)

among the natural and experimentally infected dogs was found (p = 0.0144) (Fig. 3).

Epidermal alterations were frequently found, mainly in cases with intense inflammatory reactions in the dermis. Hyperkeratosis, acanthosis (epidermal hyperplasia), parakeratosis, papillomatosis, the intercellular edema (spongiosis) and vacuolization of the epidermal cells (hydropic degeneration) were the most frequent epithelial alterations observed (Fig. 4 a-d). Foci of intense parakeratosis were associated with hyperkeratosis in 20 cases of all infected dogs (16.9%). Ulcerative lesions occurred in only 11 infected (9.3%) of the infected dogs (Fig. 4c). However, interstitial necrosis was absent in all cases.

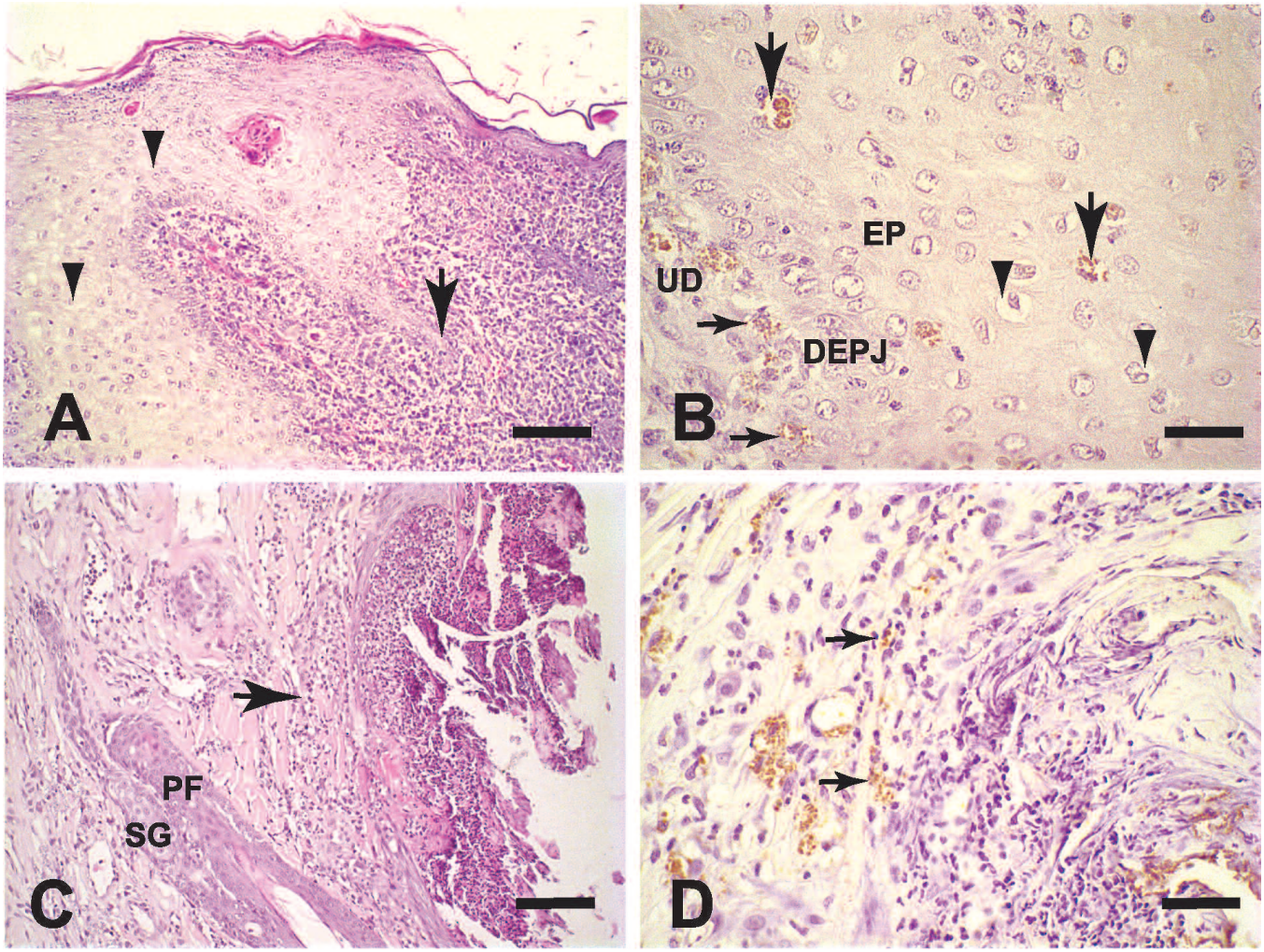

Fig. 4. Ear healthy skin section (extremity of pina) of a dog naturally infected with *L. chagasi*. **A.** Epidemic alterations such as acanthosis (arrowheads) and papillomatosis (arrow) as observed. Hematoxylin-Eosin. **B.** Immunolabelled amastigotes (arrows) could be seen in epithelial cells. Hydropic degeneration is shown by arrowheads. Note many amastigotes in macrophages localized in "the dermo-epidermal junction" (DEPJ) (thinny arrows). Streptoavidin-peroxidase. **C.** Ulcerative lesion and the chronic mononuclear exudate in the dermis (bottom of the ulcer lesion (arrow). Hematoxylin-Eosin. **D.** Immunolabelled amastigotes at the bottom of the ulcer lesion (arrows) Streptoavidin-Peroxidase. EP: epithelium; UD: upper dermis; DD: deep dermis; PF: pylus follicles; SG: sebaceous gland. Bars: A, C, 60 μm; B, D, 20 μm.

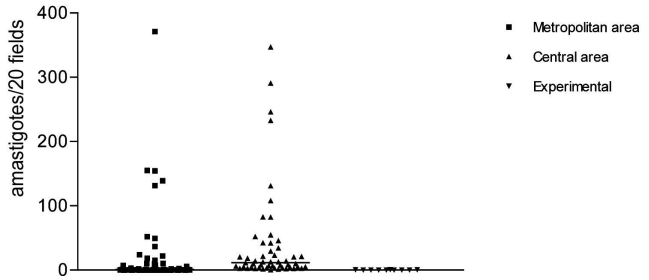

Fig. 5. Number of amastigotes (morphometrical analysis) in the dermis of healthy skin ear of infected dogs with *Leishmania (Leishmania) chagasi* in the different groups analyzed: Metropolitan area (n=55); Central area (n=53) and Experimental (n=10) *: statistical difference between all infected dogs p<0.001.

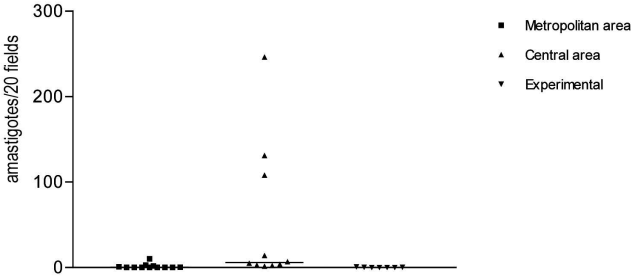

Fig. 6. Number of amastigotes (morphometrical analysis) in the dermis of asymptomatic healthy skin ear of infected dogs with *Leishmania (Leishmania)* chagasi in the different groups analyzed: Metropolitan area (n=55); Central Area (n=53) and Experimental (n=10). Statistical difference between experimentally and naturally infected dogs p<0,001.

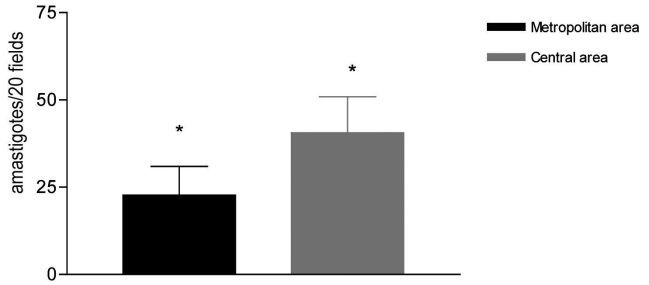

Fig. 7. Number of amastigotes (morphometrical analysis) in the dermis of asymptomatic healthy skin ear of infected dogs with *Leishmania (Leishmania) chagasi* in the different groups analyzed: Metropolitan area (n=55) and Central area (n=53) *: statistical difference between naturally infected dogs p<0.0001

Parasite load versus clinical aspects

Quantitative morphometrical analysis showed that the parasite load was statistically different among the groups, where animals from the central area showed higher parasite load than the others (p<0.001) (Fig. 5). Otherwise, considering the parasite load, the clinical status of all the animals and geographic area (origin), asymptomatic dogs from the central area harbored more parasites than asymptomatic animals from the metropolitan area or experimental infected dogs (p<0.001) (Fig. 6). However, symptomatic dogs did not show any differences between naturally infected groups from the distinct areas, this difference only occurred in comparison to the experimentally infected dogs.

In experimental dogs eight were positive by immunohistochemistry and the other two negative dogs were positive by PCR (data not shown). Moreover, experimental dogs had lower parasite loads than naturally infected animals. However, when we compared the two groups of naturally infected dogs, a higher parasite load was seen in dogs from the central area (p<0.0001) (Fig. 7).

Parasite load versus chronic inflammatory reaction

In some cases we saw an intense tissue parasitism of amastigotes forms of *Leishmania* in the skin without an intense inflammatory reaction, or vice-versa (Fig. 8a-d). A positive correlation between the parasite load and inflammatory reaction was found, but this correlation appeared to be not strong in the experimental group (r_2=0.2783), in dogs from metropolitan area (r_2=0.2491), and in dogs from central area (r_2=0.1124).

Discussion

Lymphadenopathy was the most important clinical alteration in all symptomatic animals naturally (independently of the origin) or experimentally infected with *Leishmania*, the cervical lymph nodes being the prime site involved. As discussed by Lima et al. (2004) and Costa et al. (2008) in Brazil and Ciaramella et al. (1997) in the Mediterranean, these nodes are directly related to facial lesions in canine visceral leishmaniasis. Following lymphadenopathy changes, exfoliative dermatitis was the most common cutaneous manifestation associated with alopecia, usually starting on the head (mainly ears and orbits) and extending to the rest of the body, in agreement with the findings of Saridomichelakis et al. (2007), who considered this skin lesion to be the most common clinical manifestation of canine leishmaniasis in the Mediterranean area. However, these authors also report that excessive scaling may or may not be accompanied by alopecia, as also noted by Slappendel and Ferrer (1998) and Koutinas and Koptopoulos (1993).

Under histological analysis a chronic inflammatory reaction was observed in the ears of 92.7% from the dogs of the metropolitan area, 84.9% from the central area, which is in accordance with the results of dos-Santos et al. (2004), who found chronic dermatitis in the

ears of 93% of the naturally infected dogs in northeast of Brazil. This chronic dermatitis was also more frequent in symptomatic than asymptomatic dogs (Fig. 1), in accordance with Giunchetti et al. (2006), Xavier et al. (2006) in Brazil, and Solano-Gallego et al. (2004) in Spain. Moreover, the intensity of this inflammation (semi-quantitative and quantitative results) was higher in the skin of infected dogs obtained from the metropolitan area of Belo Horizonte. This might be because these dogs live in different conditions of health care and nutrition (Alvar et al., 2006). The cellular exudate was composed by macrophages, plasma cells and lymphocytes, being diffuse in the upper dermis and focally concentrated around vessels, glands and hair follicles in the deep dermis. A description of granulomatous inflammatory pattern in the skin has been described by dos-Santos et al. (2004). Although there are various histological definitions of granulomatous inflammation (Adams, 1976; Williams and Williams, 1983; Sheffield, 1990), most investigators agree that the structure of a typical granuloma formation induced by an intracellular microorganism consists of a core of fused parasitized resident macrophages with an encircling mononuclear cell mantle containing blood monocytes (Murray, 2001). However, we did not observe this typical granuloma formation in the dermis, as described by other studies (Giunchetti et al., 2006; Xavier et al., 2006; Moura et al., 2008). This discrepancy could be explained by the interpretation drawn from our histological analysis. We found epithelioid cells,

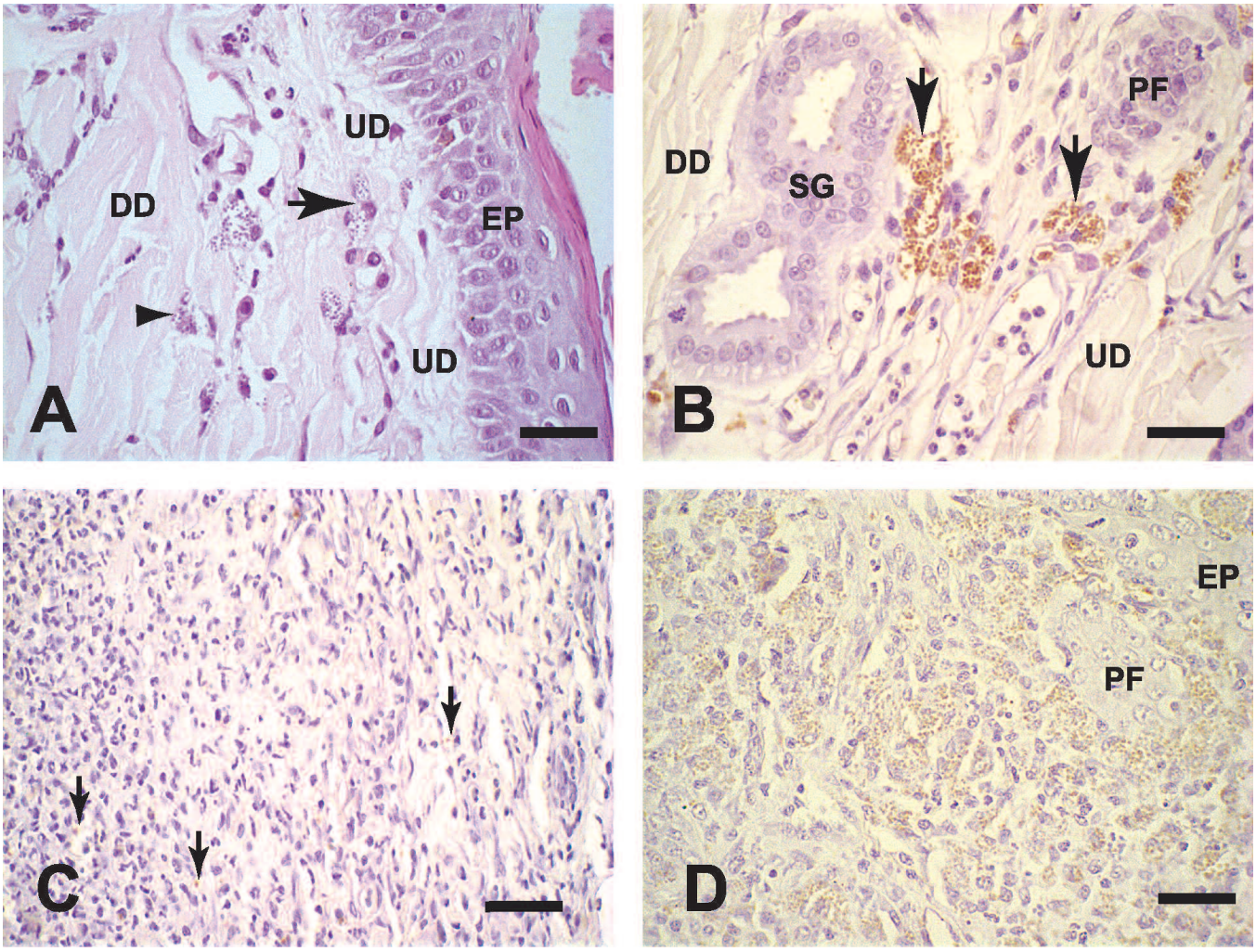

Fig. 8. Ear healthy skin section of a dog naturally infected with *L. chagasi*. **A.** Amastigotes can be observed at upper dermis (arrows) and deep dermis (arrowheads). Hematoxylin-Eosin. **B.** Immunolabeled amastigotes (arrows) could be seen in the dermis. Streptoavidin-peroxidase. **C.** An intense chronic exudate with a few immunolabeled amastigotes detected by immunohistochemistry (arrows). Streptoavidin-Peroxidase. **D.** An intense chronic exudate with a high parasite load detectable by immunohistochemistry. Streptoavidin-peroxidase. EP: epithelium; UD: upper dermis; DD: deep dermis; PF: pylus follicles; SG: sebaceous glands. Bars: 20 μm.

aggregated macrophages, or even giant cells (in sporadic presence), in chronic cellular exudate, but in contrast with the typical granuloma in our all skin tissue samples examined, the distribution of these cells was diffuse or perhaps slightly aggregated, but never encircled by a mononuclear cell mantle. The relevance of the granulomatous formation in the skin has been reported by dos-Santos et al. (2004) in canine visceral leishmaniasis. These authors discussed that the granulomatous inflammatory reaction is directly related to a lowest parasitism skin tissue burden. Our group has found this correlation in livers (Lima et al., 2007) of naturally infected dogs with *L. chagasi*, but we did not observe it in skin. In relation to the epithelial alterations, they have recently been described by others and are similar in affected and normal-looking skin of the same dogs (Solano-Galego et al., 2004; dos Santos et al., 2004). The presence of inflammatory cells in dogs without evident exfoliative dermatitis can be explained by the current hypothesis that macroscopic lesions follow the infiltration of inflammatory cells after a certain period of time (Papadogiannakis et al., 2005).

The immunohistochemistry method was chosen for the semi-quantitative and quantitative parasite burden evaluations based on previous works from our group (Tafuri et al., 2004; Xavier et al., 2006) and others (Ferrer et al., 1988; Solano-Gallego et al., 2001, 2004). Thus, we decided to use H&E studies for only histological analysis and immunohistochemistry for the parasite burden evaluation. Solano-Gallego et al. (2001) and Travi et al. (2001) considered that amastigotes are more common in the superficial dermis near or in the mid-deep dermis. In this work, independent of both the clinical status and the geographical origin of the dogs, we have found the same distribution of the parasites near the epidermis (the dermo-epidermal junction) (Fig. 4b). Interestingly, Saridomichelakis et al. (2007), in histological studies with healthy dog's muzzle skin, discussed mechanisms of the deep penetration of mammalian skin by different sandfly species. It has been calculated between 0.32 to 0.26mm against the depth of 0.54mm of the host dermis. Thus, this anatomical parameter of the amastigotes distribution in the upper dermis offers additional evidence of the importance of as reservoir of *Leishmania*. Moreover, an important fact found in this work was that the asymptomatic dogs from central area of municipality of Belo Horizonte harbor more parasites in the skin than the asymptomatic ones from metropolitan area (Santa Luzia) (statistical significance). Otherwise, a positive, but not strong, correlation exists between the parasite load and the chronic inflammatory reaction ($0.1124 < r_2 > 0.2783$). We think that it could be explained because we can find cases of dogs with an intense tissue parasitism of amastigotes forms of *Leishmania* in the skin without an intense inflammatory reaction, or vice-versa. We also think that, even though dogs from this central area must have more healthy conditions than the metropolitan area, this does not exclude the importance of these dogs in the epidemiological dimension of the disease. Nevertheless, this data is also important considering the urbanization aspect of the *Leishmania* infection in large Brazilian cities, as exemplified by the municipality of Belo Horizonte (Margonari et al., 2006). It suggests an increasing silent domestic reservoir in rich parts of the cities, and not just the periurban areas. This data also reinforces the idea that asymptomatic dogs have parasites in the skin just like symptomatic dogs, underlining the importance of asymptomatic dogs in the epidemiology of visceral leishmaniasis, as discussed by Abranches et al. (1998) in Portugal, Solano-Gallego et al. (2001) in Spain and Lima et al. (2004), Xavier et al. (2006) and Michalsky et al. (2007) in Brazil. It is noteworthy that asymptomatic animals harbouring amastigotes without any association to absence of skin macroscopically lesions were first reported in the Mediterranean by Adler and Theodor (1931) and later in Brazil by Deane and Deane (1955), the latter being a pioneering epidemiological study in northeast Brazil. This author previously considered that healthy dogs skin is nevertheless a potential source for the transmission of the *Leishmania* amastigotes to phlebotomines (females) bloodsuckers. In agreement with Adler and Theodor (1931), a scar with secondary pathogens can interfere with success at the start of the *Leishmania* infection process.

The parasite load in experimental infected dogs was statistically lower than in naturally infected dogs. Although not detectable by immunohistochemistry in the skin, probably due to the low density of the parasites or even sequestering in other organs, the possibility of the parasite remaining in the animals and providing continuous immune stimulation should be considered. The lower parasite load in organs of dogs experimentally infected was also noted by Tafuri et al. (1996) and Sampaio et al. (2007).

Some of the animals of the control groups showed an inflammatory reaction in the skin. Histological staining carried out to investigate possible infections with fungus and bacteria (Grocott and Good Pasture) (data not shown) proved negative. Thus, we think that this processes is attributable to a variety of conditions, for example, insect bites (frequent throughout the year in tropical countries), as well as trauma that might originate during fighting (Mozos et al., 1999).

In naturally infected dogs obtained from two distinct areas of municipality of Belo Horizonte, we found by immunohistochemistry that all of them had detectable parasites in the skin. The main result has indicated that asymptomatic dogs can play an important role not only in the periurban area, but also in central urban areas of the city. Moreover, an intense or higher parasite load in the dog skin does not necessarily mean an intense inflammatory reaction in the dermis or even macroscopically visible skin lesions. In addition, a profound difference in the intensity of the inflammatory reaction and the parasite load in skin of experimental infected dogs were seen.

Acknowledgements. Experimental infection was carried out at HERTAPE CALIER SAÚDE ANIMAL S/A (Juatuba, MG), Brazil. This work was financed by Conselho Nacional de Desenvolvimento da Pesquisa Tecnológica e Científica (CNPq) (Grants 477427/2006-5; 301301/2006-9) and Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) (Grant EDT 2124/03), Brazil.

References

Abranches P., Silva-Pereira M.C., Conceição-Silva F.M., Santos-Gomes G.M. and Janz J.G. (1991). Canine leishmaniasis: pathological and ecological factors influencing transmission of infection. J. Parasitol. 77, 557-561.

Abranches P., Campino L. and Santos-Gomes G.M. (1998). Canine leishmaniasis. New concepts of epidemiology and immuno-pathology: their impact in the control of human visceral leishmaniasis. Acta Med. Port. 11, 871-875.

Adams D.O. (1976). The Granulomatous inflammatory response. A review. Am. J. Pathol. 84, 164-192.

Adler S. and Theodor O. (1931). Skin infection in canine visceral leishmaniasis. Brit. Med. J. 2, 1179.

Alvar J., Croft S. and Olliaro P. (2006). Chemotherapy in the treatment and control of leishmaniasis. Adv. Parasitol. 61, 223-274.

Arias J.R., Monteiro P.S. and Zicker F. (1996). The reemergence of visceral leishmaniasis in Brazil. Emerg. Infect. Dis. 2, 145-146.

Bern C., Amann J., Haque R., Chowdhury R., Ali M., Kurkjian K.M., Vaz L., Wagatsuma Y., Breiman R.F., Secor W.E. and Maguire J.H. (2006). Loss of leishmanin skin test antigen sensitivity and potency in a longitudinal study of visceral leishmaniasis in Bangladesh. Am. J. Trop. Med. Hyg. 75, 744-748.

Brahmachari U.N. (1928). A treatise on Kala-Azar. John Bale. Sons and Danielsson Ltda. Londres. 252 pp.

Caliari M.V. (1997). Princípios de morfometria digital: Ks300 para iniciantes. Editora da UFMG, 148.

Chagas E. (1937). Leishmaniose visceral americana (Nova entidade mórbida do homem na América do Sul). Nota prévia. Revista brasileira de malariologia e doenças tropicais 11, 1-3.

Ciaramella P., Oliva G., Luna R.D., Gradoni L., Ambrosio R., Cortese L., Scalone A. and Persechino A. (1997). A retrospective clinical study of canine leishmaniasis in 150 dogs naturally infected by *Leishmania infantum*. Vet. Rec. 141, 539-543.

Costa M.M., Lima W.G., Figueiredo M.M., Michalick M.S., Tafuri W.L. and Tafuri W.L. (2008). Cervical, mandibular, and parotid lymph nodes of dogs naturally infected with *Leishmania infantum*: a histopathologic and immunohistochemistry study and its correlation with facial skin lesions. Vet. Pathol. 45, 613-616.

Deane L.M. and Deane M.P. (1954). Isolation of Leishmania in the viscera and the skin of a fox in the kala-azar endemic zone in Sobral, Ceará. Hospital (Rio J). 45, 419-421.

Deane L.M. and Deane M.P. (1955). Observações sobre a transmissão da leishmaniose visceral no Ceará. Revista brasileira de malariologia e doenças tropicais 48, 347-364.

Deane L.M. and Deane, M.P. (1962). Visceral leishmaniasis in Brazil: geographical distribution and trnsmission. Rev. Inst. Med. Trop. São Paulo. 4, 198-212.

Desjeux P. (2004). Leishmaniasis: current situation and new perspectives. Com. Immun. Microbiol. Infect. Dis. 27, 305-318.

dos-Santos W.L., David J., Badaro R. and de-Freitas L.A. (2004). Association between skin parasitism and a granulomatous inflammatory pattern in canine visceral leishmaniosis. Parasitol. Res. 92, 89-94.

Fernandes A.P., Costa M.M., Coelho E.A., Michalick M.S., de Freitas E., Melo M.N., Luiz Tafuri W., Resende Dde M., Hermont V., Abrantes C de, F. and Gazzinelli R.T. (2008). Protective immunity against challenge with Leishmania (Leishmania) chagasi in beagle dogs vaccinated with recombinant A2 protein. Vaccine 26, 5888-5895.

Ferrer L., Rabanal R.M., Domingo M., Ramos J.A. and Fondevila, D. (1988). Identification of Leishmania donovani amastigotes in canine tissues by immunoperoxidase staining. Res. Vet. Sci. 44, 194-196.

Giunchetti R.C., Mayrink W., Genaro O., Carneiro C.M., Correa-Oliveira R., Martins-Filho O.A., Marques M.J., Tafuri W.L. and Reis A.B. (2006). Relationship between canine visceral leishmaniosis and the Leishmania (Leishmania) chagasi burden in dermal inflammatory foci. J. Comp. Pathol. 135, 100-107.

Gonçalves R., Tafuri W.L., Melo M.N., Raso P. and Tafuri W.L. (2003). Chronic interstitial pneumonitis in dogs naturally infected with Leishmania (Leishmania) chagasi: a histopathological and morphometric study. Rev. Inst. Med. Trop. Sao Paulo. 45, 153-158.

Koutinas A. and Koptopoulos G. (1993). Low prevalence of feline viral infections in northern Greece. Vet. Rec. 133, 245-247.

Lainson R. and Rangel E.F. (2005). Lutzomyia longipalpis and the eco-epidemiology of American visceral leishmaniasis, with particular reference to Brazil: a review. Mem. Inst. Oswaldo Cruz 100, 811-827.

Liew F.Y. and O'Donnell C.A. (1993). Immunology of leishmaniasis. Adv. Parasitol. 32, 161-259.

Lima W.G., Michalick M.S., de Melo M.N., Luiz Tafuri W. and Luiz Tafuri W. (2004). Canine visceral leishmaniasis: a histopathological study of lymph nodes. Acta Trop. 92, 43-53.

Lima W.G., Oliveira P.S., Caliari M.V., Gonçalves R., Michalick M.S., Melo M.N., Tafuri W.L. and Tafuri W.L. (2007). Histopathological and immunohistochemical study of type 3 complement receptors (CD11b/CD18) in livers and spleens of asymptomatic and symptomatic dogs naturally infected with Leishmania (Leishmania) chagasi. Vet. Immunol. Immunopathol. 117, 129-136.

Luz Z.M.P., Pimenta D.N., Cabral A.L.L.V., Fiúza V.O.P. and Rabello A. (2001). A urbanização das leishmanioses e a baixa resolutividade diagnóstica em municípios da Região Metropolitana de Belo Horizonte. Revista da Sociedade Brasileira de Medicina Tropical 34, 249-254.

Margonari C., Freitas C.R., Ribeiro R.C., Moura A.C., Timbo M., Gripp A.H., Pessanha J.E. and Dias E.S. (2006). Epidemiology of visceral leishmaniasis through spatial analysis, in Belo Horizonte municipality, state of Minas Gerais, Brazil. Mem. Inst. Oswaldo Cruz 101, 31-38.

Maurício I.L., Gaunt M.W., Stothard J.R. and Miles M.A. (2001). Genetic typing and phylogeny of the Leishmania donovani complex by restriction analysis of PCR amplified gp63 intergenic regions. Parasitology 122, 393-403.

Melo F., Amaral, M., Oliveira P., Lima W., Andrade M., Michalick M., Raso P., Tafuri W. and Tafuri W. (2008). Diffuse intralobular liver fibrosis in dogs naturally infected with Leishmania (Leishmania) chagasi. Am. J. Trop. Med. Hyg. 79, 198-204.

Michalick M.S.M., Melo M.N., Tafuri W.L., Aguiar S.S., Nascimento P.H., Genaro O., Costa C.A. and Mayrink W. (1993). Spreading of Visceral Leishmaniasis in Urban Area of Belo Horizonte, MG, Brazil.

Mem. Inst. Oswaldo Cruz 88, -123.

Michalsky E.M., Rocha M.F., da Rocha Lima A.C., Franca-Silva J.C., Pires M.Q., Oliveira F.S., Pacheco R.S., dos Santos S.L., Barata R.A., Romanha A.J., Fortes-Dias C.L. and Dias E.S. (2007). Infectivity of seropositive dogs, showing different clinical forms of leishmaniasis, to Lutzomyia longipalpis phlebotomine sand flies. Vet. Parasitol. 147, 67-76.

Moreno E.C., Melo M.N., Genaro O., Lambertucci J.R., Serufo J.C., Andrade A.S., Antunes C.M. and Carneiro M. (2005). Risc factors for Leishmania chagasi infection in a urban area of Minas Gerais State. Rev. Soc. Bras. Med. Trop. 38, 456-463.

Moura E.P., Ribeiro R.R., Sampaio W.M., Lima W.G., Alves C.F., Melo F.A., Melo M.N., Tafuri W.L. and Michalick M.S.M. (2008). Histopathological and parasitological analysis of skin tissues biopsies from two distinct anatomical areas of the ears of dogs. Brasil. J. Vet. Pathol. 1, 10-15.

Mozos E., Perez J., Day M.J., Lucena R. and Ginel P.J. (1999). Leishmaniosis and generalized demodicosis in three dogs: a clinicopathological and immunohistochemical study. J. Comp. Pathol. 120, 257-268.

Murray H.W. (2001). Tissue granuloma structure-function in experimental visceral leishmaniasis. Int. J. Exp. Pathol. 82, 249-267.

Nicolle C. (1910). Quelques donnés nouvelles relatives au Kala-azar infantile. Bull. Soc. Path. Exot. 3, 431-432.

Oliveira C.A., Assunção R.M., Reis I.A. and Projetti F.A. (2001). Spatial distribuition of human and canine visceral leishmaiasis in Belo Horizonte, Minas Gerais State, Brazil, 1994-1997. Cadernos de Saúde Pública. 17, 1231-1239.

Papadogiannakis E.I., Koutinas A.F., Saridomichelakis M.N., Vlemmas J., Lekkas S., Karameris A. and Fytianou A. (2005). Cellular immunophenotyping of exfoliative dermatitis in canine leishmaniosis (*Leishmania infantum*). Vet. Immunol. Immunopathol. 104, 227-π237.

Ross R. (1899). Report on the nature of Kala-azar. Calcutá: Office on the Superintendent of Governement Printing.

Sampaio W.M., Moura E.P., Arruda F.C., Ribeiro R.R., Alves C.F., Melo F.A., Fernandes A.P., Michalick M.S., Melo M.N., Tafuri W.L. and Tafuri W.L. (2007). In vitro binding and survival assays of Leishmania parasites to peripherical blood monocytes and monocyte-derived macrophages isolated from dogs naturally and experimentally infected with *Leishmania (Leishmania) chagasi*. BMC Vet. Res. 3, 11.

Santos-Gomes G.M., Campino L. and Abranches P. (2000). Canine experimental infection: intradermal inoculation of Leishmania infantum promastigotes. Mem. Inst. Oswaldo Cruz 95, 193-198.

Saridomichelakis M.N., Koutinas A.F., Olivry T., Dunston S.M., Farmaki R., Koutinas C.K. and Petanides T. (2007). Regional parasite density in the skin of dogs with symptomatic canine leishmaniosis. Vet. Dermatol. 18, 227-233.

Sheffield E.A. (1990). The granulomatous inflammatory response. J. Pathol. 160, 1-2.

Slappendel R.J.F. and Ferrer L. (1998). Leishmaniasis. In: infectious deseases of dog and cat, C. E. Greene. Saunders Company, Philadelphia. pp 450-458.

Solano-Gallego L., Morell P., Arboix M., Alberola J. and Ferrer L. (2001). Prevalence of Leishmania infantum infection in dogs living in an area of canine leishmaniasis endemicity using PCR on several tissues and serology. J. Clin. Microbiol. 39, 560-563.

Solano-Gallego L., Fernandez-Bellon H., Morell P., Fondevila D., Alberola J., Ramis A. and Ferrer L. (2004). Histological and immunohistochemical study of clinically normal skin of Leishmania infantum-infected dogs. J. Comp. Pathol. 130, 7-12.

Tafuri W.L., Tafuri W.L., Barbosa A.J., Michalick M.S., Genaro O., Franca-Silva J.C., Mayrink W. and Nascimento E. (1996). Histopathology and immunocytochemical study of type 3 and type 4 complement receptors in the liver and spleen of dogs naturally and experimentally infected with *Leishmania (Leishmania) chagasi*. Rev. Inst. Med. Trop. Sao Paulo 38, 81-89.

Tafuri W.L., de Oliveira M.R., Melo M.N. and Tafuri W.L. (2001). Canine visceral leishmaniosis: a remarkable histopathological picture of one case reported from Brazil. Vet. Parasitol. 96, 203-212.

Tafuri W.L., Santos R.L., Arantes R.M., Gonçalves R., de Melo M.N., Michalick M.S. and Tafuri W.L. (2004). An alternative immunohistochemical method for detecting Leishmania amastigotes in paraffin-embedded canine tissues. J. Immunol. Methods. 292, 17-23.

Tesh R.B. (1995). Control of zoonotic visceral leishmaniasis: is it time to change strategies? Am. J. Trop. Méd. Hyg. 52, 287-292.

Torres C.M. (1941). Alterações cutâneas do cão no Kala-azar sul-americano Mem. Inst. Oswaldo Cruz 36, 37-67.

Travi B.L., Tabares C.J., Cadena H., Ferro C. and Osorio Y. (2001). Canine visceral leishmaniasis in Colombia: relationship between clinical and parasitologic status and infectivity for sand flies. Am. J. Trop. Med. Hyg. 64, 119-124.

Williams G.T. and Williams W.J. (1983). Granulomatous inflammation: a review. J. Clin. Pathol. 36, 723-733.

Xavier S.C., de Andrade H.M., Monte S.J., Chiarelli I.M., Lima W.G., Michalick M.S., Tafuri W.L. and Tafuri W.L. (2006). Comparison of paraffin-embedded skin biopsies from different anatomical regions as sampling methods for detection of Leishmania infection in dogs using histological, immunohistochemical and PCR methods. BMC Vet. Res. 2, 17.

Yakimoff W.K. (1913). Leishmaiose Canine a Taschkent. Bull. Soc. Path. Exot. 6, 432-433.

Young C.W. and Hertig M. (1926). The development of flagellates in Chinese sandfllies (Phlebotomus) fed on hamsters infected with Leishmania donovani. Proc. Soc. Experim. Biol. and Med. 23, 611-615.

Accepted January 25, 2010